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RESEARCH ARTICLE

A Comparative Study on the effect of Warfarin v/s Acenocoumarol in patients with Atrial fibrillation

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ABSTRACT:

Aim: To compare and evaluate the effectiveness of warfarin and acenocoumarol in atrial fibrillation patients. **Methods:** It was a prospective observational study for a period of 1 year. The effectiveness was determined by comparing individual mean Time in therapeutic range (TTR) calculated using "Fraction of INR in range" method as well as by evaluating stroke risk using CHA_2DS_2VASc scores. The safety of the drug therapy was assessed from the ADR occurred after the drug administration. The data was analyzed using statistical software (version 20). **Result:** A total of 218 patients were selected for the study. The mean age of patients treated with acenocoumarol was 57.01±13.07 years and that of patients treated with warfarin was 67.18±12.24 years respectively. Mean TTR was found to be 56.54% ± 19.67 vs 50.69% ± 23.57 for acenocoumarol and warfarin respectively (p value <0.05). After the drug initiation, 12 patients experienced stroke episodes in acenocoumarol group while 24 patients experienced stroke in warfarin treated group (p value <0.05). A total of 463 ADRs were observed during the study period, of which 174 belong to acenocoumarol treated patients and 289 to warfarin group. Among these, bleeding (127 patients) was the main ADR (55 vs 72) with p value of 0.001.

Conclusion: This study concluded that acenocoumarol is a better oral anticoagulant compared to warfarin while considering various factors like mean TTRs, ADRs observed, stroke incidence and QOL of patients.

KEYWORDS: Acenocoumarol, Warfarin, TTR, Atrial fibrillation, OAC.

INTRODUCTION:

Atrial Fibrillation is the most prevalent type of sustaining dysrhythmia that has evidently affected more than 33.5 million people around the world. Atrial fibrillation is the evident predecessor of stroke and thromboembolism that have substantial morbidity and mortality.[1] The prevalence rate of atrial fibrillation has remarkably increased over the previous years.[2] Patients with atrial fibrillation are at risk of developing stroke and antithrombotic prophylaxis is essential in patients with atrial fibrillation at risk of developing stroke.[3,4]

Coumadin derivatives are the anticoagulant agents preferred for this purpose which include mainly warfarin and acenocoumarol. Warfarin is an anticoagulant medication that is used to slow down the blood clotting process, whereas Acenocoumarol is a blood thinner, used to treat thrombotic disorders that obstruct the normal blood flow in blood vessel.

Theoretically warfarin is a drug with long half-life as compared to acenocoumarol, so it is expected to provide more stability in anticoagulation but there exists conflicts between published literatures regarding the effectiveness of the two. It was found that in majority of these published literatures the sample size was small and the study duration was short[5]. So the study results cannot be considered as a generalized one. Hence, by rectifying these drawbacks we had conducted this study for a comparatively longer duration on a larger sample size. Our study results have the potential to alter the general

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prescribing patterns of both the drugs providing increased treatment goal achievement chances.

A drug providing greater stability of anticoagulation is safer and ensures reduced requirement for repeated checking of INR values, hence leading to the betterment of quality of life of the patients.

The alternate anticoagulant therapies, that may be suggested to substitute the coumadin derivatives is not economically feasible by all the patients hence there is a need to ascertain the best anticoagulant among the relatively accessible warfarin and acenocoumarol.

METHODS:

Study Design:

A Prospective Observational study conducted in a multi-speciality tertiary care teaching hospital for a period of 1 year.

Inclusion criteria:

- All adult patients with age ≥18 years with confirmed diagnosis of atrial fibrillation.
- Patients who were on warfarin and acenocoumarol as anticoagulant therapy.
- Patient who were willing to provide information through phone.

Exclusion criteria:

- Patients undergoing any other anticoagulant therapy.
- Pediatric patients.
- Patients unwilling to give consent form.

Method of data collection:

The study protocol was approved by the Institutional Ethics Committee before the enrolment of patients (IEC-AIMS-2017-PHARM-371). The effectiveness of the anticoagulation therapy was evaluated by assessing parameters like time in therapeutic range (TTR) and occurrence of stroke. TTR was calculated using "Fraction of INRs in range" method. All the patients were assessed for the risk for stroke using CHA_2DS_2VASc score and the occurrence of stroke in both the groups were observed. Both these parameters were assessed for each drug and compared. The safety of the drug therapy was evaluated from the ADR occurred after drug intake and causality was assessed by using Naranjo's Causality Assessment Scale. Severity of observed ADR was assessed with Hartwig's severity scale. Compliance to anticoagulation therapy was measured using MMAS-4 during the study period. Quality of Life of patients was assessed individually by using SF-12 quality of life questionnaire for both the drugs and compared.

Statistical analysis and interpretation:

Statistical analysis was performed using IBM SPSS version 20.0 software. Descriptive statistical methods were used to calculate the mean and range. Pearson Chi-square test and paired t test were employed for the calculation of p-values and a value <0.05 was taken as significant.

RESULTS:

Demographic characteristics:

A total of 218 patients who satisfied the inclusion and exclusion criteria were selected as study sample with 109 patients in each group. In this study it was found out that males (53.25% vs 61.5%) were predominant than females (46.8% vs 38.5%) in both groups and most of the patients were in the age range of 71-80 years (36.7%). The mean age of patients treated with acenocoumarol was 57.01±13.07 years and that of patients treated with warfarin was 67.18±12.24 years respectively. In both the groups (acenocoumarol vs warfarin) hypertension (67.9% vs 61.5%) was the predominant comorbidity, followed by dyslipidemia (33% vs 48.6%), diabetes (33% vs 47.7%), stroke (11% vs 22%), COPD (14.7% vs 12.8%) hypothyroidism (17.4% vs 11%) and BPH (7.3% vs 7.3%).

INR categorization of sample population:

The total number of INRs in acenocoumarol group was found to be 922. Out of these, 263 were in sub therapeutic range, 149 in supra therapeutic and 510 were found to be in range. Total number of INRs in warfarin group was 992. Among these, 324 were sub therapeutic, 198 supra therapeutic and 470 were in range.

ASSESSMENT OF EFFECTIVENESS:

Time in therapeutic range (TTR) of sample population:

The mean TTR for acenocoumarol group was found to be 56.54% ± 19.67 and 50.69% ± 23.57 for warfarin group. The mentioned values were found to be statistically significant with a p value of 0.048.

Table 1. Comparison of mean time in therapeutic range of warfarin and acenocoumarol

Drug	Target INR	Mean TTR (%)	SD	p value
Acenocoumarol	2-3	56.54	19.67	
Warfarin	2-3	50.69	23.57	0.048

Stroke risk assessment using CHA_2DS_2VASc score:

In acenocoumarol group most of the patients (83.4%) were found to be in high risk category and only 18 patients (16.5%) come under the intermediate risk. None of the patients came to low risk category. While in the warfarin treated group the distribution is as follows, high risk (90.8%), intermediate risk (3.6%) and low risk (5.5%).

Occurrence of stroke in sample population:

The occurrence of stroke in patients were assessed after initiation of anticoagulation therapy followed by atrial fibrillation detection in the study subjects.

Table 2. Comparison of stroke occurrence in each group

Group	No. of patients with stroke occurrence (n)	%	No. of patients without stroke occurrence (n)	%	p value
Acenocoumarol	12	11.01	97	88.99	<0.05
Warfarin	24	22.01	85	77.98	

Only 12 (11.01%) patients encountered a stroke episode in acenocoumarol treated group and 24 (22.01%) patients in warfarin treated group. This data was proven to be statistically significant with a p value of less than 0.05.

ASSESSMENT OF SAFETY:

Out of 218 study patients, 463 adverse events were observed, in which 174 belong to acenocoumarol treated patients and the rest of 289 ADRs belong to the warfarin treated patients. Among them bleeding (58.25%) was the most prominent ADR in both the groups with 55(50.4%) patients in acenocoumarol group and 72(66.05%) in warfarin group. Other ADRs observed are depicted in figure 1. As per the Naranjo causality assessment scale, 211 ADRs were under definite category. Hartwig's severity assessment revealed that in acenocoumarol group 190 (65.06%) ADRs belong to mild category, 73 (25%) belong to moderate category and 29 (9.9%) in high severity category while in warfarin taking study subjects 107 (62.57%) ADRs belong to mild, 26 (15.2%) belong to moderate category and 38 (22.22%) ADRs belong to high severity category.

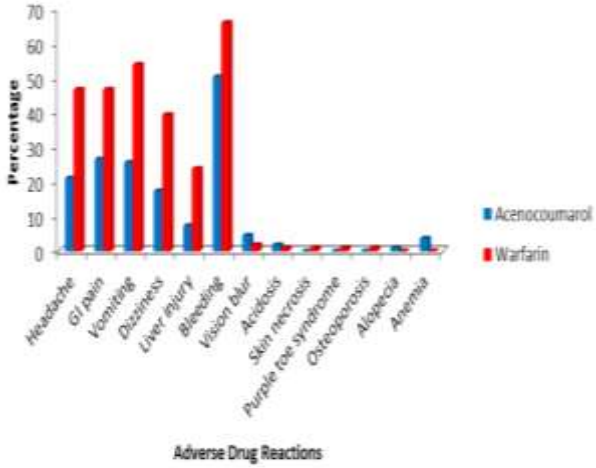

Fig1. Adverse drug reactions observed in sample population.

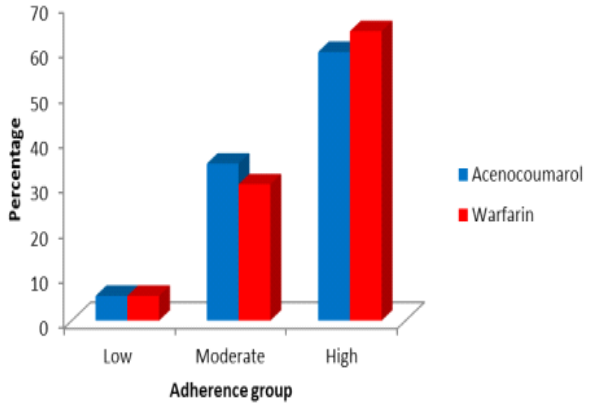

Fig 2. Medication adherence by MMAS-8 scoring.

Medication adherence of sample population:

In acenocoumarol group 65 patients (59.63%) were in high adherence category, 38 patients (34.86%) in moderate and 6 patients (5.5%) in low adherence. In warfarin treated group 70 patients (64.22%) were in high adherence category, 33 patients (30.27%) in moderate and 6 patients (5.5%) in low adherence category.

Quality of life assessment:

Statistically significant betterment of QOL was seen in patients with use of acenocoumarol compared to warfarin over a number of domains which are physical functioning (acenocoumarol: 52.98±26.07; warfarin 40.60±30.76, p value 0.002), general health (acenocoumarol 74.31±20.25; warfarin 62.84±31.29, p value 0.002) and mental health (acenocoumarol 79.35±14.16, warfarin 73.67±13.36, p value 0.003).

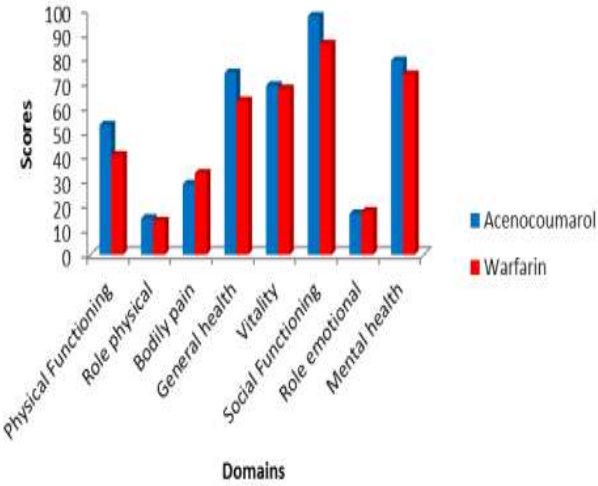

Fig 3. Comparison of quality of life of patients on use of acenocoumarol and warfarin.

Warfarin group showed statistically significant association between medication adherence and TTR as well as medication adherence and quality of life domains like, physical functioning (40.60%), role physical (13.79%), general health (62.84%) and vitality (67.77%). Also, there was a positive correlation between age and the stroke occurrence risk of patients with atrial fibrillation.

DISCUSSION:

Demographic characteristics:

In our study, male patients (53.2% and 61.5%) were more predominant than female patients. Most of the patients were in the age group of 71-80 years. From our study atrial fibrillation were mostly seen in elderly patients. Similarly another study by Go AS et al[6] and J. Heeringa et al[7] showed that elderly people have increased chances for developing atrial fibrillation. This is because ageing is associated with stiffening of the arteries, that result in high blood pressure and increased workload of the heart. Over time this increased workload may lead to ventricular hypertrophy, thus making the heart more irritable, resulting in extra beats that can become risk factors for atrial fibrillation.

Co-morbidities observed:

Hypertension (67.9% and 61.5%) was the predominant co-morbid condition among both the groups, which may be due to its high prevalence in the population. Long-standing hypertension, especially if sub-optimally controlled, leads to LVH, structural changes and enlargement of the left atrium, heterogeneity of atrial conduction and fibrosis, all of which may contribute to the development of AF. A study conducted by Hasan Kaya et al[8] also depicts hypertension as the most common co-morbidity among AF patients.

Medication adherence of sample population:

Majority of patients were in the high medication adherence category (59.63% and 64.22%) which could be explained by patient's perception of the importance of anticoagulants over other medication. In a study conducted by Patel et al[9] also, majority of patients reported medium or high adherence to oral anticoagulation. We also found that the acenocoumarol group showed no significant association between medication adherence and TTR while warfarin group showed a statistically significant association (p value < 0.05). The rate of adherence may differ by many factors, including types of population, study design, method of measurement, to quote a few[16].

INR categorization of sample population:

Majority of patients were in the therapeutic INR range (55.3% and 47.4%) that may be because of the high medication adherence shown by the study groups.

Time in therapeutic Range (TTR) of sample population:

Mean TTR was found to be high in patients receiving acenocoumarol (56.54% ± 19.67) as compared to warfarin (50.69% ± 23.57) when calculated using fraction of INRs in range method. Mean TTR was found to be high among both the groups because majority of INRs were found to be in the therapeutic range. There is a statistically significant difference in mean TTR among patients prescribed with acenocoumarol versus warfarin (p value <0.05). Similarly Kulo et al[10] performed a study in which acenocoumarol has shown significantly better anticoagulation stability with therapeutic INR values covering significantly longer time of treatment.

Assessment of risk for stroke using CHA_2DS_2VASc Score:

Stroke risk for atrial fibrillation patients was assessed using CHA_2DS_2VASc score and found that majority of patients were at high risk for stroke (83.4% and 90.8%) in both groups. For AF patients, risk for stroke increases because the rapid heartbeat allows blood to pool in the heart, which can cause clots to form and travel to the brain, resulting in stroke[11]. We also found statistically significant association between age and stroke risk which was similar to a study performed by Flegel KM, Hanley J et al,[12] that suggested increasing age (>75 years) as one of the major risk factor for stroke development in AF patients.

Occurrence of stroke in sample population:

In this study both groups encountered stroke episodes (11.01% and 22.01%) which was one of the major complications associated with under dosing of vitamin K antagonists.

Adverse drug reactions observed in sample population:

Safety of the drugs were assessed by observing the occurrence of ADRs. In our study bleeding was the most prominent ADR seen in both the groups. Vazquez Fernando J et al[13] and Ambrosi P et al[14] stated in their meta-analysis that bleeding rates were much higher with the use of vitamin K antagonists. Majority of the ADRs were found to be in definite (64.2%) category as per Naranjo assessment and were severe (51.37% and 49.54 %) as per Hartwig's severity assessment.

Quality of life of sample population:

One of our objectives was to evaluate the quality of life of patients on anticoagulation therapy using SF-12 quality of life questionnaire. Statistically significant difference in improvement of QOL was shown by patients taking acenocoumarol when compared to warfarin over various domains. This may be due to the increased number of elderly patients presented within warfarin group. Older patients are more likely to have

low medication adherence which in turn results reduction in QOL. A study conducted by Reynolds, Matthew R et al,[15] mentioned that elderly patients present worse QOL, especially in the relation to their physical activity.

CONCLUSION:

The study mainly focussed on comparing the safety and effectiveness of warfarin and acenocoumarol in AF patients. From our study, the mean TTR was found to be high among acenocoumarol treated group rather than warfarin group. Risk for stroke as well as its occurrence was found to be high in case of warfarin group.

Major ADR occurred was bleeding among both the groups and it was also high among warfarin treated group. Significant difference in improvement of QOL was observed with acenocoumarol treated group compared to warfarin, whereas medication adherence was found to be higher among warfarin treated group.

Overall, from our study we conclude that acenocoumarol is a better OAC as compared to warfarin in terms of safety and effectiveness.

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RESEARCH ARTICLE

Effectiveness of Oral Nano-particle based Vitamin D Solution in Pain Management: A Prospective Cross-sectional study

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ABSTRACT:
The aim of the present study was to evaluate the effectiveness of oral nano formulation of vitamin-D in pain management of some neurospinal and musculoskeletal disorders. The study was conducted for duration of two years on patients suffering from neurospinal disorders like generalised back pain, myofascial syndrome, osteopenia and osteoporosis. Numerical Rating scale was used to assess pain scores before and after treatment. The dose of vitamin-D formulation was 60,000IU weekly for six weeks. Non-parametric tests were employed for qualitative data and quantitative data (with non-Gaussian distribution) to find out statistically significant relation between the variables. Vitamin-D levels and pain scores within the group were compared using Wilcoxon test. p-value<0.05 was considered as statistically significant. p-value<0.01 was considered as statistically highly significant. There was a very highly statistically significant association between vitamin-D formulation and improvement in vitamin-D levels (p<0.0001) and pain scores (p<0.0001). Our study concludes that oral nano formulation of vitamin-D if given at 60,000 IU weekly for six weeks was effective in treating pain due to some musculoskeletal and neurospinal disorders.

KEYWORDS: Vitamin-D, pain, numerical rating scale, myofascial syndrome, osteopenia, osteoporosis.

INTRODUCTION:

Vitamin D is a hormone and essential nutrient for bone metabolism and neuromuscular function[1], stimulates osteoblast activity, increases intestinal absorption of calcium from diet, regulates phosphorus levels and it also potentially influences the pain pathways that are associated with changes in cortical, immunological, hormonal, and neuronal levels.[2] Without the presence of activated vitamin D, normal bone metabolism is altered so that only 10% of calcium and 60% of phosphorus is absorbed[3], this exacerbates osteoporosis and osteopenia and also might lead to osteomalacia. Vitamin D deficiency has been reported in populations with several different types of chronic musculoskeletal pain such as osteoarthritis, rheumatoid arthritis, osteoporosis, soft tissue rheumatism, low back pain, and arthralgia[4-7].

Vitamin D deficiency is associated with musculoskeletal pain, loss of type II muscle fibres, and proximal muscle atrophy.[8,9] Plotnik of and Quigley[2] found that 89% of subjects with chronic musculoskeletal pain were deficient in Vitamin D. Vitamin D insufficiency is highly prevalent in older osteoporotic patients, who are particularly vulnerable to the bone weakening due to vitamin D insufficiency.[10,11]

The majority of studies demonstrate the beneficial role of vitamin D as a therapeutic agent to maintain bone density in older patients with osteoporosis. Current guidelines recommend the prevention of osteoporosis in the elderly via pharmacological and non-pharmacological measures, including vitamin D and calcium supplementation in addition to osteoporosis treatment.[12,13,14]

The aim of the present study was to evaluate the effectiveness of oral nanosolution of vitamin D3 in pain management of some neuro spinal disorders. The objectives of the study were to evaluate the improvement in vitamin-D levels, to evaluate the improvement of pain score in patients post treatment with vitamin-D formulation.

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MATERIAL AND METHODS:

Study site and study design:

This was a panel study where in the pain scores were collected repeatedly at different times from same individuals. The study duration was two years i.e. from 2017 to 2019. The study was conducted at RK Hospitals, Gajuwaka, Visakhapatnam.

Ethical Considerations:

Permission was approved from Institutional Ethical Committee to conduct this study (RK/IEC/59/2017). The aim and objective of the study was clearly explained to the patients. A patient consent form was obtained from patients who are willing to co-operate the study.

Participants:

Inclusion criteria:

1. Patients who are diagnosed with myofacial syndrome, back pain, osteopenia and osteoporosis.
2. Patients with vitamin D deficiency.

Exclusion criteria:

1. Patients with normal vitamin D levels.
2. Patients diagnosed with other neuro spinal disorders.
3. Patients with insufficient findings.

Sampling technique and sample size:

Purposive and convenience sampling technique was employed to select the subjects. The estimated sample size was 377. But due to loss to follow up (86) and due to insufficient data (22), the sample size taken was 269.

Study Instrument:

Numeric Rating Scale (NRS) for Pain was an 11 item (0-10) pain intensity rating scale in which the respondent asked to select a numerical value between 0 and 10 that bests describe their intensity. '0' indicates "no pain" and '10' indicates "worst possible pain". Higher score indicates high pain intensity.

Data collection:

Patients satisfying inclusion criteria were selected. Patient's socio-demographic details and clinical history was noted down. The pain score was calculated from them initially using numeric rating scale for pain. Vitamin-D levels were noted down from lab reports. Again, patient's pain score was calculated after 6 weeks of follow up. The dose of oral nano solution of Vit-D3 was 60,000IU once a week on empty stomach. According to Vitamin-D council, the vitamin-D status was categorised as below:

Vitamin-D status	Blood Level
Deficient	0-30 ng/ml
Insufficient	31-39 ng/ml
Sufficient	40-80 ng/ml
Toxic	>150 ng/ml

Data analysis:

Frequency and percentages were calculated for qualitative data. Mean and standard deviation was calculated for quantitative data. Non-parametric tests were employed for qualitative data and quantitative data (with non-Gaussian distribution) to find out statistically significant relation between the variables. Vitamin-D levels and pain scores within the group were compared using Wilcoxon test. p-value <0.05 was considered as statistically significant. p-value <0.01 was considered as statistically highly significant.

RESULTS AND DISCUSSION:

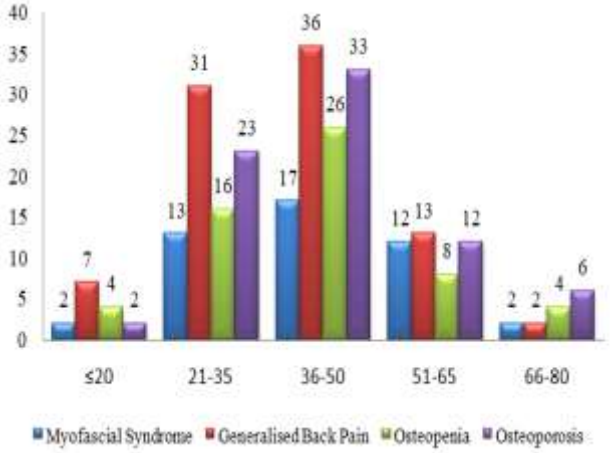

Figure 1: Age wise distribution of patients

Patients with age group between 36-50 years were high in generalised back pain (78.26%), osteopenia (44.82%), osteoporosis (43.42%) and myofacial syndrome (37%). The mean age of patients in myofacial syndrome, generalised back pain, osteopenia and osteoporosis was 41.45±13.92 years, 40.41±13.00 years, 41.34±13.88 years and 42.88±13.69 years respectively.

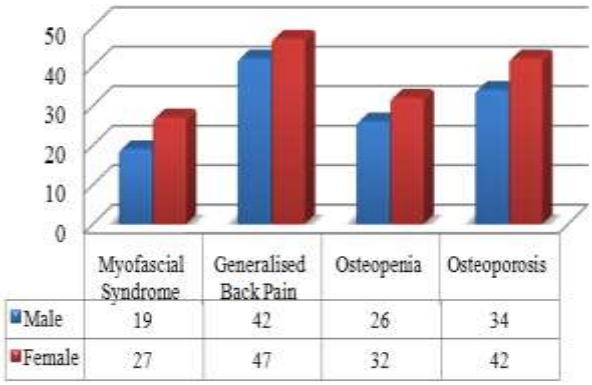

Figure 2: Gender wise distribution of patients

Females were predominantly higher than males in myofascial syndrome (58.69%), generalized back pain (52.80), osteopenia (55.17%) and osteoporosis (55.26%).

Table 1: Vitamin-D levels before and after treatment

S. No.	Name of the disease	Before (Mean± SD)	After (Mean± SD)
1	Myofascial syndrome	15.30±6.76	44.15±9.49
2	Generalised Back Pain	13.32±5.18	42.36±7.84
3	Osteopenia	21.74±7.82	46.26±8.34
4	Osteoporosis	18.54±6.04	43.76±7.83

Table 2: Pain scores before and after treatment

S. No	Name of the disease	Before (Mean±SD)	After (Mean±SD)
1	Myofascial syndrome	7.32±1.82	1.45±1.60
2	Generalised Back Pain	7.50±1.67	1.82±1.88
3	Osteopenia	7.44±1.85	1.91±1.81
4	Osteoporosis	7.10±1.86	1.73±1.68

The vitamin-D levels and pain scores had been considerably improved after treatment in all four neurospinal disorders as shown in Table 1 and Table 2.

Table 3: Comparison of Vitamin-D levels and Pain scores within the disorders

S. No	Name of disease	Vitamin-D levels		Pain Score	
		z-statistic	p-value	z-statistic	p-value
1	Generalized back pain	-8.19	**<0.0001**	8.15	**<0.0001**
2	Myofascial syndrome	-5.9	**<0.0001**	5.9	**<0.0001**
3	Osteopenia	-6.62	**<0.0001**	6.62	**<0.0001**
4	Osteoporosis	-7.57	**<0.0001**	7.57	**<0.0001**

In our study patients with age group 36-50 years were most commonly affected in the four groups. Patients with age group 41-50 years were most commonly affected in myofascial syndrome.[15] The age group showing the highest incidence was the 20-40 year age group, although children can also have MPD syndrome.[16] The average age of women suffering from osteopenia and osteoporosis was 59.2±10.5 years in a study.[17] Females were more commonly affected than males in our study.

There is increase in mean vitamin-D levels after six weeks in all the four groups. In India current recommendations for correction of vitamin D level, is by giving 60,000 IU of oral Vitamin D on a weekly basis for 8 weeks.[18,19] In a study by Manek each subject planned to receive 60,000 IU of nano particle based vitamin D, once weekly, for 8 weeks orally and it was observed that patients who have received vitamin D3 therapy for at least 4 weeks period, the improvement (serum 25 [OH] D >30 ng/ml) was seen in 84.2% (n=32) patients at the end of 4 weeks itself and 39.5% (n=15) patients at the end of 8 weeks.[20] A published literature suggests that vitamin D3 granules eight weeks of vitamin D3 60,000 IU/week oral granules supplementation increased serum 25 (OH) D to optimal levels in most of the subjects with Vitamin D deficiency.[21] It has been reported that approximately 50% of orally ingested vitamin D3 is absorbed.[22] In-vitro study done on rat intestine showed that average absorption of Nano particle-based vitamin D was 77.83±0.24%. The predicted human absorption may be more than 90%. This study also showed that nano particle-based vitamin D absorbed through various parts of rat small intestine like duodenum, jejunum, and ileum.[23]

There was very high statistically significant relationship between Vitamin –D levels and pain scores in all the four groups ($p<0.0001$). Few studies linked low levels of vitamin D and the incidence of both acute and chronic pain.[24-27] Few studies reported a link between Vitamin D deficiency and musculoskeletal symptoms like muscle weakness, pain, bone pain.[28-30] Clinical studies of vitamin D supplementation in patients with known vitamin D deficiency have shown mixed results in improving pain scores.[31] Low 25(OH) D levels have been found to be related to heightened central sensitivity (particularly augmented pain processing) upon mechanical stimulation in chronic pain patients.[32] Research suggests that nanoformulation will help improve the bioavailability of fat-soluble nutrients.[23] Few research studies suggested that nanoformulation of vitamin-D may enhance therapeutic efficacy, photo-stability and bio-degradation of vitamin D supplements. [23,33,34]

The available literature on randomized double-blind placebo-controlled trails of vitamin D supplementation in treating chronic pain conditions was limited. Mixed results were reported on the improvement of pain with vitamin-D supplementation. Infra red therapy and a session of soft tissue massage was found to be effective in the improvement of chronic low back pain.[41] Aceclofenac in combination with thiocolchicoside was effective in reducing the acute low back pain than aceclofenac monotherapy.[42]

Table 4: Literature review of some studies on Vitamin D and pain outcome

Author	Condition	Dose of Drug	Pain Outcome	Improvement with vitamin D
Warner and Arnspiger[35]	Diffuse Musculoskeletal Pain	50,000 ergocalciferol per week for 3 months	There was no correlation between vitamin D level and pain on VAS (r 0.038) or FPS (r 0.298) at 3-month follow-up	No
Di Munno et al[36]	Polymyalgia Rheumatica	35,000 IU for 25 days	Subjective pain on movement significantly decreased in both groups over time, but there was no difference between groups	No
Wepner et al[37]	Severe pain from Fibromyalgia syndrome	2400 IU or 1200 IU per day	A marked reduction in pain was noted over the treatment period in the treatment group. Variance analysis of the 2 groups at 4 time points showed a significant group effect in visual analog scale scores. This also was correlated with scores on the physical role functioning scale of the Short Form-36.	Yes
McAlindon et al [38]	Symptomatic knee OA pain	2000 IU daily with dose escalation to elevate serum levels to more than 36ng/ml	Vitamin D supplementation for 2 years at a dose sufficient to elevate 25(OH)D serum levels to more than 36ng/ml when compared to placebo, did not reduce knee pain or cartilage volume loss in patients with symptomatic Osteo Arthritis	No
Sakalli et al[39]	Pain in the elderly	300,000IU via p.o and parenteral	Megadose vitamin D administration increases quality of life, decreases pain, and improves functional mobility when given via oral or intramuscular routes in the elderly	Yes
Schreuder et al[40]	Nonspecific musculoskeletal pain	150,000IU vitamin D_3p.o for 6 weeks	Patients in vitamin D group were significantly more likely than their counterparts in the placebo group to report pain relief 6 weeks after treatment (34.9 vs. 19.5%, P= 0.04). The former was also more likely to report an improved ability to walk stairs (21.0 vs. 8.4%, P= 0.008).	Yes

CONCLUSION:

Our study concluded that oral nano formulation of vitamin-D with 60,000 IU weekly for six weeks was effective in treating pain in some musculoskeletal diseases.

CONFLICTS OF INTEREST:

The authors declare no conflict of interest.

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RESEARCH ARTICLE

LC-MS/MS and NMR Characterization of impurities in Epalrestat

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ABSTRACT:
For the identification and characterization of main impurities in Epalrestat, a responsive and rapid analysis of LC-MS/MS and NMR was created. Epalrestat is used in diabetic neuropathy therapy, which in patients with diabetes mellitus is one of the most severe long-term complications. Epalrestat is a derivative of carboxylic acid and a non-competitive and reversible inhibitor of the reductase of the aldose. It reduces intercellular sorbitol deposition that is thought to be causing diabetic neuropathy, retinopathy, and nephropathy. Aldose reductase is the key enzyme in the polyol pathway, the enhanced activity of which is the basis for diabetic neuropathy this enzyme is targeted by the aldose reductase inhibitors (ARI). Epalrestat is the only commercially available API. It is easily absorbed in the neutral tissues and has minimal side effects inhibiting the enzyme. At 5.1 RT in Epalrestat, one of the primary impurities was observed in the analytical HPLC process. Preparative HPLC technique was used to further characterize the impurity. To predict the structure, isolated impurity was subjected to amass and NMR analysis of LC-MS/MS was performed using a reverse-phase column of C18 and with a mobile step consisting of methanol and ammonium type in a ratio of 70:30 to pH 4.6. The injection volume is 10 µL, and the binary gradient system was used to isolate. The flux rate was maintained at 1.0 ml/min. The temperature of the column was held at 45ºC. For this study, the MS conditions were adopted with a scan range of m/z= 50 to 500 with a dwell time of 3 seconds.

KEYWORDS: LC-MS/MS, NMR, HPLC, Epalrestat, Diabetic.

INTRODUCTION:
Epalrestat is a compound 2-[(5Z)-5-[(E)-2-methyl-3-phenylprop-2-enylidene]-4-oxo-2-sulfanylidene-1, acetic acid 3-thiazolidin-3-yl]. 1] It is a derivative of carboxylic acid and is a non-competitive and reversible aldose reductase inhibitor. It is used in diabetic neuropathy care which is one of the most common long-term complications in diabetes mellitus patients. 2] This decreases the deposition of intercellular sorbitol which is thought to be causing diabetic neuropathy, retinopathy, and nephropathy. Aldose reductase is the main enzyme in the polyol pathway, the enhanced activity of which is the basis for diabetic neuropathy 8, 10] Aldose reductase inhibitors (ARI) target this enzyme. [3] Epalrestat is the only ARI commercially available. It is easily absorbed into the neutral tissues and inhibits the enzyme with minimum side effects. [4] (Figure. 1).

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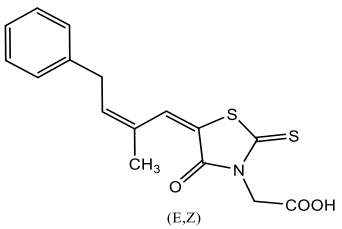


Figure. 1: Structure of Epalrestat

EXPERIMENTAL:
Chemicals and Reagents:
Epalrestat (99% pure) samples provide by TCI Chemicals Pvt. Ltd. HPLC grade Acetonitrile was procured from Merck. Analytical reagent grade Ammonium acetate was purchased from Loba Chemie.

Instrumentation:
Shimadzu LC-20AD with PDA detector were used for chromatographic separations. LC solution software was used for data analysis. Shimadzu electronic weighing balance was used for weighing samples and Mark ultra sonicator was used for sonication of mobile phases and samples.

LC-MS/MS was performed by using Shimadzu LC-MS/MS 8030 system with triple Quadra pole mass spectrometry. NMR was performed on FT NMR spectrometer system – 400MHz (Make: Agilent USA model: 400 MRDD2).

RESULTS AND DISCUSSION:

LC-MS/MS study of Epalrestat key impurity:

The LC-MS spectrum of Epalrestat key impurity (Figure. 2) showed peak at RT 5.1 minutes displayed the protonated molecular ion (M^++H) at m/z = 319, this is corresponding to the molecular formula $C_{15}H_{13}NO_3S_2$. And there Fragmentation pathway. [5-9]
(Figure. 2, 3 and 4).

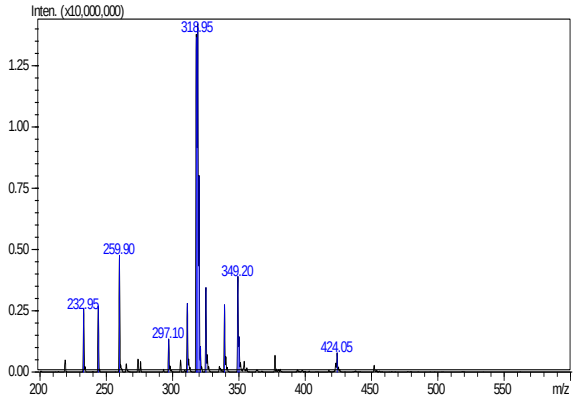


Figure. 2: Epalrestat Pure Drug MS/MS spectra

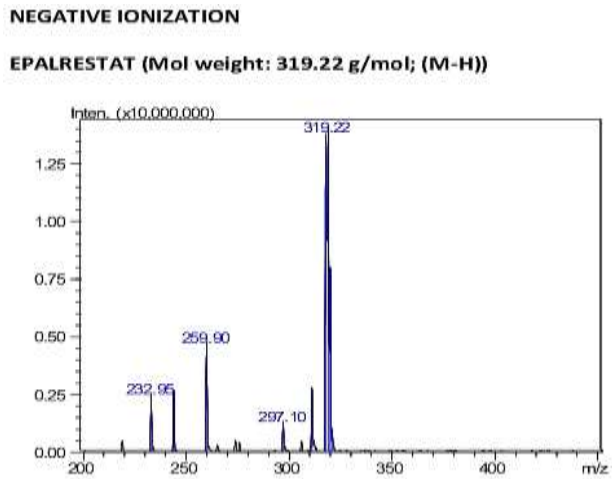


Figure. 3: Epalrestat impurity MS/MS spectra

Table 1: m/z Values of Ionization in LC-MS/MS

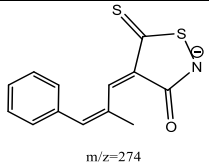
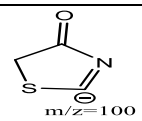
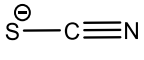
Peak	m/z values
M^+ peak	319
 m/z=274	274
 m/z=100	100
 m/z=58	58

Fig 3: Fragmentation Pathway

NMR Study of Epalrestat key impurity:

The NMR data ($_1H$, $_{13}C$) of Epalrestat impurity was noted using $CDCl_3$ as solvent at 400MHz for H_1 and 100MHz for $_{13}C$ on Agilent FT-NMR 400MHz spectrometer.

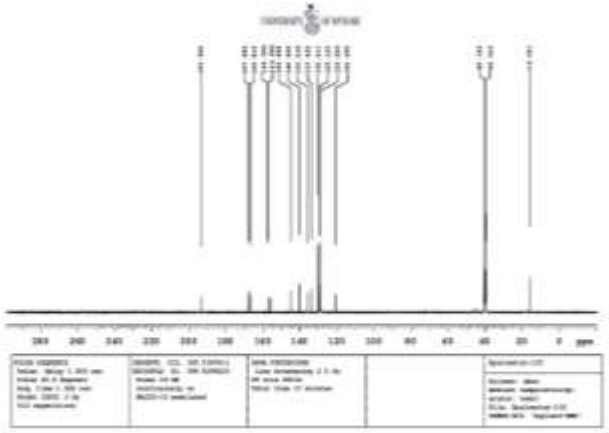


Fig 4: ^{13}C NMR of Epalrestat

The ^{13}C NMR spectrum (Fig 4) of Epalrestat impurity has showed R-CH_2-R peaks at 16.1ppm and 40.1ppm. The peak at 193.6 is due to R-CH=O and the peak at 40.144ppm is due to C-N. The additional peaks from 100 to 150ppm are due to C=C.

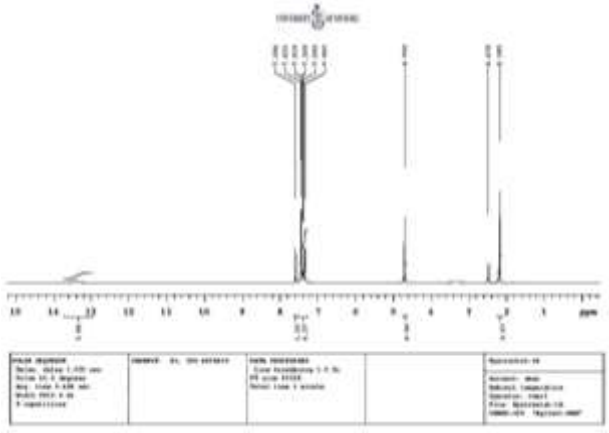


Fig 5:^1H NMR of Epalrestat

The ^1H NMR spectrum (Fig 5) of Epalrestat impurity has showed aromatic peaks in between 6.5 and 7.5ppm. Peaks from 4.5 to 6.5 are due to C=C bonds and the peak at 2.2ppm and 2.4ppm are due to R-OOR

Negative Ionization
Epalrestat Impurity (Mol weight: 319.22g/mol; (M+H))

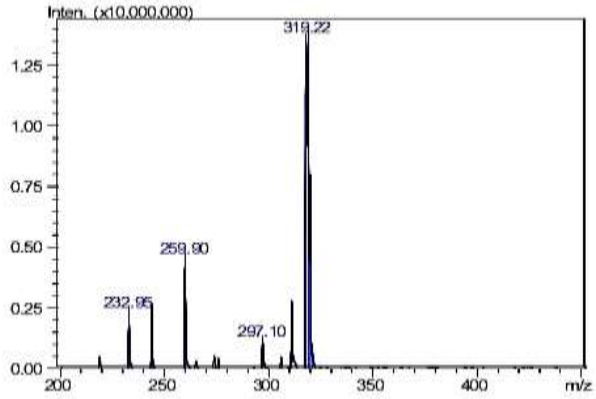


Fig 6: Epalrestat impurity MS/MS Spectra

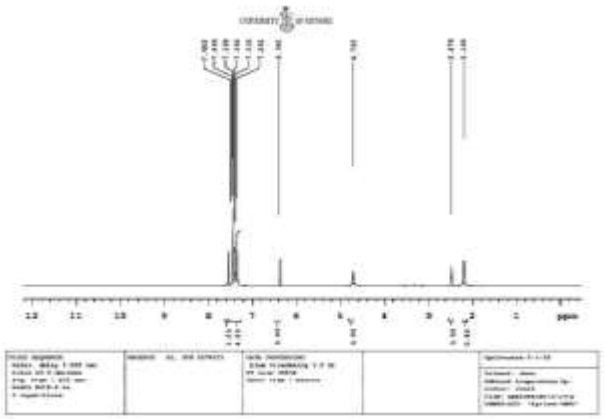


Fig 7: ^{1}H NMR Spectrum of Epalrestat impurity in $CDCl_3$.

The ^{1}H NMR spectrum (fig. 7) of Epalrestat impurity has showed aromatic peaks in between 6.5 and 7.5ppm. Peaks from 4.5 to 6.5 are due to C=C bonds and the peak at 2.2ppm and 2.4ppm are due to R-OOR.

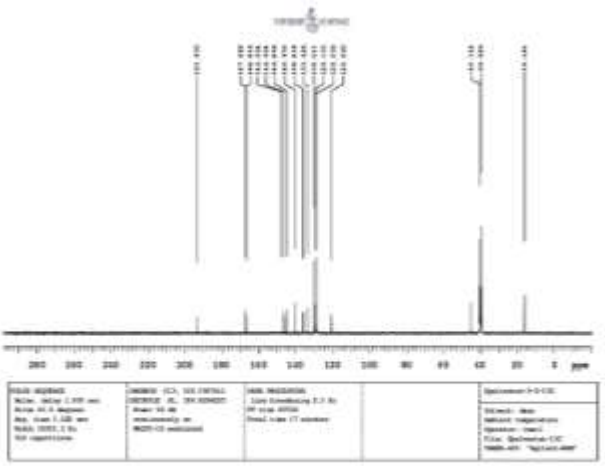
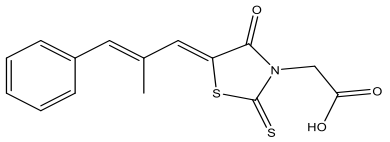


Fig 8: ^{13}C NMR Spectrum of Epalrestat Impurity in $CDCL_3$.



2-((*Z*)-5-((*E*)-2-methyl-3-phenylallylidene)-4-oxo-2-thioxothiazolidin-3-yl)acetic acid
Chemical Formula: $C_{15}H_{13}NO_3S_2$
Exact Mass: 319.03
Molecular Weight: 319.39
m/z: 319.03 (100.0%), 320.04 (16.2%), 321.03 (9.0%), 322.03 (1.5%), 321.04 (1.2%)
Elemental Analysis: C, 56.41; H, 4.10; N, 4.39; O, 15.03; S, 20.08

Fig 9: Structure for Epalrestat Impurity

The ^{13}C NMR spectrum (Fig. 8) of Epalrestat impurity has showed R-CH_2-R peaks at 16.1ppm and 40.1ppm. The peak at 193.6 is due to R-CH=O and the peak at 40.144ppm is due to C-N. The additional peaks from 100 to 150ppm are due to C=C.

CONCLUSION:

A rapid and sensitive LC-MS/MS characterization method for predicting the structure of key impurity was developed. The predicted structure was based on mass and NMR data.

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CONFLICTS OF INTEREST:

The authors affirm no conflicts of interest.

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RESEARCH ARTICLE

UV Spectrophotometric Estimation of Pioglitazone using Multivariate method in Bulk drug and Pharmaceutical Dosage Form

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ABSTRACT:
Multivariate calibration technique makes the use for determination of Pioglitazone in bulk drug and pharmaceutical dosage form. This technique is constructed to manipulate statistical calculations, in correlating the relation between concentrations against absorbance at five separate wavelengths. λmax of the Pioglitazone was found to be 218nm. The results were statistically performed and offer ideal outcome by removing variations emerging from the instrumental or experimental conditions. The multivariate technique was approved according to ICH guidelines as well as originated to be sensitive, linear, accurate, precise, and consistent. The graph have been constructed to be straight-line over a concentration ranging from 8–16µg/mL with acceptance value of correlation coefficient (*r*2) of about 0.9969. Equation of linear regression has shown Y= 0.0486x + 0.1856 and both LOQ as well as LOD was originated to be 1.2765 and 3. 86831µg/mL individually. While %RSD for precision intraday as well as interday have been in range of 0.7343–0.9543 and 0.837–0.901 respectively. The percentage recovery was found within the range of 92.87–99.78% w/w. The results shows that the multivariate technique is simple, linear, precise, accurate, sensitivity and reproducible for the estimation of Pioglitazone in pharmaceutical dosage form.

KEYWORDS: ICH guidelines, Insulin-sensitizing, Multivariate calibration, Pioglitazone, Pharmaceutical dosage form, UV spectrophotometry.

INTRODUCTION:
Pioglitazone is a member of the class of thiazolidenediones that is 1,3-thiazolidine-2,4-dione substituted by a benzyl group at position 5 which in turn is substituted by a 2-(5-ethylpyridin-2-yl) ethoxy group at position 4 of the phenyl ring. It shows hypoglycemic activity. It has a role as an insulin-sensitizing drug, an EC 2.7.1.33 (pantothenate kinase) inhibitor and a xenobiotic. It is a member of thiazolidenediones, an aromatic ether and a member of pyridines[1].

Pioglitazone triggers the nuclear peroxisome proliferator activated receptor-γ (PPAR-γ), which assist to the increased transcription of various proteins regulating glucose and lipid metabolism[2-3]. These proteins escalates the post-receptor actions of insulin in the liver and peripheral tissues, which assist to improved glycaemic control with no increase in the endogenous secretion of insulin[4-5].

The drug has been well tolerated by adult patients of all ages in clinical studies. Oedema has been reported with mono therapy, and pooled data have shown hypoglycaemia in 2 to 15% of patients after the addition of pioglitazone to sulphonylurea or insulin treatment. There have been no reports of hepatotoxicity[6].

Pioglitazone is an orally administered insulin sensitizing thiazolidinedione agent that has been developed for the treatment of type 2 diabetes mellitus[7].

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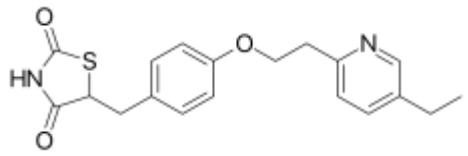

Figure 1 Pioglitazone chemical structure

In multivariate technique it shows the change of solitary species analysis out-of one reliant changeable to a self-reliant changeable, since the wavelengths are concurrently comprise in the calibration model. Therefore estimation of species is feasible in the analytical system[8].

In optimization state, the mathematical technique applied gives a significant resolution, sensitivity, rapidity and minimum cost for the quality analysis for concerning of mixtures. Statistical perspective are elaborated below.

Assuming that analyte (X) absorbance was calculated at five wavelengths stand (λ = 214, 216, 218, 220 and 222nm), subsequent equations are noted as follows to all wavelengths.

$A\lambda 214 = f \times C_x + k1$……..(1)
$A\lambda 216 = g \times C_x + k2$……(2)
$A\lambda 218 = h \times C_x + k3$……(3)
$A\lambda 220 = i \times C_x + k4$……..(4)
$A\lambda 222 = j \times C_x + k5$……..(5)

Where, Aλ appear for climax zone of the analyte; f, g, h, i and j are slopes functions of linear regression of the analyte; k1, k2, k3, k4 and k5 are intercepts functions of linear regression at five chosen wavelengths and C_x appears for concentration of analyte. Equations shown above 1 to 5 can be noted as follows:

$AT = f \times C_x + g \times C_x + h \times C_x + i \times C_x + j \times C_x + KT$ which can be simplified as……(6)

$AT = C_x (f + g + h + i + j) + KT$……...(7)

Where, AT and KT appears for total climax zone gained and total of intercepts of regression equations at five-wavelengths stand individually. The concentration of analyte (X) in the solution of unspecified concentration can be measured using equation below:

$$C_x = \frac{(AT - KT)}{(f+g+h+i+j)} \quad \text{……(8)}$$

In case such as multivariate technique it contains the application of regression equations which is based on two variables i.e absorbance against concentration where it's restored for the forecast of an unspecified concentration of analyte against the absorbance[9].

So far in the literature for the estimation of Pioglitazone in biological samples and pharmaceutical formulations techniques like HPLC[9-13], HPTLC[14-19], RP-HPLC[20-29] UV[30-45] spectroscopy, potentiometric[46-49] and LC-MS/MS[50] was useful for analysis, from which HPLC method have been mostly used. Multivariate method using UV spectrophotometer have been useful for determination of Pioglitazone in pharmaceutical dosage form. The main objective of this method was to evolve fast, simple, sensitive and reliable, multivariate technique. This method is used for quality analysis of Pioglitazone in pharmaceutical dosage form.

MATERIAL AND METHODS:

Chemicals and reagents

1. Sodium Hydroxide 0.1M (NaOH):

Solubility:

1. Sodium Hydroxide 0.1M: Freely Soluble
2. Water: practically insoluble

Instrumentation:

1. Perkin-Elmer UV-Visible spectrophotometer
2. Electrical balance
3. Sonicator

METHOD DEVELOPMENTS:

Selection of solvent:

The suitable solvent of Pioglitazone was found to be 0.1M NaOH. Therefore solvent 0.1M NaOH was chosen to solubilize the sample.

Standard solution preparation:

Standard solution has been formulated in which 100mg of Pioglitazone drug powder is dissolved into 100ml of 0.1MNaOH to get a concentration of 1mg/1mL.

Sample solution preparation:

Ten tablets of Pioglitazone was grinded using pastle with motar and weight equivalent to 100mg of the drug was transferred into 100ml of 0.1M NaOH to get concentration of (1mg/1mL). The solution was further processed for working concentration ranging from 8-16 µg/mL and scanned in UV spectrophotometry, where chart with absorbance against concentration was plotted to get linearity chart.

Estimation of λ_{max}: Standard solution of Pioglitazone was dissolved into 0.1M (NaOH) to make concentration of (10µg/mL) which have been examined in the region from 200-400nm using UV.

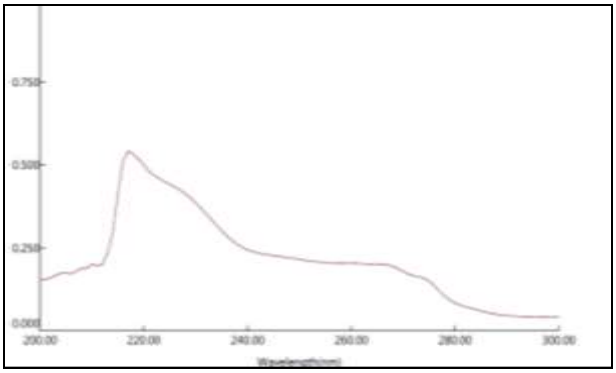

Figure. 2 UV Spectrum of Pioglitazone at 218 nm.

METHOD VALIDATION:

The technique was approved as per ICH Q2B guidelines for linearity, precision, accuracy and reproducible.

Linearity:

Standard solution of Pioglitazone was dissolved into 0.1M NaOH to make concentration in the range of 8-16µg/mL. Where absorbance was recorded over a range surrounding 218nm i.e., 214, 216, 218, 220 and 222nm, in orderly to enhance good correlation and reduce instrumental variations. A graph was constructed showing the absorbance of five separate concentration was noted at five separate wavelengths and curves were shown below.

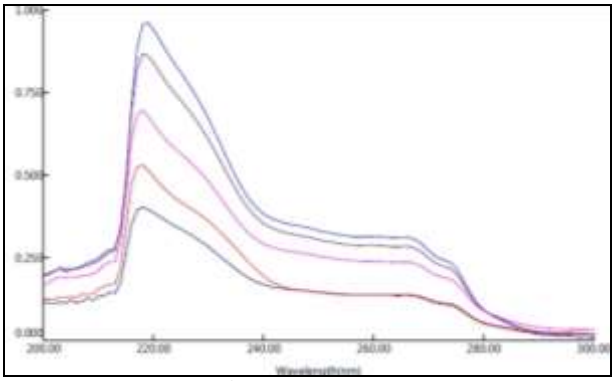

Figure 3 UV Spectrum of Pioglitazone showing linearity at 218 nm.

The responsiveness of multivariate calibration was estimated in calculation of LOQ and LOD using the following formula.

LOQ = 10 S/σ and
LOD = 3.3 S/σ
Where, S represent concentration of standard deviation while
σ appears to be standard curve of the slope.

Precision:

10ml of standard solution of drug was arranged and dissolved into a conical flask containing 100ml of 0.1M NaOH as solvent. To these mixture, 1.2mL was taken using pipette and transferred into 10mL standard flasks, the volume is filled up with sodium hydroxide to make concentrations of 12µg/mL. The aliquots were scanned six times a day for intraday precision and six days at same time for interday precision at five wavelengths using UV spectroscopy.

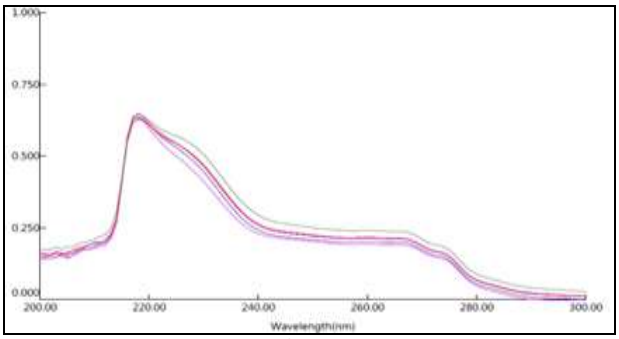

Figure 4: UV Spectrum of Pioglitazone showing intra-day precision

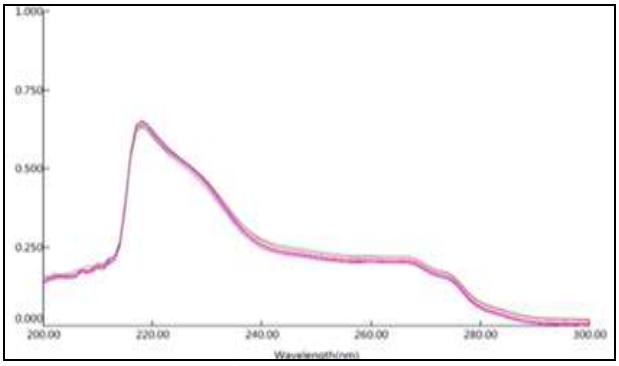

Figure 5: UV Spectrum of Pioglitazone showing intraday precision

Accuracy:

The accuracy of multivariate technique have being estimated using standard addition method. An already arranged stock solutions of standard and sample, 10mL of the solutions was taken using pipette and dissolved into 100mL standard flask with solvent to make a concentration of 100µg/mL. 1mL of the standard solution were pipetted into three 10mL standard flasks and 0.8, 1.2, 1.6mL of the sample solution was poured to the above standard flasks and mixed. Standard flasks was filled with solvent. The aliquots were scanned using UV spectroscopy.

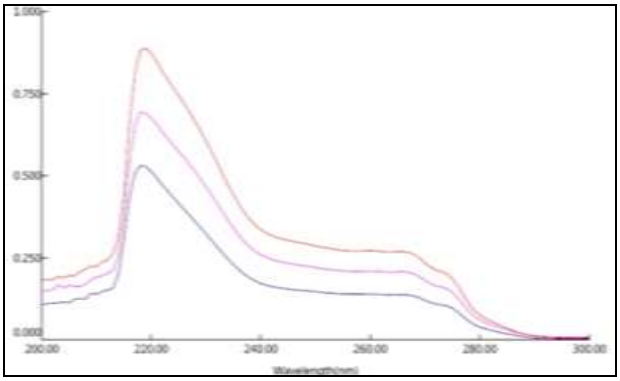

Figure 6: UV spectrum of pioglitazone showing accuracy

RESULTS AND DISCUSSION:

Pioglitazone λmax was found to be at 218nm with solvent 0.1M NaOH. The curves were found to be linear for the concentration ranging from 8-16µg/mL. Therefore the analytical data of linear regression for the curves shows great linear relations with R^2 = 0.9969. Equation of linear regression has shown Y = 0.0486x + 0.1856 while LOD as well as LOQ was originated in the range of 1.2765-3.8683 µg/mL and 2.490-3.045µg/mL. Finally the %RSD for the precisions was found to be 0.734-0.954, at all the five wavelengths, where according to ICH guidelines it is within the acceptance limits.

Linearity:

Five separate concentrations 8-16 µg/mL was measured at five different wavelengths, 214, 216, 218, 220 and 222nm, for linearity studies. The table was tabulated and shown below. Figure 7-16 shows calibration graphs and residual plots respectively.

TABLE I: UV calibration of five different wavelengths:

Conc. In (µg/ml)	(nm)				
	214	216	218	220	222
8	0.336	0.398	0.434	0.587	0.539
10	0.515	0.609	0.684	0.735	0.814
12	0.564	0.684	0.768	0.867	0.958
14	0.496	0.626	0.742	0.867	0.972
16	0.439	0.612	0.790	0.868	0.986

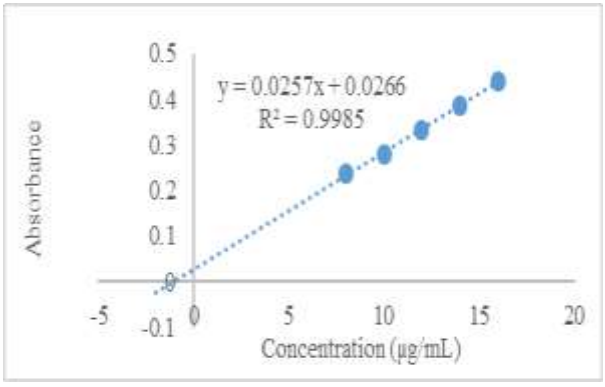

Figure 7 Calibration graph at 214 nm.

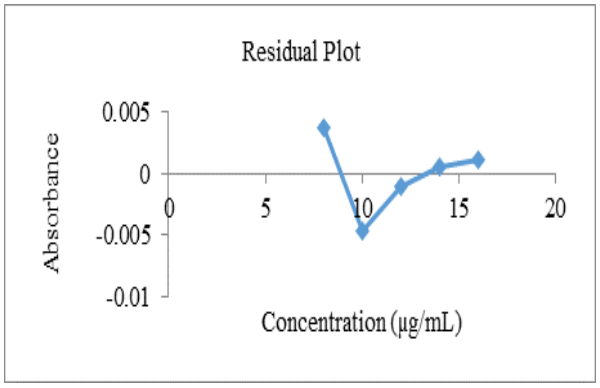

Figure 8 Residual plot at 214 nm

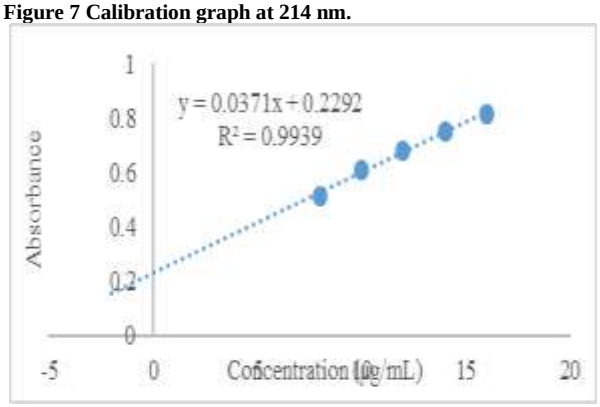

Figure 9 Calibration graph at 216 nm

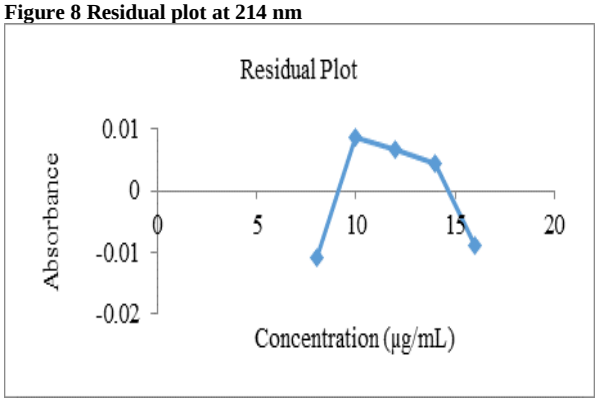

Figure 10 Residual plot at 216 nm

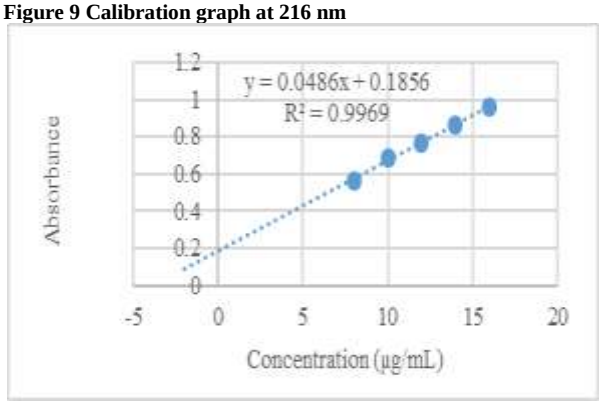

Figure 11 Calibration graph at 2148nm

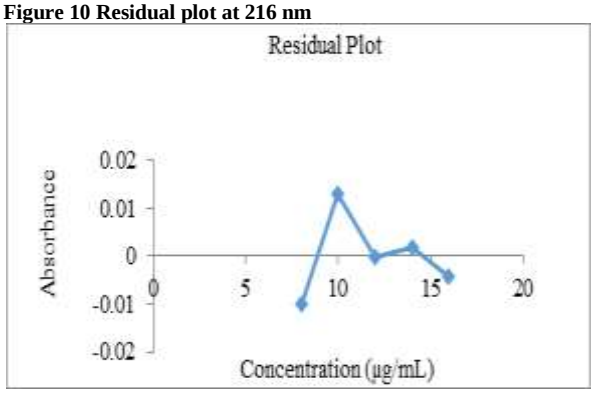

Figure 12 Residual plot at 218 nm

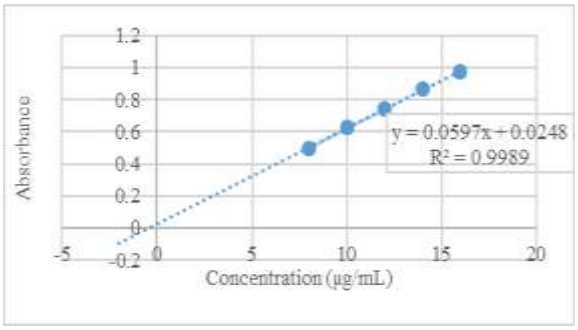

Figure 13 Calibration graph at 220 nm

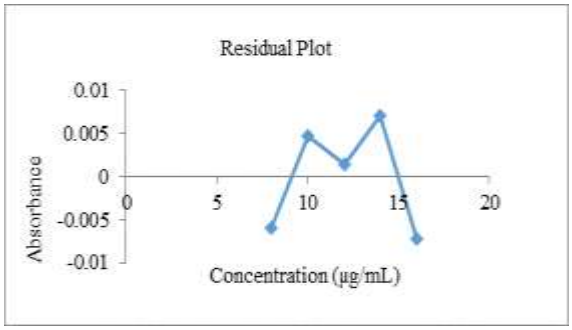

Figure 14 Residual plot at 220 nm

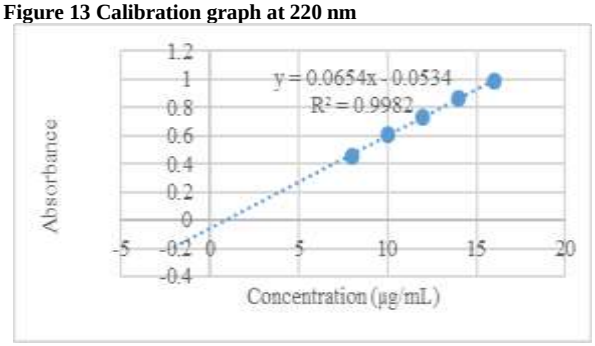

Figure 15 Calibration graph at 222 nm

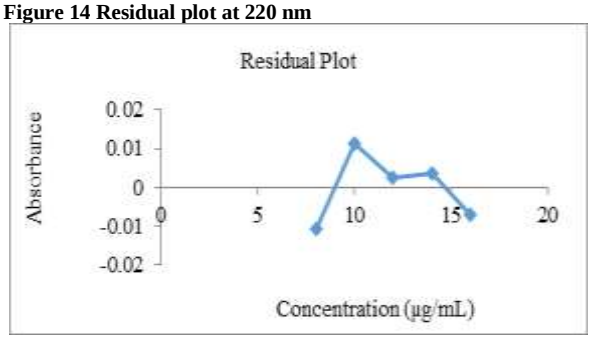

Figure 16 Residual plot at 222 nm

Minimum value of standard deviation for five wavelengths shows the technique have being accurate, moreover calculations of LOD and LOQ was shown in table II below.

Table II: Showing linearity of LOD and LOQ for all wavelengths.

(Abs)	Equation of regression	R^2	Standard deviation	LOD (µg/mL)	LOQ (µg/mL)	% RSD
214	Y = 0.0257x + 0.0266	0.9985	0.0128	1.6436	4.9805	2.55
216	Y = 0.0371x + 0.2292	0.9939	0.0226	2.0102	6.0916	4.52
218	Y = 0.0486x + 0.1856	0.9969	0.0188	1.2765	3.8683	3.77
220	Y = 0.0597x + 0.0248	0.9982	0.0208	1.1497	3.4841	4.16
222	Y = 0.0654x + 0.0248	0.9989	0.0231	1.6559	3.5321	4.63

Precision:

The low standard deviation value for all wavelengths shows that the method was precise. The % RSD value for the intraday and interday precision was in the range of 0.734-0.954, therefore it is within the acceptance criteria of less than 3% for all wavelengths. Minimum value of the %RSD shows that the technique is precise.

Table III: Showing Intra-day precision:

Day	*(nm)*				
	214	**216**	**218**	**220**	**222**
1	0.557	0.561	0.617	0.608	0.572
2	0.565	0.562	0.625	0.597	0.571
3	0.551	0.563	0.619	0.603	0.583
4	0.559	0.553	0.612	0.607	0.574
5	0.552	0.559	0.615	0.607	0.576
6	0.553	0.567	0.619	0.613	0.576
SD	0.0053	0.0047	0.0044	0.0054	0.0042
%RSD	0.9543	0.8319	0.7123	0.8883	0.7343

Table IV: Showing Inter-day precision:

Days	**(nm)**				
	2% RSD 14	**216**	**218**	**220**	**222**
1	0.558	0.543	0.643	0.603	0.583
2	0.554	0.533	0.632	0.601	0.582
3	0.547	0.544	0.632	0.612	0.576
4	0.555	0.538	0.637	0.605	0.586
5	0.561	0.545	0.631	0.607	0.574
6	0.551	0.548	0.635	0.614	0.585
SD	0.0079	0.0077	0.0083	0.0084	0.0081
%RSD	0.9014	0.7989	0.7113	0.8410	0.8374

Table V: Showing study recovery:

(Abs)	Quantity available in (µg/ml)	Quantity added (µg/ml)	Absorbance	Quantity recovered (µg/ml)	% Recovery
214	8	0	0.329	7.671	95.89
		4	0.561	11.973	99.78
		8	0.939	15.761	98.51
216	8	0	0.386	7.614	95.18
		4	0.609	11.144	92.87
		8	0.984	15.641	97.76
218	8	0	0.434	7.565	94.56
		4	0.684	11.535	96.13
		8	0.968	15.577	97.36
220	8	0	0.487	7.548	94.35
		4	0.735	11.532	96.10
		8	0.967	15.547	97.17
222	8	0	0.439	7.571	94.35
		4	0.714	11.533	96.11
		8	0.958	15.581	97.38

Recovery:

Recovery percentage of the drug from synthetic compound have been in the range of 92.87 – 99.78 %w/w, it is within the acceptance limit 94-104%w/w according to ICH guidelines.

CONCLUSION:

The proposed UV spectrophotometric using multivariate technique was approved by assessing diverse approved parameters. Moreover the developed method does not require any complicated solvents and it is simple, sensitive, precise, accurate and reproducible for determination of Pioglitazone in pharmaceutical dosage form. The multivariate technique can be strongly suggested for quality analysis of Pioglitazone in pharmaceutical formulation.

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CONFLICTS OF INTEREST:

There is NO conflict of interest on the study reported by authors.

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RESEARCH ARTICLE

Ameliorated Solubility and Dissolution of Flurbiprofen using Solubilizer Sepitrap 80 and Sepitrap 4000

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ABSTRACT:
The capability of novel solubilizer sepitrap 80 and sepitrap 4000 to enhance the solubility, dissolution was assessed in current investigation through the formation of simple physical mixture with a poorly aqueous soluble drug Flurbiprofen, phenylalkanoic acid derivative from non-steroidal anti-inflammatory drugs (NSAIDs).The physical mixtures were prepared in 1:1, 1:2 and 1:3 proportions with sepitrap 80 and sepitrap 4000 and characterized for saturation solubility, dissolution, and stability studies. The physicochemical properties of physical mixtures with solubilizer were confirmed by DSC, PXRD, and SEM. Saturation solubility was carried in order to determine solubility of drug in distilled water. The physical mixtures exhibited solubility of 261% and 369.87%with sepitrap 80 and sepitrap 4000. The dissolution rate and solubility were undoubtedly improved by physical mixtures as compared to model drug alone. Physical mixtures incorporated with sepitrap 4000 at 1:2 ratio proved better thansepitrap 80. Hence, the sepitrap could be exploited as a solubilizer to improve the solubility of Flurbiprofen.

KEYWORDS: Poor water soluble, Flurbiprofen, Solubilizer, Sepitrap80, Sepitrap 4000.

INTRODUCTION:
Flurbiprofen [2-(3-fluoro-4-biphenyl) propionic acid], is a phenylalkanoic acid derivative and non-steroidal anti-inflammatory agent, with analgesic and antipyretic activity and has chiral center. It is one of the most potent inhibitors of platelet aggregation, generally prescribed for rheumatoid arthritis, osteoarthritis, gout, musculoskeletal disorders, rheumatic diseases, post-operative pain, sunburn, and dysmenorrhoea.[1-3] The model drug exerts its effect by reversible inhibition of cyclooxygenase (COX), the enzyme that bring out conversion of arachidonic acid to prostaglandin G2 (PGG2) and PGG2to prostaglandin H2 (PGH2) this results in adequately declining the concentration of prostaglandins involved in inflammation, pain, swelling and fever. Flurbiprofen, most potent NSAIA in terms of prostaglandin inhibitory activity is a non-selective COX inhibitor and inhibits the activity of both COX-1 and COX-2.

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FLB belongs to BCS Class II and exhibit low solubility and low dissolution rates and suffers from poor oral bioavailability constraints due to its poor water solubility of 5-10µg/mL.[4-6] Generally drugs with low aqueous solubility (usually lower than 100µg/ml) show dissolution-limited, incomplete absorption from the gastrointestinal tract of animals and humans and are hydrophobic.[7] Poor aqueous solubility is prime limiting factor with many new drugs in their successful set up in market despite of their full potential pharmacokinetic activity. Molecules that would have highly constructive effect on their physiological target would not be further developed if their bioavailability is limited by their aqueous solubility.

Thus solubility is a most valued contributor in the formulation of finished pharmaceuticals. These molecules need enhancement in low solubility, dissolution rate and bioavailability which is featured to drug's success. Among all solubility enhancement technique use of solubilizer is a valuable approach for enhancing the solubility, dissolution rate and bioavailability of BCS Class II drugs due to simplicity of process.[8-9]

Literature survey put forward various approaches such as use of hydrophilic carriers[10-11], liquisolid compact technique[12-13], supercritical carbon dioxide[14], use of surfactant[15-17] microemulsions[18], complexation with cyclodextrins[18-20], solid dispersion[21-28], adsorption of nifedipine on porous calcium silicate[29] and the use of conventional solubilizer such as polysorbates, PEG 400to elevate the solubility of Flurbiprofen. But there is no focus on novel solubilizer such as sepitrap, soluplus, povacoat, dendrimers for FLB.

Thus considering the supremacy of adding novel solubilizer such as sepitrap to drug the present investigation was carried out to contribute the promotion of the solubility of poorly water-soluble drug, FLB via solubilization using sepitrap 80 and sepitrap 4000 as an auxiliary substance, which to our knowledge has not been reported yet.

MATERIALS AND METHODS:

FLB was obtained as a gift sample from FDC Limited, Ahmadabad, India. Sepitrap 4000, Sepitrap 80 were gifted by Seppic, Mumbai. All other chemicals and solvents used were of pharmaceutical and analytical grade.

Determination of maximum wavelength of FLB:

Flurbiprofen, 10mg was dissolved in 10ml of methanol and then the volume was made up to 100ml with distilled water. The concentration of the resulting solution was 100µg/ml and this stock solution was diluted with distilled water to get 2µg/ml. The absorbance of the resulting solution was measured using UV-Visible spectrophotometer in the wavelength range of 200-400 nmby a method previously developed and validated.[30-31]

Preparation of FLB and sepitrap physical mixture:

The 100mg of FLB weighed accurately and physical mixtures of FLB with sepitrap 80 and sepitrap 4000 was prepared by simple spatulation method in the various proportions like 1:1,1:2 and 1:3[32-33] The various proportion used for physical mixtures is given in table no.1

Table No 1: Composition of various physical mixtures of FLB with sepitrap 80 and sepitrap 4000

Batch No	Drug (mg)	SEPITRAP 80 (mg)	SEPITRAP 4000 (mg)
PM1	100	100 (1:1 ratio)	---
PM2	100	200 (1:2 ratio)	---
PM3	100	300 (1:3 ratio)	----
PM4	100	---	100 (1:1 ratio)
PM5	100	---	200 (1:2 ratio)
PM6	100	---	300 (1:3 ratio)

Drug content determination:

Physical mixtures of Flurbiprofen and sepitrap 80 and sepitrap 4000 were transferred to 100ml volumetric flask. Add 50ml methanol and shake the flask thoroughly. Make the volume with 100ml with methanol. Dilute the solution if required and estimate the drug content spectrophoto metrically at 248nm. Drug content was calculated from the standard curve of Flurbiprofen.

Saturation solubility studies:

A saturation solubility study was performed to determine increase in the solubility of pure drug compared with the physical mixtures of FLB with Sepitrap 80 and sepitrap 4000. Excess amount of the drug and physical mixtures of were separately introduced into the 250mL stoppered conical flasks containing 25mL of double distilled water. Then flasks were covered with cellophane membrane to avoid loss of solvent and kept in rotary shaker for 48 h at 37±0.5^{0}C. Aliquots were then withdrawn and filter through What man filter paper. The concentration of FLB was determined by using UV visible spectrophotometer at 248nm (Shimadzu UV spectrophotometer 1800) after appropriate dilution. To calculate the solubility of FLB three determinations were carried out for each physical mixture. The solubility of FLB and percent increase in solubility by the solubilizer were determined.[30]

Fourier transform infrared spectrophotometer studies:

FT-IR has been employed as a useful means to identify drug excipient interaction. Samples were analyzed by the potassium bromide pellet method in an IR spectrophotometer (Alpha T Bruker) in the region from 4000 to 400 cm^{-1}. The FT-IR spectra of pure Flurbiprofen, physical mixture of Flurbiprofen-sepitrap 80 and physical mixture of FLB- sepitrap 4000 are given in figure No.2

Differential scanning calorimeter (DSC) analysis:

The solid state interaction of drug with physical mixtures examined very well by differential scanning calorimetry (DSC) one of the most widely used calorimetric techniques. Samples of the pure drug and physical mixture of drug sepitrap were taken in flat-bottomed aluminum pans and heated over a temperature range of 30 to 300°C at a constant rate of 10°/min with purging of nitrogen (50 ml/min) using alumina as a reference standard in a differential scanning calorimeter (Mettler Toledo, Staresw 920). The DSC thermogram of pure Flurbiprofen, physical mixture of FLB- Sepitrap 80 and physical mixture of FLB- Sepitrap 4000 are shown in Figure No.3

Powder X-ray diffractometry (PXRD) analysis:
The PXRD study was carried out by using X-ray diffractometer (Miniflex 600 X-ray diffract to meter, Rigaku Corporation Japan). The Powder X-ray diffraction technique has been utilized broadly along with DSC to study the interaction and to obtain the changes in the crystallanity of the physical mixtures of drug and sepitrap prepared. For this the samples of pure drug, physical mixtures of Flurbiprofen-sepitrap 80 and FLB-sepitrap 4000 were irradiated with monochromatised CuKα radiation and analyzed between from 5˚ to 60˚ **(**2θ). The PXRD diffract to grams of pure Flurbiprofen, physical mixture of FLB with sepitrap 80 and sepitrap 4000 are shown in figure no.4

Scanning electron microscopy (SEM) studies:
A scanning electron microscope (VEG A3 TESCAN), under accelerating voltage of 15 keV was used to study the surface morphology of pure FLB and physical mixture of FLB with Sepitrap 80 and sepitrap 4000 by fixing samples on SEM stub with double-sided adhesive tape and then coated in a vacuum with thin gold layer before investigation. The SEM images of FLB and its physical mixtures with sepitrap 80 and sepitrap 4000 are shown in figure no.5

Dissolution studies:
The FLB and its physical mixture equivalent to200 mg of FLB were placed into dissolution tester, USP Type-II (EDT 08LX Electrolab). The drug and its physical mixture were placed in 900 ml of dissolution medium and maintained at 37±0.5°C, stirring rate at 50 rpm using 0.1 N HCl (pH 1.2) and distill water pH(7). At appropriate intervals, 5 ml of samples were taken and filtered through a 0.45 micron filter (Millipore, USA) and analyzed at 248nm by UV-Visible spectrophotometer. The withdrawn volume was replenished immediately with the same volume of the prewarmed (37°C) dissolution medium to maintain sink condition. The mean of three determinations was used to calculate the drug release from each of the formulations.[34-35]

Zeta potential:
Surface charge on sepitrap loaded physical mixture was determined using Zetasizer, (HORIBA scientific SZ-100). Physical mixture was diluted in deionized water (1/10 w/v) and was plaecd in measument cell for 60 sec for detemination of average zeta potential and charge on the physical mixture.

Stability study:
Stability study for selected physical mixture PM5 was carried out with the help of stability chamber (Remi SC-19 Plus) by storing 1gm of physical mixture in an ambered colored screw capped glass bottles at accelerated and controlled temperatures 40^0C and relative humidities (75%) for a period of 3 months.[36-40]Physical mixture was evaluated for physical appearance and in-vitro dissolution at the end of three months.

RESULT AND DISCUSSION:

Determination of maximum wavelength of FLB:
The absorbance measured using UV-Visible spectrophotometer of the 2µg/ml in the wavelength range of 200-400 nm and maximum wavelength was found at248nm.

Drug content determination:
The drug content of physical mixtures was determined using previously reported method.[33]The practical drug content of physical mixtures was found to be 48.56±1.13,32.12±1.32and23.91±1.63respectively which corresponded to the ratio of the drug with solubilizer. The theoretical drug content, practical drug content is summarized in table No.2

Table No. 2 % drug content, solubility data of physical mixture of FLB with sepitrap 80

Batch No	Sepitrap 80 Theoretical % Drug content	Sepitrap 80 Practical % Drug content	Solubility in water (µg/mL)
PM1	50	48.56±1.13	14.32±0.04
PM2	33.33	32.12±1.32	20.90±0.05
PM3	25	23.91±1.63	22.60±0.03
Batch No	**Sepitrap 4000 Theoretical % Drug content**	**Sepitrap 4000 Practical % Drug content**	**Solubility in water (µg/mL)**
PM4	50	49.32±1.59	20.23±0.07
PM5	33.33	32.56±1.46	29.59±0.03
PM6	25	24.65±1.58	30.25±0.04

% drug content, solubility data of physical mixture of FLB with sepitrap 4000* Represents mean ± S.D. (n = 3)

Saturation solubility studies:
The solubility of pure FLB in distilled water was determined to be only 8.15±0.03µg/mL. Remarkable enhancement in the solubility of FLB in presence of sepitrap 80 and sepitrap 4000 compared to pure FLB alone was observed in the saturation solubility studies. The physical mixtures of FLB with sepitrap 80 in the proportion of 1:1, 1:2 and 1:3 exhibited a solubility of 14.32±0.04, 20.90±0.05 and 22.60±0.03µg/mL respectively. The physical mixtures of FLB with sepitrap

4000 in the proportion of 1:1,1:2 and 1:3 exhibited a solubility of 20.23±0.07, 29.59±0.03 and 30.25±0.04µg/ mL respectively. 261% increase in the solubility in case of physical mixture of FLB with sepitrap 80 with proportion 1:2 whereas 369.87% increase in the solubility in case of physical mixture of FLB with sepitrap 4000 with proportion 1:2 was observed. The solubility increased sharply upon addition of the solubilizer, reaching a maximum at solubilizer proportion between 1:1 and 1:2 but then it declined somewhat upon further addition of solubilizer. The enrichment in solubility of FLB in presence of sepitrap 80 and sepitrap4000 clearly expresses the novel solubilizer in powder form that is polysorbate 80(sepitrap 80) and polyoxyl 40 hydrogenated castor oil (sepitrap 4000) is promising approach for enhancement of solubility of poorly soluble drug FLB. The solubility of FLB and percent increase in solubility due to use of sepitrap 80 and sepitrap4000 are given in figure no.1

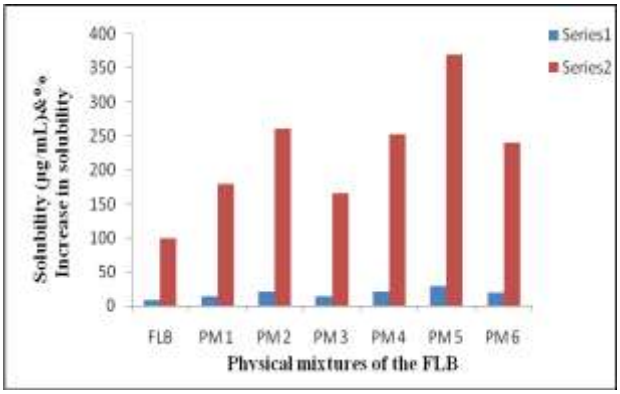

Figure No. 1: Saturation solubility of FLB in distilled water with different proportions of sepitrap 80 and sepitrap 4000

Fourier transform infrared spectrophotometer studies:

The FT-IR analysis has been carried out in order to characterize the possible interactions between FLB and solubilizer in solid state. FTIR spectrum of pure FLB exhibited IR absorption bands at 1706 cm-l (C=O stretching of carboxylic acid), 958 cm−1 (O-H bending), 1219cm−1 (C-F stretching of an halogen), 2860cm−1 (CH-CH_3stretching), 2934 cm−1 (C-H stretching), 2980cm−1 (C-H stretching),and broad peak of FLB in the range of 2500-3300cm−1 due to hydrogen bonding. The physical mixture did not show new peaks indicating that no chemical bonds were created in. The FT-IR spectrum of physical mixture retains principle IR absorption bands of drug with no substantial shifting of the position of the functional groups and indicating no major interaction between FLB and solubilizer, sepitrap 80 and sepitrap 4000 and also may be expressive of uniform FLB dispersion as a consequence of the physical mixing with solubilizer.

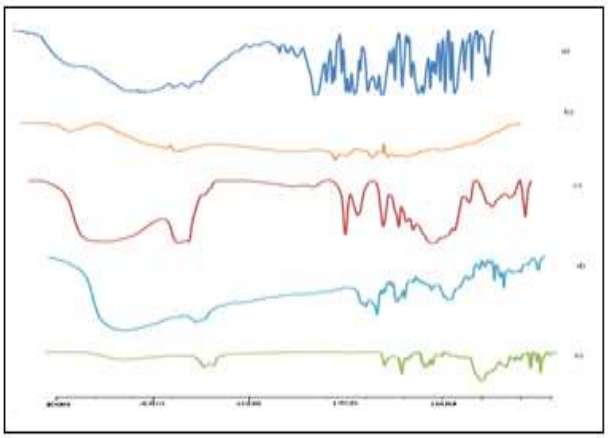

Figure No. 2 FT-IT Spectra of A] Pure Flurbiprofen, B] sepitrap 80 C] sepitrap 4000D] Physical Mixture of FLB - sepitrap 80 E] Physical Mixture of FLB –sepitrap 4000

Differential scanning calorimeter (DSC) analysis:

Thermal analysis was also performed using DSC technique to evaluate parameter of the physical mixture determined as a function of temperature and to demonstrate any unexpected interaction between FLB and solubilizer. The DSC thermogram of pure FLB and sepitrap 4000 are shown in fig no. 3. Crystalline nature of FLB was indicated bya sharp endothermic peak with an onset of 120^0C and peak at 122.18^0C with end set at 125^0C recorded as function of temperature representing melting point of drug. The sharp endothermic peak appeared in drug almost completely disappeared and shown with reduced intensity in physical mixture of sepitrap 4000. The characteristic peak of FLB was unchanged, illustrating an absence of strong interaction between drug and solubilizer in physical mixture. There were no appearance of new peaks; this revealed that there is no significant and unexpected interaction between drug and sepitrap 4000.

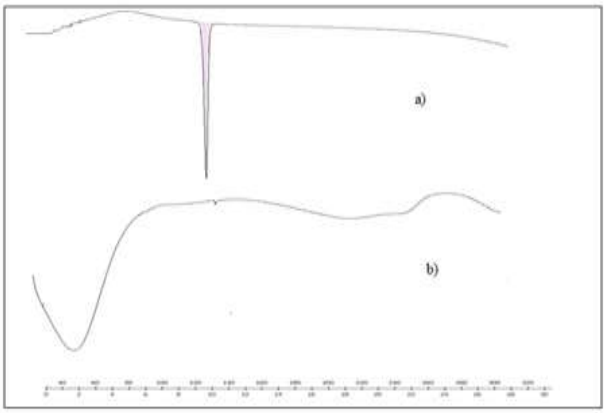

Figure No. 3: DSC thermogram of A] Pure Flurbiprofen B] Physical Mixture of FLB –sepitrap 4000

Powder X-ray diffractometry (PXRD) analysis:

The Powder X-ray diffractometry (PXRD) is used as structural analysis technique to assess the degree of crystallanity of the given sample. Powder X-ray diffraction pattern of FLB displayed sharp peak at diffraction angle representing typical crystalline nature. Crystallanity of the sample was reduced with its conversion into amorphous nature with the generation of physical mixture of the sample with the solubilizer sepitrap 80 and 4000. Hence physical mixture showed few, less intense peaks. Change of crystalline nature to amorphous ensured solubility amelioration of poorly soluble FLB.

The powder X-ray diffract to grams of pure FLB showed various distinctive peaks at 11.68, 11.70, 11.72, 11.74, 16.12 etc that indicated a high crystallanity. No new peak was detected and hence there was no unfavorable interaction of the drug with sepitrap. The physical mixtures of FLB with sepitrap 80 and sepitrap 4000 demonstrated distinctive peaks but reduced peak intensity in terms of counts. IR and DSC studies support the same data, which is confirmed by x-ray diffractometry. The PXRD Diffract to grams of pure FLB, physical mixture of FLB with sepitrap 80 and sepitrap 4000 are shown in figure no.4

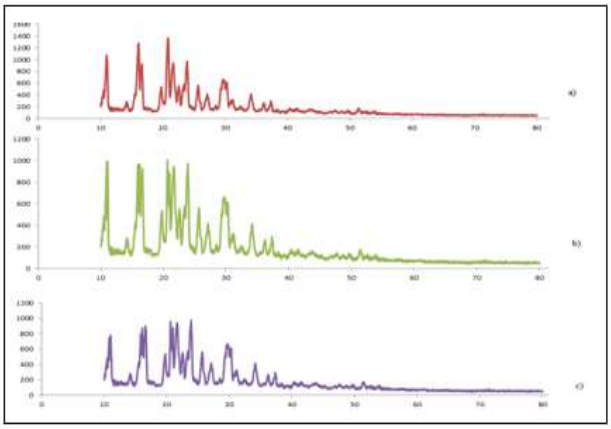

Figure No.4: PXRD patterns of A] Pure Flurbiprofen, B] Physical Mixture of FLB - sepitrap 80 C] Physical Mixture of FLB –sepitrap 4000

Scanning electron microscopy (SEM) study:

The SEM was executed to investigate surface morphology of the drug and its significant changes when mixed with solubilizer in powder form in various proportions as a physical mixture. The scanning electron micrographs of FLB powder appeared smooth and exhibiting loose aggregates of rectangular shape. The SEM images of sepitrap 4000–drug physical mixture shows slight changes in its surface structure due to polyoxyl40 hydrogenated castor oil incorporated in solid form. The slight change in surface morphology suggest that pure drug and solubilizer, sepitrap were mixed thoroughly and may be responsible for the enhancement of solubility when it comes in contact with fluid medium. The scanning electron micrographs of FLB and its physical mixture with sepitrap 4000 are displayed in Figure 5

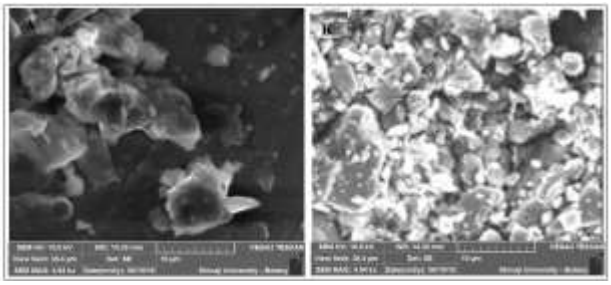

Figure No. 5 SEM images of A] Pure FLB, B] Physical Mixture of FLB –sepitrap 4000 1:2 ratio

***In-vitro* dissolution studies in SGF and Distilled water:**

FLB and its physical mixtures were evaluated for dissolution properties and compared with the pure FLB alone. The in-vitro dissolution results were assessed on the basis of cumulative percentage drug release, dissolution efficiency and correlation coefficient (r). Figure No.7 and 8 shows the dissolution profiles of FLB and various physical mixtures in SGF and distilled water pH 7 respectively. Dissolution of pure FLB at pH 7 was comparatively greater but decreased at acidic pH 1.2 and hence rapidly absorbed from small intestine and not well absorbed from upper GIT considering poor solubility in acidic environment. Physical mixtures of FLB showed enhanced dissolution in the lower pH with sepitrap 4000improving its absorption from upper gastro intestinal track. The pure FLB showed drug dissolution of 16.24 and 19.63% in 30 min in SGF and distilled water respectively, physical mixture of FLB with sepitrap 80 in 1:2 proportion showed 49.36 and 55.29% drug dissolution in 30 min while physical mixture of FLB with sepitrap 4000 in 1:2 proportion showed 60.23 and 63.96% drug dissolution in SGF and distilled water respectively. The dissolution of FLB was significantly enhanced by use of solubilizer sepitrap in 1:2 proportions. Increase in both wettability and solubility may be attributed to enhancement in FLB dissolution. The solubility of pure FLB increased due to localized solubilization because in minimal time more than80% of the solubilizer is desorbed from sepitrap and is available for solubilizing the drug. Sepitrap 80 as well as 4000 is free flowing with good ability to settle, and have particle size lesser than 200µm and widely chosen for their solubilization potential. Sepitrap 4000 is more potential and effective solubilizer as compared to sepitrap 80 in exactly half proportion to that of sepitrap80 because physical mixture containing FLB–sepitrap 80 in 1:2 proportion showed identical dissolution compared to the physical mixture containing FLB–sepitrap 4000 in 1:1.

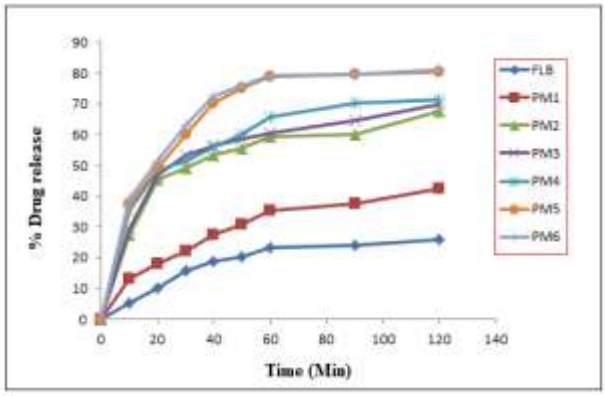

Fig. 7. The dissolution profile of pure drug and physical mixtures in SGF

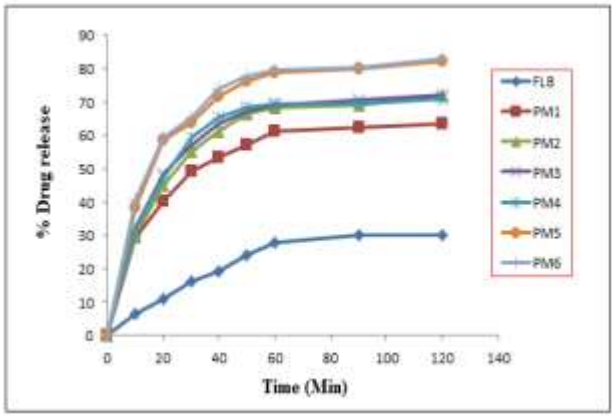

Fig. 8. The dissolution profile of pure drug and physical mixtures in distilled water

Zeta potential determination:

The zeta potential is highest measured parameters for determination of overall charges gain by particles in specific medium and which is considered as important factors for stability. Value away from zero is considered as optimum one (±30 mV), providing maximum stability to particle in dispersed medium. The zeta potential of physical mixture of flurbiprofen with novel soilubilizer sepitrap 4000(1:2) was found to be −19.1mV Figure 9 indicate zeta potential of physical mixture.

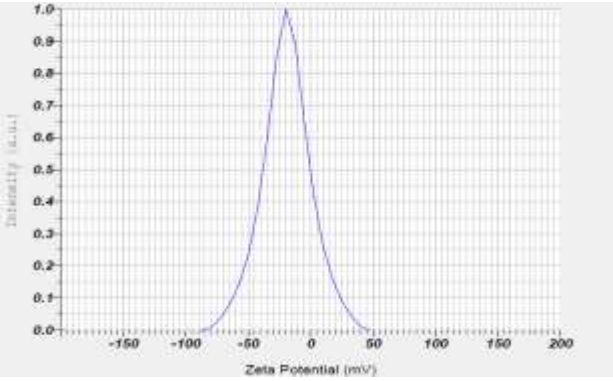

Figure 9: Zeta potential of physical mixture (PM5) flurbiprofen with novel soilubilizer sepitrap 4000(1:2)

Stability study:

There was no significant change in the physical appearance, drug content and percent drug dissolution in the FLB physical mixtures. A stability results clearly indicate that the physical mixtures were sufficiently stable under accelerated and controlled conditions.

CONCLUSION:

Poor water solubility has been attributed to almost half of the new molecular entities manufactured annually by pharmaceutical sector, and is claimed to lessen the performance of more than 10% of successfully marketed drugs. Novel solubilizer, sepitrap helps to find out solutions to utilize more effectively poorly water soluble new chemical entities (NCE) and develop these API into effective new drugs. It increases bioavailability of Flurbiprofen by solubilization of API in the medium and acts by increasing solubility.

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RESEARCH ARTICLE

In vitro Antioxidant potential and Anticancer activity of *Ceratophyllum demersum* Linn. extracts on HT-29 human colon cancer cell line

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ABSTRACT:

Objective: To decide the phytochemical constituents, antioxidant and anticancer potential of *Ceratophyllum demersum* Linn. extracts on HT-29 human colon malignant growth cell line. **Methods:** The whole plant was exposed to Hot Soxhlet continuous extraction with expanding polarity of solvents viz., pet ether, chloroform, ethanol, and aqueous maceration. Phytochemical screening was finished utilizing distinctive phytochemical tests. The antioxidant potential was tried utilizing 2, 2-diphenyl-1-picrylhydrazyl, ferric ion reducing power assay and phosphomolybdenum assay. In vitro anticancer action tried on HT-29 human colon malignant growth cell line and it was assessed by (3-(4, 5-dimethyl thiazole-2yl)- 2, 5-diphenyl tetrazolium bromide) MTT test. **Results and Discussion:** Preliminary Phytochemical screening affirmed the presence of phytoconstituents like alkaloids, flavonoids, glycosides, saponins, sterols, tannins, and reducing sugar. Antioxidant potential was demonstrated most noteworthy in ethanol extracts dependent on the test performed. The ethanol extracts were seen as specifically cytotoxic to HT-29 human colon malignant growth cell line. **Conclusion:** The outcomes show that *Ceratophyllum demersum* Linn. was a promising antioxidant; and anticancer agent for HT-29 human colon malignancy cell line. In any case, further examinations are expected to presume that the particular constituent liable for its antioxidant action and cancer prevention agent.

KEYWORDS: Phytochemical; antioxidant; anticancer; colon cancer; *Ceratophyllum demersum.*

1. INTRODUCTION:

Cancer is a disease described by uncontrolled engendering of cells that have changed from the typical cells of the body. The malignant growth cells can attack the neighbouring and distant tissues via the circulation. In advanced stages, a malignant growth patient may die because of either ill-advised finding or treatment disappointment. Malignancy is one of the push zones for which powerful medications at reasonable costs are not accessible until now presumably because of an absence of understanding the disease pathophysiology. For such a ghastly infection hostile to malignancy drugs have been created from an assortment of sources extending from normal items (plants and organisms) to synthetic particles.

The broadly utilized medications that are malignant growth chemotherapeutic specialists experience the ill effects of the downside of high danger, for example, bone marrow concealment, alopecia, queasiness and spewing and are not inside the compass of a typical man [1,2].

Medicines acquired from plants have assumed a central job in the social insurance of ahead of schedule and late societies. Ayurveda, the Indian arrangement of medication for the most part utilizes plant based medications or formulations to treat different sicknesses including malignancy. About 60% of medications allowed for cancer treatment are of natural source. Vincristine, Etoposide, Irinotecan, Taxanes and Camptothecines are instances of plant-derived anti-cancer compounds. [3,4]

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A few malignancies inquire about investigations accompanied using traditional medicinal plants in solidarity to find new helpful specialists that do not have the dangerous reactions connected with current chemotherapeutic agents and the medications under clinical trials, phytomedicines have improved strongly in the previous two decades. [5,6]

Current treatment of malignant growth has significantly more side effects to defeat from that; natural remedies are helpful, adequate and demanding by the disease patients because of its less or no toxic effect.

Henceforth here is a significant need to create substitute restorative techniques against this deadly illness. There is consistently the expectation that the pursuit among the traditional therapeutic plants may furnish strong and safe prescriptions with few or no poisonous impacts.

Reactive oxygen species are thought to assume a significant job in numerous human maladies. Radical scavenging power is essential because of the lethal job of free radicals in biological systems. Numerous secondary metabolites like phenols, polyphenols, and flavonoids fill in as wellsprings of antioxidants and perform scavenging activity. [7]

In spite of assuming a key job in cell forms, reactive oxygen species promptly join and oxidize biomolecules, for example, sugars, proteins, and lipids and in this manner making them inactive with sub-sequent harm to cells, tissues, and organs prompting beginning of numerous maladies including malignancy. In this manner, by diminishing free radicals and oxidative pressure, antioxidants assume a job in improving DNA harm, decreasing the rate of abnormal cell division, and lessening mutagenesis. Hence, numerous antioxidant-rich plants possess cancer prevention activity. [8,9]

Ceratophyllum demersum L., having a place with the family Ceratophyllaceae [10], is one of the major appealing aquatic therapeutic plant that has been utilized as pain relieving, antipyretic, anti-inflammatory and in the treatment of ulcer, looseness of the bowels, wounds, burning sensation, hemorrhoids or piles, inherent hemorrhages, hyperdipsia, epistaxis, hematemesis [11,12]. Furthermore, it was accounted for that the aqueous, chloroform, ethanol and methanol extracts of C. demersum have an antimicrobial impact against confined strains of bacteria and fungi [13]. Moreover, other chemical compounds, for example, tricin-7-O-β-D-glycoside, naringenin-7-O-β-D-glycoside, esculetin, β-sitosterol, 7α-hydroxyl-β-sitosterol,7α-methoxyl-β-sitosterol, and palmitic acid have likewise been isolated from C. demersum [14].

The assessment of the range of biological activities (antineoplastic and anti-inflammatory) with a prediction of activity spectra for substances (PASS) for the significant parts of essential oil of *C. demersum* alongside different plants extracted with hexane was studied. The anticipated estimation of anti-inflammatory and antineoplastic activities with probability above 0.8 was detected for 12 compounds (2Z,4Z)-Hepta-2,4-dienal; 2-Phenylacetaldehyde; (3E,5E)- Octa-3,5-dien-2-one; 2,6-Dimethylcyclohexan-1-ol; geranylacetone; α-muurolene; β-ionone; β-eudesmol; α- eudesmol; biformen; kaurene and manool [15].

C. demersum has been generally utilized as bioindicators of heavy metals in air contamination, radioactivity indicators [16,17], biomonitoring [18] in the aquatic condition, genetic engineering [19]. It is likewise one of the well-known plants in the aquatic industry due to its resistance in a wide range of aquatic conditions [20,21]. It additionally gives a phenomenal living condition for shelter to fish and aquatic organisms. It is likewise utilized as a wellspring of food for some livestock, poultry [22]and fish [23]. *C. demersum* is astringent, bitter, sweet, oleaginous, fragrant and purgative [24]

However, this plant has not been studied for anticancer action on colon cancer and there is a just a single accessible examination study on antioxidant properties of this plant. Therefore, we have made an endeavour to utilize herbal plant to check adequacy against colon malignant growth cell lines. The present investigation was aimed meant to assess preliminary phytochemical evaluation, in vitro antioxidant potential, and in vitro cytotoxicity against HT-29 colon malignant growth cell lines.

2. MATERIAL AND METHOD:

2.1 Chemicals:

All solvents were analytical grade. Pet Ether, Chloroform, ethanol, Ascorbic acid, ferric chloride, aluminium chloride, potassium acetate, DPPH reagent, sodium carbonate, was obtained from Loba Chemie Pvt. Ltd. Mumbai. Shimadzu 1800 UV-Vis Spectrophotometer was utilized in all spectrophotometric estimations.

2.2 Cell culture:

The human colon cancer cell line (HT-29) was obtained from National Centre for Cell Sciences (NCCS), Pune, India. The cells were kept up at 37°C in a humidified atmosphere (90%) containing 5% CO_2 and afterword, cultured in (Dulbecco's Modified Eagle Medium) with low glucose, Thermo fisher scientific (Cat No-11965-092) with 10% (v/v) FBS, 100 units/mL penicillin, and 100 µg/ml streptomycin.

2.3. Strategy for Collection of plant:
The plant *Ceratophyllum demersum* Linn. chosen for the investigation was collected from the Kolhapur district of Maharashtra. The plant was authenticated and a Voucher specimen was kept at the herbarium of the Department of Botany, Shivaji University Kolhapur, Maharashtra, India. (Voucher reference numbers: SSA-01).

2.4. Phytochemical Evaluation:
The whole plant of *Ceratophyllum demersum* Linn. were collected, washed with water, shade dried, and powdered. The powdered material was exposed to successive solvent extraction with a selected order of polarity i.e. pet ether, chloroform, ethanol and water maceration. The resulted successive extracts i.e. Pet ether Extract [PECD], chloroform Extract [CECD], Ethanol Extract [EECD], Aqueous Extract [AECD], were exposed to phytochemical investigation for the identification of phytoconstituents present.

2.4.1 Tests for carbohydrates:
The carbohydrates were tried by using Benedict's test, Fehling's test and Molisch test.

2.4.2 Test for proteins:
Various extracts were dissolved in a few ml of water and treated with Million's reagent.

2.4.3 Tests for Fats and Oils:
Fats and oils were tested with Translucent Spot test, Acrolein test,

2.4.4 Tests for sterols:
The sterols were tested by using the Libermann-Burchard test and the Salkowski test.

2.4.5 Tests for glycosides:
Keller Kiliani Test, Borntrager's test were utilized for the analysis of glycosides.

2.4.6 Test for saponins:
Foam test was performed for the presence of saponins.

2.4.7 Test for Flavonoids:
The flavonoids were tested by the Shinoda test and Ferric chloride test.

2.4.8 Tests for alkaloids:
The alkaloids have been tested by using the Dragendroff 's test and Wagner's test.

2.4.9 Tests for tannins:
Test for tannins, ferric chloride test, and lead acetate test were performed. [25, 26, 27]

2.5. Pharmacological Evaluation:
2.5.1. In vitro Antioxidant Activity:
The *in-vitro* antioxidant activity of different extracts of *Ceratophyllum demersum* Linn. was finished by utilizing Ferric reducing antioxidant power (FRAP) assay, Phosphomolybdenum assay and 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging ability assay**.** [28]

2.5.1.1 Ferric ion reducing antioxidant power assay (FRAP):
Ferric reducing antioxidant power (FRAP) assay is a broadly utilized technique that utilizes antioxidants as reductants in a redox-connected colorimetric response, Ferric iron (Fe^{3+}) is initially reduced by electron-giving antioxidants present inside the sample to its ferrous form (Fe^{2+}). The iron colorimetric test complex builds a dark blue color product upon reduction which can be measured at 700nm. Antioxidants are molecules that go about as reducing agents by giving electrons to free radicals to stabilize them and limit the harm brought about by free radicals to DNA, cells and organ systems. Antioxidant incorporates substances such as polyphenols; flavonoids; vitamins and enzymes like glutathione peroxidase and superoxide dismutase. [29]

FRAP assay was utilized to quantify the total antioxidant potential of the extracts. Antioxidant activity assays were performed by the technique portrayed by Benzie and Strain with slight drugs. All extract of *Ceratophyllum demersum* Linn. and Ascorbic acid in various concentrations ranging from 12.5µg/ml to 200µg/ml, and were blended with 2.5ml of 0.2mM phosphate buffer (pH 7.4) and 2.5ml of potassium ferricyanide [1% weight/volume (W/V)]. The resulting mixture is incubated at 50^{o}C for 20 minutes, followed by the addition of 2.5mL of trichloroacetic acid (10% W/V) and centrifuged at 3000 rpm for 10 minutes. At that point, 2.5ml of distilled water was included and later 0.5 ml of ferrous chloride (0.1% W/V). At long last, the absorbance was estimated at 700nm. Ascorbic acid was utilized as a positive reference standard. [30]

2.5.1.2. Phosphomolybdenum assay (PM):
The antioxidant assay depends on the reduction of Phosphate-Molybdenum (VI) to Phosphate-Molybdenum (V). The incubation of extracts with the Molybdenum (VI) will communicate the presence of antioxidant constituents in the extract, which can be assessed by recording the absorbance at 695nm (to distinguish the reduced green molybdenum complex). In this way, this assay is very helpful to conceive the antioxidant capability of crude extracts.

The total antioxidant action was evaluated by Phosphomolybdenum (PM) assay utilizing the standard procedure of Prieto et al. All extracts of *Ceratophyllum demersum* Linn. and Ascorbic acid in different concentrations ranging from 12.5µg/ml to 200µg/ml were added to each test tube individually containing 3ml of distilled water and 1 ml of molybdate reagent. These tubes were kept incubated at 95ºC for an hour and a half. After incubation, they are kept at room temperature for 20–30 minutes and the absorbance was estimated at 695 nm. The positive reference standard was utilized in this assay was Ascorbic acid. [30,31]

2.5.1.3. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging ability assay:

The anti-oxidant capability of any compound can be resolved based on its scavenging activity of the stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical. DPPH is a steady free radical containing an odd electron in its structure and generally used for the detection of the radical scavenging activity in chemical analysis. [32] The lambda max of a stable DPPH radical in methanol was at 517nm. The decline in absorbance of DPPH radical is brought about by antioxidants, in view of the reaction between antioxidant particles and radical progresses, which brings about the scavenging of the radical by hydrogen donation. [33,34]

Free radical scavenging effect of plant extract was resolved utilizing the 2-diphenyl-1picrylhydrazyl (DPPH) with slight meds of the technique portrayed by Brand-Williams et al. Briefly; the concentrations of extracts ranging from 12.5µg/ml to 400µg/ml were set up in ethanol. DPPH solution (0.004%) was prepared in ethanol and 1ml of this solution was blended in with a similar volume of extracts and standard ascorbic acid solution separately. The mixture was incubated for half an hour in the dark at room temperature and the absorbance was measured at 517nm. The level of DPPH purple decolourization to DPPH yellow showed the scavenging effectiveness of the extract. Lower absorbance of the reaction mixture showed higher free radical-scavenging activity. The scavenging activity against DPPH was determined utilizing the equation:

DPPH scavenging activity (%) = AC-AT/AC× 100

AC: Absorbance of Control, AT: Absorbance of sample

The results were analyzed in triplicate. The IC50 value is the concentration of the sample required to inhibit 50% of the DPPH free radical. [30,35]

2.5.2. In-vitro Anticancer Activity:

The viability assay most as often as possible utilized all through the world is the MTT assay. This colorimetric assay utilizes a reduction of a yellow tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, or MTT) to measure cellular metabolic activity as a proxy for cell viability. Viable cells contain NAD(P)H- dependent oxidoreductase enzymes which reduce the MTT reagent to formazan, an insoluble crystalline product with a deep purple shading. Formazan crystals are then dissolved utilizing a solubilizing solution and absorbance is estimated at 500-600 nm utilizing a platereader. The darker the solution, the more the quantity of viable and metabolically active cells. [30, 36, 37]

The *in-vitro* anticancer activity of different extracts of *Ceratophyllum demersum* was was finished by utilizing HT-29 human colon malignant growth cell lines.[38]

The cells were seeded at a density of around 5×10^3cells/well in a 96-well flat-bottom microplate and kept at 37^0C in 95% humidity and 5% CO_2 overnight. Distinctive concentration (800, 400, 200, 100, 50, 25 µg/ml) of samples were treated. The cells were incubated for extra 48 hours. The cells in well were washed twice with phosphate buffer solution, and 20µl of the MTT (3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide), staining solution (5mg/ml in phosphate buffer solution) was added to each well and plate was incubated at 37^0C. After 4 hours, 100µl of Dimethyl sulfoxide (DMSO) was added to each well to dissolve the formazan crystals, and the absorbance at 570 nm was measured with UV- Spectrophotometer using DMSO as the blank. Measurements were performed and the concentration required for a 50% inhibition (IC50) was determined. The % cell viability was determined utilizing the following formula:

% Cell viability = A_{570} of treated cells / A_{570} of control cells × 100

A_{570}: Mean absorbance at 570nm. [30, 39, 40]

3. STATISTICAL ANALYSIS:

All the determinations of the experimental work were carried out in triplicates and the acquired information were classified and analyzed by using Microsoft Office Excel 2013. The results are expressed as mean values and standard Error Mean (SEM).

4. RESULTS:

4.1 Phytochemical evaluation:

The existences of numerous secondary metabolites such as sterols, flavonoids, saponins, glycosides were detected in ethanol and aqueous extracts. These significant metabolites were identified based on color changes, the formation of precipitation or formation of persistent forms qualitatively. On the other hand, a lower amount of carbohydrate, alkaloids and tannins were identified.

Traces of fats and oils were found in Pet ether extract. **(Table 1)**

Table 1: Phytochemical evaluation of *Ceratophyllum demersum* L. extracts

Compounds	PECD	CECD	EECD	AECD
Carbohydrates	-	-	+	+
Proteins	-	-	-	+
Fats and Oil	+	-	-	-
Sterols	-	++	+++	++
Glycosides	-	-	+	++
Saponins	-	-	+	++
Flavonoids	-	-	++	++
Alkaloids	-	+	+	+
Tannins	-	-	+	+

+++: Strongly present, ++: Moderately present, +: Present in traces, -: Absent

4.2 In vitro antioxidant activity:

Antioxidant capacities were shown highest in ethanol extracts based on the FRAP assay, PM assay and DPPH radical scavenging ability assay.

4.2.1. Ferric ion reducing antioxidant power assay:

In the present investigation, extracts of *Ceratophyllum demersum* L. were exposed to FRAP assay alongside standard ascorbic acid. In the results obtained, Ethanol extract showed higher activity than other extracts which was comparable to standard Ascorbic acid. **(Table 2)**

4.2.2 Phosphomolybdenum assay:

The Pet Ether (PECD), Chloroform (CECD), Ethanol (EECD) and Aqueous (AECD) extracts of *Ceratophyllum demersum* L. were subjected to PM assay along with standard ascorbic acid. In the results obtained, Ethanol extract showed higher activity than other extracts which was comparable to standard Ascorbic acid. **(Table 3)**

4.2.3. 2, 2-Diphenyl-1-picrylhydrazyl radical scavenging ability assay:

In the present study, Pet Ether (PECD), Chloroform (CECD), Ethanol (EECD) and Aqueous (AECD) extracts of *Ceratophyllum demersum* L. were subjected to DPPH free radical scavenging assay. The antioxidant potential of the extract was compared with ascorbic acid as the standard antioxidant. Ethanol extract showed higher activity than other extracts which was comparable to standard Ascorbic acid. **(Table 4)** IC50 values of DPPH scavenging activity of extracts of *Ceratophyllum demersum* L. for antioxidant activity are represented in **Figure 1.**

Table 2: Mean absorbance of *Ceratophyllum demersum* L. extracts for FRAP assay.

Concentration (µg/ml)	PECD	CECD	EECD	AECD	AS
200	0.354±0.025	0.412±0.021	0.643±0.017	0.467±0.015	0.962±0.009
100	0.275±0.017	0.265±0.018	0.444±0.028	0.312±0.020	0.612±0.001
50	0.161±0.025	0.138±0.026	0.295±0.039	0.217±0.027	0.42±0.002
25	0.094±0.023	0.077±0.007	0.18±0.035	0.079±0.009	0.266±0.007
12.5	0.052±0.014	0.06±0.008	0.086±0.017	0.054±0.011	0.164±0.009

Data expressed as Mean ± Standard error of the mean.

Table 3: Mean absorbance of *Ceratophyllum demersum* L. extracts for PM antioxidant assay.

Concentration (µg/ml)	PECD	CECD	EECD	AECD	AS
200	0.645±0.033	0.721±0.021	0.923±0.011	0.732±0.020	1.199±0.011
100	0.447±0.011	0.541±0.017	0.696±0.007	0.413±0.011	0.903±0.009
50	0.208±0.014	0.274±0.016	0.412±0.009	0.321±0.004	0.658±0.014
25	0.121±0.019	0.133±0.009	0.196±0.006	0.164±0.008	0.442±0.009
12.5	0.08±0.012	0.098±0.004	0.164±0.008	0.114±0.005	0.237±0.004

Data expressed as Mean ± Standard error of the mean.

Table 4: DPPH scavenging activity of *Ceratophyllum demersum* L. extracts for antioxidant activity.

Concentration (µg/ml)	PECD	CECD	EECD	AECD	AS
400	59.52±0.83	66.62±1.20	79.58±1.15	72.91±1.17	96.71±0.85
200	47.95±1.42	57.83±0.07	68.6±1.03	62.39±2.39	87.57±1.76
100	37.06±1.40	33.28±0.24	53.08±1.34	42.17±0.43	77.86±2.06
50	27.84±3.46	21.79±1.18	43.78±1.00	32.83±1.09	63.95±1.38
25	14.55±1.10	12.01±1.53	33.94±1.71	23.61±1.00	46.83±1.91
12.5	8.75±0.52	5.98±0.45	20.39±0.96	14.37±1.50	27.14±3.41

Data expressed as Mean ± Standard error of the mean.

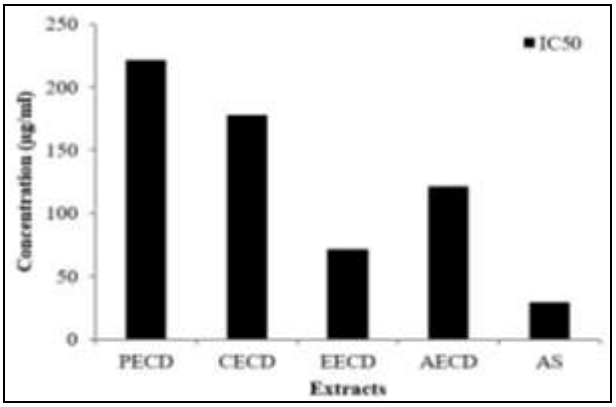

Figure 1: IC50 values of *Ceratophyllum demersum* L. extracts for DPPH antioxidant activity

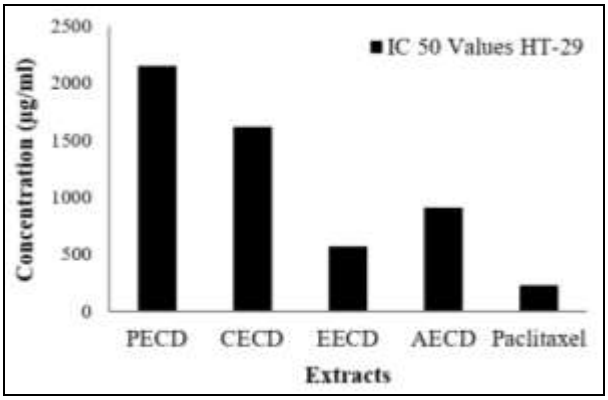

Figure 2: IC50 values of *Ceratophyllum demersum* L. extracts for cytotoxicity on HT-29 colon cancer cell line

Table 5: Percentage cell viability of *Ceratophyllum demersum* L. extracts for cytotoxicity on HT-29 colon cancer cell line.

Conc. µg/ml	PECD	CECD	EECD	AECD	Paclitaxel
25	96.18±0.66	95±0.26	95.74±0.78	94.71±0.79	92.6±0.47
50	90.35±0.77	91.77±0.46	88.47±1.11	86.76±0.35	82.01±0.83
100	84.56±0.34	86.03±0.64	80.26±0.57	76.62±1.26	68.83±0.41
200	79.79±1.57	79.44±0.34	63.68±0.45	66.32±0.39	50.53±0.50
400	74.29±0.032	73.65±0.70	60±0.64	55.44±0.52	37.39±0.43
800	64.53±0.75	61.21±0.58	46.18±0.61	57.5±0.60	21.87±0.67
NC	100				

Data expressed as Mean ± Standard error of the mean.

4.3. In vitro anticancer activity:

The result of MTT assays revealed that the ethanol extract was found to be selectively cytotoxic to HT-29 human colon cancer cell line as compared to other extracts compared with mostly used Paclitaxel as cytotoxic and normal control. **(Table 5)**. IC50 values of extracts of *Ceratophyllum demersum* L. against HT-29 Colon cancer cell lines are represented in **Figure 2**

5. DISCUSSION:

These days, the pharmaceutical ventures were concentrating in the therapeutic plants as a wellspring of lead bioactive specialists to deliver novel medications. Around 25-50% of the modern drugs were acquired from therapeutic plants. Numerous medicinal plants were interesting in their biological activities however it has been utilized by various tribes or nations for various ailments, this shows plants have a wide scope of healing powers which are recognized to their synthetic composition. [41]

In recent years, the use of herbal medicines in cancer treatment has received increasing attention due to their varied Phyto-metabolic contents with multiple biological activities [42]. The plant collected from the Western Ghats was recognized by their taxonomical characters as *Ceratophyllum demersum* L. and analyzed for the presence of phytochemicals with four solvent extracts. Preliminary phytochemical analysis revealed the presence of secondary metabolites in the selected extracts of the plant **(Table 1).** These secondary metabolites are reported to have many biological and therapeutic properties [43]. Among the various phytochemicals, sterols and flavonoids have picked up the regard for various zones of utilizations such as pharmaceutical, health, food, and cosmetic industries. These composites basic in the plant space as piece of our everyday eating routine and are appealing as attractive natural antioxidants. [44,45]

Reactive oxygen species (ROS) are thought to assume a significant job in numerous human illnesses. Free Radical scavenging activities are exceptionally vital because of the poisonous job of free radicals in biological systems. Numerous secondary metabolites like flavonoids fill in as origins of antioxidants and do scavenging activity. [46] ROS unreservedly consolidates and oxidizes biomolecules, for example, proteins, sugars, and lipids in this manner making them lethargic with consequent harm to cells, tissues, and organs prompting disease progression. [47,48]

In the present work, FRAP, PM and DPPH strategies were utilized to assess the entire antioxidant capacity of pet ether, chloroform, ethanol, and aqueous extracts.
The Ferric ion reducing capability of the extract may help as a significant presentation of its antioxidant activity. The presence of antioxidants, which have been shown to be an impart antioxidant action by breaking the

free radical chain by donating a hydrogen molecule [49, 50].

The presence of antioxidants in the extract would bring about the reduction of ferricyanide Fe^{3+} to ferrocyanide Fe^{2+} by giving an electron which was estimated spectrophotometrically at 700nm. In this assay, the yellow shade of the test solution changes to various shades of green and blue, depending on the reducing power of plant extract. The reducing power decreased with the decrease in the extract concentrations. This might be filled in as a critical marker of its antioxidant potential. The ethanol extract indicated more absorbance at 700nm than other extracts (0.643±0.017); subsequently this investigation assumed that ethanol extract of *Ceratophyllum demersum* L. may have a high measure of antioxidant property than other extracts which was comparable to that of the synthetic antioxidant standard used Ascorbic acid **(Table 2).**

By utilizing phosphomolybdenum technique, the absolute antioxidant activity of the sample was investigated. It is a colorimetric quantitative technique that estimates the reduction of Phosphate-Mo (VI) to Phosphate-Mo (V) by the sample and resulting development of a pale blue green shaded Phosphate-Mo (V) complex [30]. It helps to watch the reduction rate among antioxidant and molybdenum ligand. In the present investigation, ethanol extract displayed higher absorbance at 695nm than other extracts (0.923±0.011) **(Table 3).** Henceforth this examination perceived that ethanol extract of *Ceratophyllum demersum* L. may have a high measure of antioxidant property than other extracts which was practically comparable to that of synthetic antioxidant standard used Ascorbic acid

DPPH is steady nitrogen focused free radical which is ordinarily used to decide free radical scavenging activities of antioxidants present in plant extracts or synthetic compound. [51,52,53] The reduction capacity of DPPH radical is dictated by the decline in absorbance at 517nm prompted by antioxidants. Greater absorbance of the reaction mixture specified lesser free radical scavenging activity. [54] In the present examination, ethanol extract displayed higher free radical scavenging activity than other extracts (79.58±1.15) **(Table 4)**. Thus this examination accepted that ethanol extract of *Ceratophyllum demersum* L. may have a high measure of antioxidant property than other extracts which was practically comparable to that of the standard synthetic antioxidant agent Ascorbic acid. The outcome indicated that ethanol extract significantly scavenges the free radical and was the most potent extract with an IC50 value of 71.52µg/ml **(Figure 1).**

The evaluation of the cytotoxicity of plant extracts is important for innocuous treatment. It empowers the identification of the inborn toxicity quality of the plant. [55,56] The MTT assay is utilized in screening the crude extracts to assess the toxicity. It could likewise give a sign of conceivable cytotoxic properties of the tested plant extracts. MTT assay depends on the reduction of MTT by mitochondrial dehydrogenase by purple formazan product. It is often utilized as an in vitro model framework to measurement of cytotoxic impacts of plant extracts against malignant growth cell lines. [57,58,59]

In vitro cytotoxicity test using HT-29 colon malignant cell lines was performed to screen potentially toxic compounds that affect basic cellular functions and morphology. The four extracts (Pet ether, Chloroform, Ethanol and Aqueous) of *Ceratophyllum demersum* L. appeared in vitro development hindrance consequences for the malignant growth cell lines (HT-29), while there was no impact on the development of normal cells. Such specific impacts were focus just as, incubation period subordinate, regarding concentration (25, 50, 100, 200, 400, 800µg/ml) of each extract were assessed in triplicates by sequential dilutions. Among these six concentrations, 800µg/ml of ethanol extract was the best in creating percentage growth inhibition (Cell viability: 46.18±0.61) trailed by aqueous extract (Cell viability: 57.5±0.60) as compared to other extracts. However, the standard paclitaxel drug showed significant inhibition (Cell viability: 21.87±0.67) on the cancer cell lines **(Table 5)**.

The outcome indicated that ethanol extract is most potent extract with an IC50 value of 571.3µg/ml **(Figure 2).**

6. CONCLUSION:

It was seen that the plant *Ceratophyllum demersum* L. contains a wide assortment of secondary metabolites that hold strong antioxidants and anticancer capability dependent on the analyses performed, which gives a logical proof to direct further examinations and research the lead compounds present in the plant and assess its anticancer potential on other cancer cell lines and on in vivo animal models and set forward an endeavour to carry out trials on human beings.

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8. CONFLICT OF INTEREST:

The authors declare no conflict of interest.

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RESEARCH ARTICLE

In vitro Gastro-intestinal digestion of combined *Zingiber officinale* and *Terminalia chebula* associated with Antioxidant capacity and α-Glucosidase Inhibition

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ABSTRACT:
The rhizome of *Zingiber officinale* and the fruit of *Terminalia chebula* are widely used in traditional foods and medicines. This particular combination holds great significance in most ayurvedic formulations. However, studies on the bioaccessibility of this combination are yet to be elucidated. Thus in the present study, we aimed to evaluate the polyphenolic content, antioxidant activity and α-glucosidase inhibition before and after gastro-intestinal digestion. The bioaccessible fraction of total phenolic and flavonoid contents after intestinal digestion was found to be 46% and 33% respectively. Quantification of individual bioactive compounds present in the extract (6-shogaol, gallic acid, ellagic acid, corialgin, chebulinic acid, and chebulagic acid) was estimated by high-performance liquid chromatography. The antioxidant capacity was increased and the inhibition of α-glucosidase was reduced after intestinal digestion when compared to gastric digestion. Overall, the results indicated the bioaccessibility of polyphenols after gastro-intestinal digestion and its bioactivity, which needs to be further studied to understand the stability of bioactive compounds. This finding is valuable for food technologists and alternative medicine practitioners to lay the foundation to know the effects and bioactivity of foods and herbs upon gastro-intestinal digestion.

KEYWORDS: Ginger, Haritaki, Bioaccessibility, Antioxidant activity, α-glucosidase.

INTRODUCTION:

Since ancient times, medicinal plants have been used as therapeutic agents in Ayurveda. Research on herbal medicine has gained importance in the last few decades due to its effectiveness in various illnesses. Although some of the plants used in Ayurveda have been studied for their effectiveness, scientific validation is yet to be elucidated. Two of these herbs are *Zingiber officinale* (rhizome part) and *Terminalia chebula* (fruit part). The combination of these two herbs was observed to be present in 15% of ayurvedic churnas (reference from the National Library of Ayurved Medicines-NLAM).

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Z. officinale (ZO) commonly known as ginger, is a spice used in culinary and traditional medicine for more than 2000 years[1]. The extract from ginger rhizome contains polyphenols (gingerol and its derivatives) that have high antioxidant activity[2]. It contains volatile oils (10%), fat (10%), starch (40-60%), and resinous matter (5-8%)[3,4]. It is also proved to be effective for nausea, vomiting[5] and dysmenorrhoea[6,7]. Certain spices like ginger and its active compounds stimulate the secretion of bile acid, which aids in digestion and absorption of lipids. A study on rats fed with a diet containing ginger showed increased levels of pancreatic and intestinal lipase[8]. *T. chebula* (TC) is known as "king of herbs" in ayurveda that have been used extensively in various formulations including "Triphala" a well-known formulation used for chronic disorders. The tannis and polyphenols (chebulinic acid, chebulagic acid, corilagin, and ellagic acid, etc) present in *T. chebula* possess anti-bacterial[9] antimutagenic[10] anti-inflammatory[11,12] and anti-oxidant [13,14] activities. The bioaccessibility study on tannins revealed the maximum absorption in the gastric phase and slightly decreased absorption in the intestinal

phase[15]. Also TC exerts antimicrobial action on gastro-intestinal tract by soothing the mucosal lining[16].

In the present study, we evaluated the simulated *in vitro* digestion of the combination of ZO and TC on total phenolic and flavonoid contents, antioxidant and anti-diabetic activities. The results of this study will be more useful to understand the therapeutic effects of ZOTC after digestion.

MATERIALS AND METHODS:

Extract Preparation:

The decoction of ZO and TC was prepared according to the method depicted in Ayurvedic Book[17]. The ratio of 1:4 (ZO:TC) was taken from the reference, Ayurvedic Pharmacopeia of India. Precisely, 30grams (7.5 grams of ZO and 22.5grams of TC) was taken in 1000mL of water and decocted until the volume was reduced to one by fourth. The resulting mixture was then centrifuged to get rid of residues, and the supernatant was subjected to lyophilization for further analysis.

Simulated *In Vitro* Gastro-intestinal Digestion:

The in vitro gastro-intestinal digestion of ZOTC was performed following the method reported by Minekus et al[18]. The sample was subjected to gastric and intestinal digestions using an electrolyte stock solution, namely simulated gastric fluid and simulated intestinal fluid along with the corresponding enzymes and $CaCl_2$. At the end of each digestion, the samples were freeze-dried and kept for further analysis.

Clean-up of the Digested and Undigested Samples:

Digested and non-digested samples were mixed with 80% methanol, sonicated for 15 minutes and centrifuged at 8000g for 10 min. The supernatant was collected and the pellet fraction was mixed with 70% acetone and the same process was repeated. Both the supernatants were mixed and dried. The samples were mixed with methanol for further analysis[19].

Qualitative and Quantitative Determination of Phytochemicals:

The total phenolic content (TPC) and flavonoids (TFC) were estimated by the Folin-Ciocaltue method [20] and Alumium chloride method[19] respectively. The reference standards used for phenolics and flavonoids were gallic acid and quercetin.

The major polyphenolic compounds in ZO (6-shogaol) and TC (gallic acid, ellagic acid, corilagin, chebulinic acid, and chebulagic acid) were quantified using Shimadzu modular high-performance liquid chromatography (Kyoto, Japan, Model: CBM-20A) by two different methods for ZO and TC. The amounts of standard compounds in digested and non-digested samples were quantified to calculate the recovery index. It is calculated by the following formula:

% Recovery = (Polyphenolic content after digestion/ Polyphenolic content in non-digested samples) X 100

Radical Scavenging Activity:

Radical scavenging of digested and non-digested samples was measured using the DPPH (2,2-diphenyl-1 picrylhydrazyl) assay. Different concentrations of samples were mixed with 0.1M DPPH and incubated in the dark for 30 min. Ascorbic acid was used as a positive control. The radical scavenging was estimated spectrometrically at 518 nm[21].

Inhibition of Anti-Diabetic Related Enzyme:

Inhibition of α-glucosidase, which is related to diabetes was measured for different concentrations of digested and non-digested ZOTC and were estimated according to the method described by Adrian et al[22]. The standard drug acarbose was used as a positive control in both assays.

Statistical Analysis:

The results are expressed as mean±Standard deviation. The difference between antioxidant capacity and α-glucosidase inhibition of different phases of in vitro digestions were analyzed by one-way analysis of variance (ANOVA) followed by tukey's multiple comparisons using Graph Pad Prism version 7.

RESULTS AND DISCUSSION:

Total Phenolic and Flavonoid contents in digested and non-digested extracts of ZOTC and the recovery Index:

The insoluble fractions in each digest were measured to be 10% (gastric digest) and 30% (intestinal digest). The insoluble indigestible fraction might contain bound phenolic compounds, insoluble dietary fibre, resistant protein, resistant starch, hemicelluloses lignin, cellulose, and minerals[23]. ZO contains 27.65% insoluble fiber[24] that moves through the digestive tract without any change. Since the insoluble fiber stays long in the digestive tract, it holds the capacity to quench the radicals formed by interacting with surface proteins and it could change the micro flora in the gut[25]. It could also have an effect on bioaccessibility of digested polyphenolic contents[26].

The percentage recovery of digested polyphenolic contents was calculated based on the amount of phenolic and flavonoid contents in the non-digested, gastric digest, and intestinal digest (Figure 1). The values of the non-digested fraction were kept at 100% recovery of TPC and TFC. Gastric digestion decreased significantly ($P<0.05$) in both TPC and TFC recovery, and the intestinal digestion of TPC increased significantly ($P<0.05$), whereas TFC was reduced when compared to

gastric digestion. *=P<0.05, a=comparison of phenolic content of non-digest with gastric digest; b= comparison of phenolic content of gastric digest with intestinal digest. A= comparison of flavonoid content of non-digest with intestinal digest. The recovery of TPC increased after gastric digestion, which could be ascribed to chemical structure modification, solubility, and interactions with other compounds during the digestion process. The reason behind reduction in TFC after gastric digestion might be due to the breakdown of flavonoids to fiber, protein, or sugar residues[25].

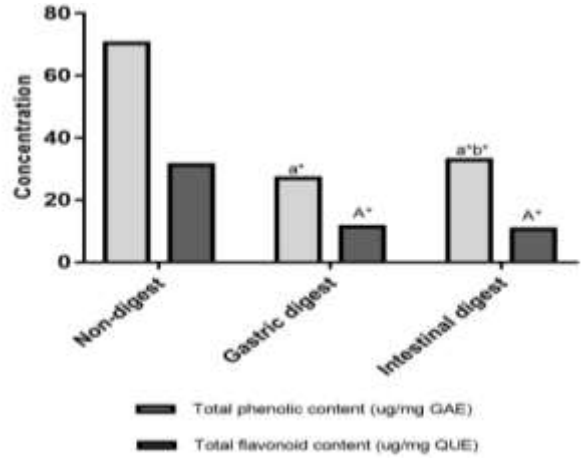

Fig. 1: Total phenolic and flavonoid contents before and after gastro-intestinal digestion.

Stability of Polyphenolic compounds in ZOTC after gastro-intestinal digestion:

The major pungent compound of dried ZO is 6-shogaol and some of the major phytocompounds in TC are gallic acid, ellagic acid, chebulagic acid, chebulinic acid, and corilagin. Table 1 shows the concentration of recovered compounds after the digestion process. Chebulinic acid was found to have the highest concentration followed by ellagic acid, chebulagic acid, gallic acid, 6-shogaol, and corilagin. Flavonoids and tannins are more stable during gastrointestinal digestion[27]. This might be due to the compound bound to fiber or proteins and released after digestion process to the colon. The non-accessible polyphenols could be fermented by the gut microbiota in the colon, resulting in short-chain fatty acids, microbial metabolites, and phenolic precursors. This transformation could create an antioxidant environment in the colon that helps in maintaining colon health[28]. An earlier report on polyphenols from TC revealed that its intake is associated with the formation of urolithins, the bioactive colonic metabolite[29]. Except for gallic acid and chebulinic acid, the concentrations of all other compounds were greatly reduced after gastric and intestinal digestions. As mentioned in a previous report, acidic pH in the gastric environment can cause a reduction in polyphenolic content due to its ability to form gels from soluble dietary fibre through some carbohydrates that entrap the polyphenols and affect their release in the gastric phase[30]. Since both ZO and TC contain dietary fibers and carbohydrates, the formation of a gel could have possibly entrapped polyphenols and released in the small intestine based on the chemical structure of the compounds.

Table-1: Polyphenolic profile obtained before and after gastro-intestinal digestion by HPLC

	Non-digested ZOTC(µg/mg)	Digested ZOTC (µg/mg)
6-Shogaol	0.195±0.06	0.027±0.00
Gallic acid	0.076±0.01	0.077±0.00
Ellagic acid	0.272±0.19	0.193±0.08
Chebulagic acid	0.320±0.05	0.130±0.00
Chebulinic acid	0.915±0.05	0.733±0.00
Corilagin	0.085±0.00	0.010±0.00

Radical scavenging Activity before and after in vitro Digestion:

The antioxidant capacity of ZOTC before and after gastro-intestinal digestion is described in figure 2. The antioxidant activity of lipophilic compounds present in ZOTC was measured by DPPH radical scavenging assay. Since 6-Shogaol, gallic acid are lipophilic and ellagic acid has lipophilic moiety, it could exert high radical scavenging activity after digestion. The IC50 value of the standard ascorbic acid was 0.89µg/ml, whereas non-digested ZOTC showed IC50 of 11.56µg/ml. The gastric digest and intestinal digest showed IC50 of 16.92µg/ml and 20.63µg/ml respectively. The antioxidant capacity of ZOTC after gastric and intestinal digestions were reduced significantly (P<0.05) when compared to the non-digested sample. This may be because of the slow release due to acidic conditions in the gastric phase. There was an increase in the antioxidant activity of the intestinal digest when compared to the gastric digest, but no significant change was observed. This may be due to the possible release of compounds from the matrix or chemical transformation of compounds that exert high antioxidant activity[31].

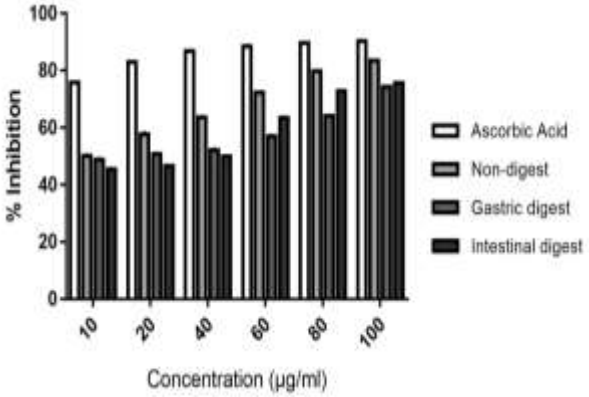

Fig.2: DPPH scavenging activity of standard Ascorbic acid, Non-digested, Gastric digested and Intestinal digested ZOTC

Inhibition of α-glucosidase in Cell-free System before and after in vitro Digestion:

Diabetes is a chronic disorder that affects glucose metabolism in the body. Type 2 diabetes constitutes 90% of the diabetes cases and major reason for the cause of deaths related to diet. Although α-glucosidase inhibitors are widely used in the treatment of type 2 diabetes (Acarbose, voglibose, and miglitol), it has major drawbacks of known side effects like flatulence and diarrhoea[32]. Therefore, natural inhibitors of food and its components can alleviate hyperglycemia through inhibition of digestive enzymes. The hypoglycaemic effect of ZOTC polyphenols has the ability to inhibit the carbohydrate hydrolyzing enzyme α-glucosidase (Figure 3). The IC50 value of the standard drug acarbose was presented to be 85.19µg/ml, whereas the non-digested ZOTC inhibited 50% of the enzyme at 36.42µg/ml. Even though the IC50 value of non-digested ZOTC was lower than standard drug, after gastric (IC50 of 108.3µg/ml) and intestinal digestion (IC50 of 209.6µg/ml), it increased significantly ($P<0.05$). Previous reports on the polyphenols from ZO[33] and TC[34] have shown to inhibit α-glucosidase enzyme. In our study, we found the inhibition of α-glucosidase enzyme by combined ZOTC after gastro-intestinal digestion.

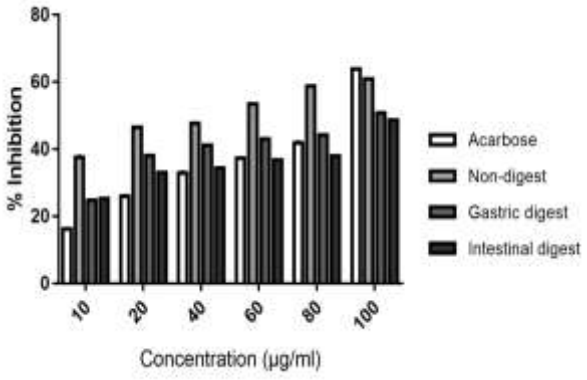

Fig.3: α-glucosidase inhibitory activity of standard drug Acarbose, Non-digested, Gastric digested and Intestinal digested ZOTC

CONCLUSION:

This work demonstrated that the polyphenolic compounds of ZOTC were released during gastric and intestinal digestions, and their bioaccessibility allowed the polyphenols to exert their bioactivity. The major bioactive compounds of ZOTC (6-Shogaol, gallic acid, ellagic acid, chebulagic acid, chebulinic acid, and corilagin) before and after digestions were measured by HPLC. The non-bioaccessible fractions indicate that they would be available for absorption in the intestine and convert to bioactive microbial metabolites in the colon by colonic microbiota. These non-bioaccessible fractions from gastro-intestinal digestion are related to the presence of gallic acid and ellagic acid. Although the digestion process reduces the polyphenolic concentration and α-glucosidase inhibition, it increases the antioxidant capacity after intestinal digestion when compared to gastric digestion, which shows the availability of bound polyphenols after intestinal digestion. This analysis helps us to improve our knowledge on how ZOTC exerts its bioactivity in vivo through bioavailability studies.

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CONFLICT OF INTEREST:

The authors declare that there are no conflicts of interest.

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RESEARCH ARTICLE

In vitro Cytotoxic studies of *Saraca asoca* bark extracts on HT-29 cancer cell Line

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ABSTRACT:

Asoka-*Saraca asoca* (Roxb.) de Wilde is one of the traditional plants with well-known medicinal values prescribed widely to treat haematuria, dyspepsia, fever, tumor, burning sensation, visceromegaly, colic, ulcers, menorrhagia, metropathy leucorrhoea, skin infection along with many other pharmacological activities. As globally, we look towards the use of non-toxic plant based products with conventional medicinal usage and the developing countries depends on herbal medicines for their primary health care needs. And so, the aim of this study is to explore the the in vitro cytotoxicity of the bark extracts of *Saraca asoca* on HT-29 cancer cell line using MTT and SRB assays. The results show a significant inhibition percentage on HT-29 cancer cell line was observed in both assays. In MTT assay the IC50 values was 174.1 and 163.2µg/ml on alcohol and aqueous bark extracts, respectively. And the SRB assay shows the IC50 values 94.76 and 200.5µg/mL on the crude ethanolic and aqueous bark extracts, respectively. Thus, both MTT and SRB assays can be used for cytotoxic screening of Saraca asoca herb towards the development of modern drug.

KEYWORDS: MTT assay, SRB assay, HT-29 cancer cell line, IC_{50} value, cytotoxic activity.

INTRODUCTION:

Over the past 100 years, chemically synthesized drugs radically changed the health care scenario in most parts of the world. However, almost 70% of population in the developing countries like India depends on traditional herbal medicines to meet out their primary health care needs[(1)]. The use of traditional medicines is not only restricted to developing countries, but also greatly increased in industrialized countries due to the public interest towards natural therapies for the treatment of various diseases. Medicinal plants constituents have an important role towards growth control of tumour cells[(2)].

Plant and phytoproducts plays a vital role in curing wide range of diseases including cancer. Many plant origin substances reported to have antitumor activity and its possible application in cancer prevention[(3)]. This emphasize the importance of ethnobotanicals in pharmacological research and drug development, though either we use plant constituents directly as therapeutic agents or the starting material for the synthesis of drugs and active pharmacological compounds[(4)].

The GLOBOCAN 2018 data states that colorectal cancer (CRC) is the third and fourth most deadly and commonly diagnosed cancer in the world, respectively. Adoption to "western" way of life, especially, in developing countries shows a constant increasing incidence of CRC. The driving sources for the rise of CRC were obesity, static lifestyle, consumption of redmeat and social drinking. Recent advances in early detection screenings and treatment can reduce CRC mortality rate in developed nations[(5)].

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Human cancer cell lines were used commonly for experimental models, as it continue to have the characteristics features of cancer cells that can be propagated and genetically modified to provide consistent results.[6-10]. Thus, we believe there is a strong correlation between the uses of appropriate cell line for *invitro* preclinical studies of cancer drug responses and to the cancer properties.

Cytotoxicity assays has different parameters associated with cell death and multiplication[11]. which is intensively utilized for the evaluation of toxicology under in-vitro condition[12]. Two major techniques are used to assess the cell growth. The first one is the MTT assay[13] used to measure the activity of living cells through mitochondrial dehydrogenases. The key component is 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) is converted to an insoluble purple formazan. The amount formazan formed is directly proportional to the number of active cells[14]. Thus, MTT investigation indicates the anticancer potential of the test samples against cancer cell lines[15]. The second technique is sulforhodamine B (SRB) assay developed in 1990 investigate the cytotoxicity in cell based studies[16] [17]. The amount of bound dye can be used to measure the cell proliferation. Ashoka i.e. *Saraca asoca* Roxb.de Wilde, syn. *S.indica* belongs to Caesalpinaceae family is an ancient scared tree seen throughout India[18]. According to Ayurvedic system of medicine, *Saraca asoca* is an excellent source of herb that claims to treat several diseases. Several studies has reported that this legendry evergreen tree is useful in treating gynaecological disorders and also possesses antimicrobial, anti-progestational, anti-ulcer, anti-oxidant and anti-cancer activity[19]. As colon cancer cell has a very limited availability of drugs in the market, the current study focus on the bark extracts of *Saraca asoca* aganist HT-29 colon cancer cell line, which resembles the real colon tissues[20] for the evaluation of *invitro* anticancer activity[21] to formulate plant based drug source[22].

MATERIALS AND METHODS:

Collection of sample:

The bark of *Saraca asoca* tree was collected from Anaikatti, Coimbatore, Tamil Nadu, India. The stem bark sample was authenticated by Dr. John Britto, Professor, Department of Botany and Director, Rapinat Herbarium, St. Joseph's College, Tiruchirappalli, Tamil Nadu, India.

Processing of plant material:

The collected bark part was manually cleaned to remove course impurities and washed thoroughly with distilled water and shade dried. The dried bark sample was uniformly grinded using mechanical grinder to make a crude powder stored in airtight container for further use [23][24].

Preparation of extracts:

Extract from the dried stem bark was prepared using ethanol and water by soxhlet extraction method. The residue was left to air dry at room temperature for about 72 hours. The dry residue was stored at 4°C in air-tight bottle.

In vitro cytotoxcity:

Invitro cytotoxcity activity was determined by the standard MTT[25] and SRB assay. The colon cancer cell line HT-29 was procured from ATCC, stock cells was cultured in DMEM supplemented with 10% inactivated Fetal Bovine Serum (FBS). All cells were maintained in a humidified atmosphere of 5% CO_2 at 37°C until confluent.

Cell treatment procedure:

The monolayer cells were trypsinized and the cell count was adjusted to 5.0×10^5 cells/ml with the respective media containing 10% FBS. About 100µl of the diluted cell suspension was seeded to each of the 96 well microtiter plates and incubated for 24 hours to allow cell attachment at 37°C, 5% CO_2, 95% air and 100% relative humidity. After incubation the cells were treated with serial two fold dilutions prepared using DMEM plain media for treatment to obtain final concentrations of 10, 20, 40, 80, 160, 320µg/ml. The final volume in each well was maintained as 200µl. The medium with DMSO alone served as control.

MTT assay:

MTT is a water soluble tetrazolium salt yielding a yellowish solution when prepared in media or salt solution that lacks phenol red. MTT is converted to insoluble purple formazan. The amount of formazan formed indicates the degree of effects caused by the test material. After incubation the test solutions in the wells were discarded and 100µl of MTT (5mg/10ml of MTT in PBS) was added to each well and incubated for 4 hours at 37°C in 5% CO_2 atmosphere[26]. The supernatant was removed and DMSO of 100µl was added and to solubilise the formed formazan the plates were gently shaken. Then the absorbance was read at 590nm using a microplate reader.

SRB assay:

Sulforhodamine B assay does not rely on the metabolic activities of the cell, instead used for the determination of cell density, based on the measurement of cellular protein content[27]. After incubation the test solutions in the well were discarded and 25µl cold 50% (wt/vol) TCA was added gently to each well directly to medium supernatant and incubated at 4°C for 1 hr. Then, 50 µl of

0.04% (wt/vol) SRB solution was added to each well to incubate at room temperature for 1 hr. Now the plate was rinsed adequately with 1% (vol/vol) acetic acid to remove unbound dye. Followed by air-dried and 50% of 19mM Tris base solution (pH 10.5) was added to each well and the plate under agitation in orbital shaker for 10 min to solubilise protein-bound dye. Absorbance was measured at 560nm in a plate reader.

Calculation of inhibition:

The percentage of growth inhibition can be calculated by the following formula:

$$\% \text{ Inhibition} = \frac{\text{OD of control} - \text{OD of sample}}{\text{OD of control}} \times 100$$

For cytotoxicity, lower the IC 50 higher the cytotoxicity[28].

RESULT:

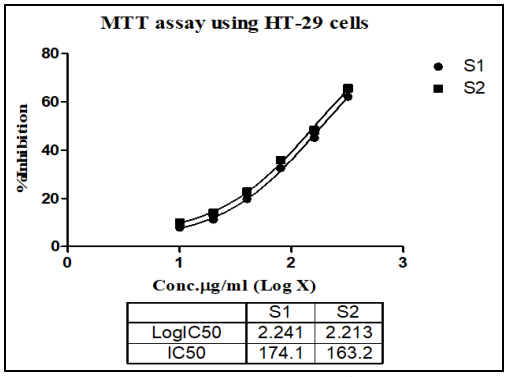
Natural product substances have historically served as the most significant source of new leads for pharmaceutical development[29]. HT-29 colon cancer cell line were seeded and exposed to *Saraca asoca* ethanolic and aqueous extracts at 10, 20, 40, 60, 80, 160, 320 µg/ml of concentration. In MTT assay shows dose-dependent inhibition of cell proliferation in HT-29 cell line by the ethanolic and aqueous bark extracts of *Saraca asoca* (Figure 1). Table1shows the extracts exposure demonstrated a maximum decrease in cell growth by MTT assay on HT-29 cell line of 62.19% and 65.61% of inhibition at 320µg/ml concentration by ethanolic (S1) and aqueous (S2) bark extract, respectively, compared to DMSO control treatment. The IC50 value for MTT has been determined for ethanolic and aqueous extract as 174.1 and 163.2µg/ml, respectively using Graph Pad Prism 6 software.

Table 1: Percentage of cell inhibition of *S.asoca* against HT-29 cancer cell line

Compound name	Conc. µg/ml	OD at 590nm	% Inhibition	IC50 µg/ml
Control	0	0.739	0	
S1	10	0.681	7.88	**174.1**
	20	0.656	11.27	
	40	0.593	19.76	
	80	0.498	32.57	
	160	0.405	45.13	
	320	0.279	62.19	
S2	10	0.666	9.90	**163.2**
	20	0.636	13.97	
	40	0.571	22.77	
	80	0.474	35.83	
	160	0.382	48.31	
	320	0.254	65.61	

Table 2: Percentage of cell inhibition of *S.asoca* against HT-29 cancer cell line

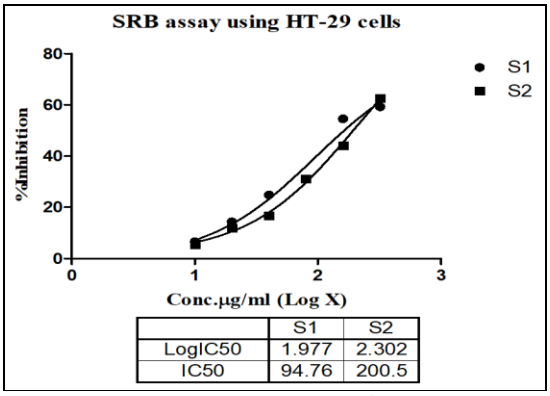
Compound name	Conc. µg/ml	OD at 560nm	% Inhibition	IC50 µg/ml
Control	0	0.439	0.00	
S1	10	0.410	6.58	**94.76**
	20	0.376	14.35	
	40	0.330	24.83	
	80	0.303	30.98	
	160	0.199	54.57	
	320	0.179	59.23	
S2	10	0.415	5.47	**200.5**
	20	0.387	11.94	
	40	0.365	16.86	
	80	0.302	31.12	
	160	0.245	44.15	
	320	0.164	62.55	



	S1	S2
LogIC50	2.241	2.213
IC50	174.1	163.2

Figure 1- Growth inhibition percentage of various *S.asoca* bark extracts concentrations

The SRB assay with the ethanolic and aqueous extracts of *Saraca asoca* bark sample shows maximum cell inhibition at 59.23% and 62.55%, respectively, at 320 µg/ml concentrations (Figure 2). Table 2 presents the IC50 value of both ethanolic (S1) and aqueous (S2) *Saraca asoca* bark extracts as 94.76 and 200.5µg/ml, respectively with Graph Pad Prism software



	S1	S2
LogIC50	1.977	2.302
IC50	94.76	200.5

Figure 2- Growth inhibition percentage of various *S.asoca* bark extracts concentrations

DISCUSSION:

Humankind have been benefitted by plants, herbs, and ethnobotanicals since early days and still used worldwide for health promotion and to treat diseases. Herbal medicines, due to less toxicity, have great demand across the globe[30]. Natural sources and plants are the basis of modern medicine; thereby largely contribute to the preparation commercial drugs[31]. At present the most reliable and available invitro screening techniques used to evaluate the anticancer activity of herbal formulations on cancer cell lines are MTT and SRB assay. The MTT and SRB is used for quantitative and for qualitative analysis, respectively[32][33][34]. The MTT assay is colorimetry method of analysing the color reduction of reagent to estimate cell viability. It determines the cytotoxicity by mitochondrial dehydrogenase activities in living cells. The better linearity of cell number with higher sensitivity is achieved by cell independent staining of SRB assay. In this assay the cell debris does not get stained and so the drug sensitivity data will not get affected [35]. It measures whole protein content that is proportional to the cell number[36].

Cancer is one of the most dreadful diseases worldwide that increases at a progressive rate. Public awareness towards phytopharmaceuticals gains the importance of phytoconstituents as therapeutic agents to provide defensive mechanism[37]. Every system of medicine emphasized the importance of most secondary metabolites from herbal plants were used to cure enumerous diseases like diabetics, cancer, arthritis etc. Hence, phytoconstituents can continue to be used as ideal sources for anticancer drug formulations. In this study, the MTT and SRB methods were used to evaluate the cytotoxicity potential of the *Saraca asoca* bark extracts. Results from those assays revealed that the bark extracts of *S.asoca* explored a very good percent of cell inhibition with increasing concentration of the bioactive component of the test material.

CONCLUSION:

We conclude that the *in vitro* studies reduce the usage of animals for clinical trials. It helps to assess the larger number of compounds with minimum quantity quickly. The findings suggest that the bark extracts of *Saraca asoca* have a potential promising novel anticancer activity against HT-29 cancer cell line. Thus, the overall results indicate the potential use of this indigenous tree as a novel chemotherapeutic agent for the treatment of Colon cancer.

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RESEARCH ARTICLE

Evaluation of Anti-inflammatory and Anti-diabetic activities of *Actinodaphne madraspatana* bedd leaves

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ABSTRACT:

The present study was carried out to find out the *in-vivo* anti-inflammatory and anti-diabetic activities of ethanol extract (200 and 400mg/kg) of leaves of *Actinodaphne madraspatana* (*A. madraspatana*) on Swiss Albino rats. The anti-inflammatory activity was investigated in carrageenan induced rat paw edema model which was compared with standard drug indomethacin at a dose level of 10mg/kg and the parameter measured being the paw volume by mercury displacements at 0, 15, 30, 60, and 120 minutes. The edema was induced in rats by administration of 1 % w/v solution of carrageenan in normal saline solution (1%w/v). The anti-diabetic activity was investigated in streptozotocin induced diabetic rat, which was compared with standard drug glibenclamide at a dose level of 4 mg/kg and the parameter measured being the blood glucose level on 0, 7, 14, 21 days. Diabetes was induced in rats by administration of streptozotocin (60mg/kg) in ice cold citrate buffer (pH 4.3). Results of pharmacological activities revealed that the ethanol extract of the plant leaves showed the significant ($p<0.001$) anti-inflammatory and anti-diabetic activities in a dose of 200mg/kg and 400 mg/kg body weight. The ethanol extract of leaves of *A.madraspatana* possess the anti-inflammatory and anti-diabetic activities.

KEYWORDS: *A.madraspatana* Leaves, Acute toxicity study, Ethanol extract, *In-vivo* pharmacological activities.

INTRODUCTION:

The use of medicinal plants and traditional medicines in developing countries as therapeutic agents for the maintenance of good health is well known in the literature. Medicinal plants containing various phytoconstituents are used to treat animal and human diseases and are considered as a rich resource of pharmacologically active ingredients which can be used in the development and synthesis of new drugs[1].

Medicinal plants play a critical role in the development of human cultures and moreover, medicinal plants, considered as a source of nutrition, and are rich in fiber and antioxidants. Antioxidants compound possesses anti-atherosclerotic, anti-inflammatory, anti-bacterial, anti-viral, anti-carcinogenic, and anti-tumour activities to greater or lesser extent[2]. Medicinal plants have a promising future because there are about one million of plants around the world and most of their biological activities have not investigated yet and their biological activities could be decisive in the treatment of present or future studies[3].

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A.madraspatana is one the most used herbal remedy in the natural medicine. It belongs to the family Lauraceae in the major group of angiosperms (flowering plants). It is commonly known as 'Putta Thali' in Tamil, 'Ray Laurel' in English, 'Irolimarom', 'Mungali' in Malayalam, 'Kovangutti' in Telugu[4]. It is a medium-sized evergreen tree and Shrub, widely distributed on the Rock Hill slopes at higher elevations, Aruku Valley, Vishakhapatnam District, Talakona, Dharmagiri, Microwave station, on the way to Thumburu Theertham. Leaves, flowers and fruits of *A.madraspatana* constitute the phytoconstituents. It is a precursor of vitamin A. The benzene extract of the Heartwood was reported to contain 5,7,8-Trimethoxyflavone[5]. The Leaves of the plant are used traditionally to cure wounds, cure mania, fickle minded behavior and diabetes[6]. Literature survey revealed that there's no scientifically proved report on the anti-inflammatory and anti-diabetic activities of *A.madraspatana*. Hence, the purpose of the current study had been designed to evaluate the anti-inflammatory and anti-diabetic activities of ethanol extract of leaves of *A.madraspatana* experimentally by *in-vivo* methods such as carrageenan-induced rat paw edema model for anti-inflammatory activity and streptozotocin induced diabetic in rats for anti-diabetic activity.

MATERIALS AND METHODS:

Collection and authentication of plant material:

The leaves of *A.madraspatana* were collected from Talakona forest near to Tirupathi and were authenticated by Dr. K. Madavachetty, S. V. University, Tirupati, Andhra Pradesh. A voucher specimen (ACD) has been kept in the Herbarium.

Drugs and chemicals:

Streptozotocin (STZ) was purchased from sigma Aldrich Chemicals, Germany. Carboxymethyl cellulose (CMC) was purchased from M/S Hi-media Ltd, Bombay. Glibenclamide and indomethacin were obtained as gift samples from Accent Pharma, Pondicherry, India. All other chemicals and reagents used in the study were of analytical grade.

Preparation of Plant Extract:

The fresh leaves of *A.madraspatana* were washed thoroughly with tap water and then in distilled water. The washed leaves were a shade dried at room temperature and powdered by the electronic grinder. About 200g of dry powder was extracted in the ethanol by continuous hot percolation using soxhlet apparatus. The extraction was continued for 72 h. The ethanol extract was filtered and concentrated to a dry mass by using rotary evaporator.

Experimental Animals:

Swiss Albino rats (190-250gms) of either sex used for this study. The animals were housed in polypropylene cages in a controlled room temperature 22±10°C and relative humidity of 60-70%. They were kept under standard conditions of 12/12 h light and dark cycle. The animals were maintained with standard pellet diet (Kamadenu Enterprises, Bangalore) and water *ad libitum*. The animals were acclimatized to laboratory condition for seven days before commencement of the experiment. The experimental protocol was subjected to the scrutiny of the Institutional Animal Ethical Committee (Reg.No. 1558/PO/a/11/CPCSEA) and was cleared by the same before starting the experiment.

Acute toxicity study:

Acute toxicity study of ethanol extract of *A.madraspatana* was carried out in Swiss Albino rats of either sex (190-250g) according to OECD (Organization for Economic Cooperation and Development) guidelines No. 423. Extract at different doses upto 2000mg/kg p.o. was administered and the animals were observed for behavioral changes, toxicity, and mortality upto 48 hours[7,8,9].

Evaluation ofanti-inflammatory activity:

Rats of either sex were randomly divided into four groups (I-IV) with six animals in each group (n = 6) and treated as follows. Group I, received a 1ml of 0.5% CMC; Group II, received an ethanol extract (200mg/kg); Group III, received an ethanol extract (400mg/kg); Group IV, received a standard drug indomethacin (10mg/kg). Thirty minutes after drug administration, edema was induced by the injection of 0.1ml of 1% carrageenan solutions in normal saline into the subplantar tissue of the right hind paw. The hind paw volume of the rats was measured using a plethysmograph before injection and at 0 min, 30min, 1 h, 2 h, 3 h, 4 h, 5 h, and 6 h after carrageenan injection[10,11,12,13]. The difference between the initial and subsequent paw volume reading gave the actual edema volume. The percent inhibition of inflammation was calculated using the formula

$$\text{Inhibition (\%)} = \frac{\text{Control - Test}}{\text{Control}} \text{ X } 100$$

Experimental induction of diabetes:

Rats were fasted for 24h prior to the induction of diabetes and diabetes was induced by a single intraperitoneal injection of a freshly prepared solution of streptozotocin (60mg/kg) in ice cold citrate buffer (pH 4.3). 5% Dextrose solution was administered orally for 24 h to prevent mortality due to initial hypoglycemia. After 72 h, fasting blood levels was tested using glucose oxidase-peroxidase reactive strips (One Touch, Life

Scan India, Mumbai, India). Rats showing the fasting blood glucose more than 250mg/kg were considered as diabetic and used for further study[14,15,16,17].

Evaluation of antidiabetic activity:

The diabetic rats fasted overnight and were randomly divided into five groups (I-V) with six animals in each group (n =6) and treated as follows. Group I rats served as normal control and received a citrate buffer (pH 4.3); Group II, received a citrate buffer (pH 4.3); Group III, received an ethanol extract (200mg/kg); Group IV, received anethanol extract (400mg/kg); Group V, received a standard drug glibenclamide (4mg/kg). After administration, anti-diabetic activity was evaluated by collecting blood samples on 0, 7, 14, 21st day of study respectively. Blood samples were collected from snipping tail of rat with the help of sharp razor and each time the tail of the rat was sterilized with spirit. Blood glucose levels were determined using glucometer (One Touch, Life Scan India, Mumbai, India).

Histopathological study:

After the study, rats were sacrificed under anesthesia. Their pancreas was excised immediately, washed in ice-cold normal saline (0.9%w/v) and fixed overnight in 10% formalin solution. Pancreas section was made by microtome, dehydrated in graduated ethanol (50-100%), cleared in xylene and embedded in paraffin. The sections (4-5µm) were stained with hematoxylin and eosin dye and examined with a photomicroscope.

Statistical analysis:

The data are expressed as the mean ±SEM. The statistical analysis was carried out using one-way analysis of variance (ANOVA) followed by Dunnett's test for the multiple comparisons using prism GraphPad version 5.0. The p values less than 0.001 were considered statistically significant.

RESULTS AND DISCUSSION:

Acute toxicity study:

The oral administration of ethanol extract of *A.madraspatana* did not show any behavioral changes, toxicity, and mortality even after 48 hours. The extract was found to be safe at the dose of 2000mg/kg. From the dose response curve, 1/10th and 1/5th of LD_{50} value showed good therapeutic efficacy. Hence 1/10th and 1/5th of doses were selected for the present study[18,19].

Evaluation of anti-inflammatory activity:

Anti-inflammatory activity of ethanol extract of leaves of *A.madraspatana* on on carrageenan induced rats paw edema model were tabulated (Tables 1 and 2). Carrageenan induced rat paw edema is a widely used animal model to screen the anti-inflammatory activity. Carrageenan induced rat paw edema model is used for evaluation of pain at the site of inflammation without any damage or injury to the inflamed paw. There are several mediators involved in inflammation. Serotonin, bradykinin, and histamine are the mediators in the early phase of carrageenan induced inflammation. Prostaglandins (PGs) are the mediators in the late phase of inflammation. Local and systemic inflammation is associated with enhanced levels of the pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin-1 (IL-1), and interleukin-6 (IL-6)[19].

Table 1: Anti-inflammatory activity of ethanol extract on carrageenan induced paw edema

Groups	Initial Paw volume	Mean Paw volume after induction in ml						
		30 minutes	1 Hour	2 hours	3 hours	4 hours	5 hours	6 hours
I	0.09±0.007	0.22 ±0.006	0.29 ±0.009	0.42 ±0.010	0.59 ±0.008	0.63 ±0.008	0.69 ±0.009	0.72 ±0.010
II	0.10±0.006	0.18 ±0.008*	0.22 ±0.010*	0.30 ±0.006*	0.37 ±0.009*	0.36 ±0.007*	0.37 ±0.009*	0.33 ±0.007*
III	0.10±0.007	0.16 ±0.006*	0.20 ±0.006*	0.26 ±0.007*	0.33 ±0.007*	0.33 ±0.004*	0.32 ±0.007*	0.30 ±0.010*
IV	0.11±0.007	0.13 ±0.004*	0.17 ±0.004*	0.20 ±0.010*	0.26 ±0.006*	0.25 ±0.006*	0.26 ±0.006*	0.25 ±0.007*

All values are expressed as mean ± SEM for six animals; p<0.001 compared to negative control; statistically significant

Table 2: Percentage inhibition of paw edema of ethanol extract on carrageenan induced paw edema

Group	Dose in mg/kg	Percentage inhibition of paw edema						
		30 minutes	1 hour	2 hours	3 hours	4 hours	5 hours	6 hours
II	200	18.18	24.14	28.57	37.29	41.27	46.38	54.17
III	400	27.27	31.03	38.10	44.07	47.62	52.66	58.33
IV	10	40.91	41.38	52.38	55.93	58.73	62.80	65.28

The present study revealed that the significant (p<0.001) reduction of paw edema was observed in rats after the administration of both doses of ethanol extract (200 mg/kg and 400mg/kg) and the standard drug indomethacin compared to carrageenan treated rats. The potential mechanism for the anti-inflammatory activity will probably due to the inhibition of release of mediators like Serotonin, bradykinin, histamine, and

prostaglandins. A previous research study with some other plants like *Aegle* marmelo[11], *Trigonella foenum-graecum*[19] and *Zhumeria majdae*[19] also showed the same activity in this model.

Evaluation of antidiabetic activity:

To induce diabetes in animals, streptozotocin is commonly used which produces moderate hyperglycemia with similar clinical symptoms of to type 2 diabetes mellitus. Streptozotocin causes alkylation of pancreatic DNA strands by entering to the islet β-cell *via* low affinity glucose transporter 2 and induces activation of poly (ADP-ribosylation) that causes depletion of cellular adenosine triphosphate, and nicotinamide adenine dinucleotide. As a result, the generated free radicals cause pancreatic β-cells necrosis[20,21].

Table 3 reveals the blood glucose levels of normal, diabetic control, ethanol extract, and drug treated rats. The present study revealed that the administration of streptozotocin to rats significantly ($p<0.001$) increased the blood glucose level when compared to normal control rats due to pancreatic β-cells necrosis. A significant ($p<0.001$) reduction of blood glucose level was observed in diabetic rats after the administration of both doses of ethanol extract (200mg/kg and 400mg/kg) and the standard drug glibenclamide from first to third week compared to diabetic control rats due to regeneration of damaged pancreatic β-cells. The variations in blood glucose level in normal and experimental groups were also recorded. The body weight of the rats was reduced after the streptozotocin administration significantly ($p<0.001$) than normal control rats (Fig. 1), and this action may be due to degradation of structural protein, and fats. A significant ($p<0.001$) increased body weight in diabetic rats was observed after administration of both doses of ethanol extract (200 and 400mg/kg) and in glibenclamide when compared to diabetic control rats[22,23,24,25].

Table 3: Effect of ethanol extract on glucose level in streptozotocin induced diabetic in rats

Groups	Blood glucose level in mg/dl			
	0 day	7th day	14th day	21st day
I	76.5±1.72	77.8±1.70	81.6±1.62	82.3±2.02
II	a299.3± 3.46*	a305.0± 2.98*	a307.5± 2.78*	a313.0± 1.65*
III	b301.5± 1.92ns	b269.8± 2.84*	b239.1± 3.04*	b196.6± 2.98*
IV	b306.0± 2.29ns	b257.3± 2.30*	b213.5± 2.32*	b162.0± 1.75*
V	b301.8± 2.70ns	b244.5± 3.62*	b183.8± 3.62*	b114.1± 1.99*

All values are expressed as mean ± SEM for six animals; a$p<0.001$ compared to normal control; b$p<0.001$ compared to diabetic control; *statistically significant; and ns-non significant

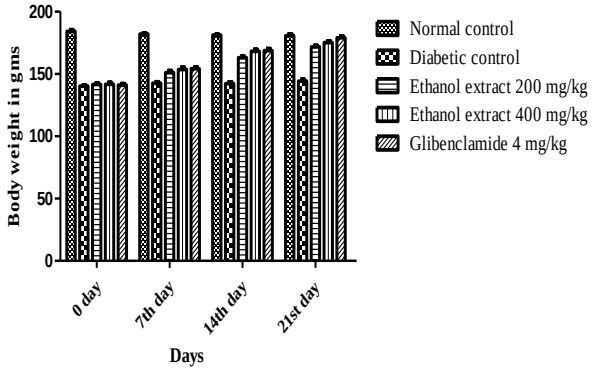

Fig.1: Effect of ethanol extract of leaves of *A.madraspatana* on body weight of streptozotocin induced diabetes in rats.

This action may due to the preventive effect of ethanol extract on structural protein degradation. There are many reports available to support the multiple mechanisms of anti-diabetic plants exerting their blood glucose lowering effects, such as regeneration of damaged pancreatic islet β-cells, inhibition of carbohydrate metabolizing enzymes, enhancement of insulin secretion, release, and sensitivity. The ethanol extract of leaves of *A.madraspatana* may exert the lowering blood glucose level, possibly by above mechanism (s) and the anti-diabetic activity of both doses of the extract was comparable to that of standard drug glibenclamide. The similar anti-diabetic activity produced by some other plants like *Merremia emarginata*[26], *Ipomoea mauritiana*[27], *Chloroxylon swietenia*[28], Phytosaponin[29], *Praecitrullus fistulosus*[30] in streptozotocin induced diabetic model.

Histopathological study:

The anti-diabetic activity of ethanol extract was further confirmed by a histopathological study of the pancreas (Figure 2A-2D). Histology of the pancreas sections of the control rats showed the normal pancreatic β-cell. The pancreas sections of carbon streptozotocin treated rats showed the complete destruction of pancreatic β-cell due to the induction of streptozotocin when compared to normal control rats. The pancreatic sections of ethanol extract treated rats showed an increase in pancreatic β-cell count and remodeling of the structure of the pancreas when compared to the glibenclamide treated and control group's rats.

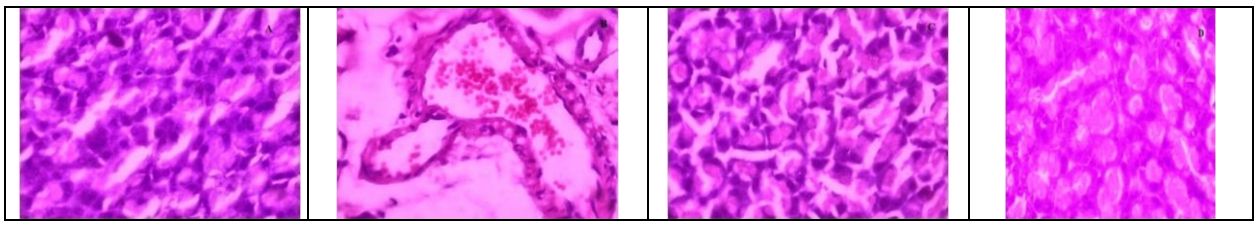

Fig.2: Histopathological slides of pancreas of different animal groups, Normal control (A); Diabetic control (B); Test group (C); Standard control (D)

CONCLUSION:

The results of the present study indicate that the ethanol extract of leaves of *A.madraspatana* possess the anti-inflammatory, and antidiabetic activities and further studies are required to isolate and characterize the active phytoconstituents, which are responsible for the anti-inflammatory and antidiabetic efficacy. This investigation will be a valuable platform for identifying the lead molecules for anti-inflammatory and anti-diabetic activities in future.

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RESEARCH ARTICLE

Investigation of *in vitro* Anthelmintic activity of *Ocimum basilicum* Linn. (Lamiaceae)

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ABSTRACT:
Virudhunagar region of Tamil Nadu is inhabited by common people who are using several plants or plant- based preparations for the treatment of various ailments in their traditional system of medicine. During our course of study on ethnomedicine in this region, it was found that the plant being used as anthelmintic is seed of *Ocimum basilicum* Linn., (Lamiaceae). This plant has a wide reputation among natives of being curative for intestinal-worm infection in the form of alcoholic extract. Based on this an attempt has been made to evaluate the anthelmintic potential of this plant. The Chloroform and Ethanolic extracts of *Ocimum basilicum* Linn, (Lamiaceae) seeds were investigated for anthelmintic activity using earthworms (*Pheretima posthuma*). Various concentrations (25mg/ml, 50mg/ml and 75mg/ml) of plant extract were tested. Albendazole (25mg/ml, 50mg/ml and 75mg/ml) was used as reference standard drug whereas distilled water was used as control. Determination of paralysis and death time of the worms were recorded. The extract exhibited significant anthelmintic activity at a concentration of 50mg/ml. The results show that the ethanolic extract possesses vermicidal activity and found to be effective as an anthelmintic.

KEYWORDS: Ethnomedicine, *Ocimum basilicum Linn.*, *Pheretima posthuma,* Anthelmintic, Vermicide.

INTRODUCTION:
Helminth infections are among the most widespread infection in humans, distressing a huge population of the world. The majority of infection due to helminths are generally restricted to tropical regions and cause enormous hazard to health and contribute to the prevalence of undernourishment, anaemia, eosinophilia and pneumonia[1]. Parasitic diseases cause ruthless morbidity affecting principally population in endemic areas[2]. The gastro-intestinal helminths have become resistant to currently available anthelmintics drugs, therefore there is a foremost problem in treatment of helminths diseases[3]. Hence there is an increasing demand for natural anthelmintics.

Ocimum basilicum Linn.., belongs to Lamiaceae family commonly known as sweet basil. It is 10-30cm long with hairy stems, growing in normal Tropical areas[4]. Leaves are simple, opposite, strongly scented and have a petiole and are ovate up to 5cm long, usually toothed. Seeds are enclosed with flowers, dark brownish to balck in colour, oblong in shape and have a mucilaginous nature[5]. Leaves were useful in inflammation, anthelmintic antioxidant, antimicrobial and snake bite[6]. Seeds are used as antihyperlipidemic[7]. Seeds contain Triterpenoids, Phenyl propanes, Steroids, Omega-3 Fatty acids, Eugenol, 1,8-cineole, Tannins, Saponins and Mucilage[9].

Virudhunagar region of Tamil Nadu is inhabited by common people who are using several plants or plant-based preparations for the treatment of various ailments in their Traditional system of medicine. *Ocimum basilicum* Linn., (Lamiaceae) has a wide reputation among natives of being curative for intestinal-worm infection in the form of alcoholic extract by hot percolation. In the present study, an attempt has been made to evaluate the anthelmintic potential of *Ocimum basilicum* Linn.,

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MATERIALS AND METHODS:

Plant Material:

The seeds of *Ocimum basilicum* Linn, was procured from Virudhunagar region (Tamil Nadu) in March 2018. The plant and plant material were identified and authenticated by Dr. K.N. Sunil kumar, Research officer and HOD of Pharmacognosy, Department of Siddha Central Research Institute, Arumbakkam, Chennai -600 106.The seeds were shade dried, powdered and stored in air tight container and used for further extraction.

Preparation of Extract:

The coarse powder of the seeds were successively extracted with solvents of increasing polarity like n-Hexane, Chloroform and Ethanol by continuous percolation process using Soxhlet apparatus. It was dried and the percentage yield was calculated. Appearance and consistency of the extract were alsonoted.

Worms:

Anthelmintic activity was studied in adult earthworms (*Pheretima posthuma*) by *in- vitro* studies. Earthworms were collected near the farms. The average size of earthworms were 5-7 cm.

Drugs and Chemicals:

Albendazole was used as a standard for the experimental protocol.

Anthelmintic Activity:

The anthelmintic study was carried out by the method of Ajaiyeoba et al[10]. *In- vitro* study was performed using adult earthworms (*Pheretima postuma*) owing to its anatomical and physiological resemblance with the intestinal roundworm parasites of human beings for preliminary evaluation of anthelmintic activity[11,12]. Test samples of the extract was prepared at the concentrations 25mg/ml, 50mg/ml and 75mg/ml in distilled water and two worms i.e., *Pheretima posthuma* of approximately equalsize (same type) were placed in each 9cm petri dish containing above test solution of extracts. Albendazole (25mg/ml, 50mg/ml and 75 mg/ml) was used as reference standard and distilled water as control[13,14]. All the test solutions and standard drug solutions were prepared freshly before starting the experiments. Observations were made for the time taken for paralysis when no movements of any sort could be observed except when the worms were shaken vigorously. Time for death of worms were recorded after ascertaining that the worms neither moved when shaken vigorously nor when dipped in warm water (50^oC)[15]. All the results are furnished in Table 1.

Table 1: Results of Anthelmintic activity

Type of solvent	Concentration used	Time take (min) For Paralysis For Death
Control	--	-- --
Standard	25 mg	32.30±0.46 36.54±1.20
	50 mg	**28.28±1.10 31.40±1.14**
	75 mg	25.20±1.50 29.10±1.40
Chloroform	25 mg	24.25±0.56 25.45±1.10
	50 mg	21.50±1.34 23.10±1.32
	75 mg	17.15±1.56 20.45±1.40
Ethanol	25 mg	19.50±0.52 22.00±1.24
	50 mg	**15.05±1.48 19.06±1.30**
	75 mg	13.40±1.54 17.20±1.52

RESULTS AND DISCUSSION:

From the observations made, higher concentrations of extract produced paralytic effect much earlier and the time of death was shorter for all worms. Ethanolic extract showed anthelmintic activity in a dose dependent manner giving shortest time of paralysis (15 min) and death (19 min) with 50mg/ml concentration. Evaluation of anthelmintic activity was compared with reference standard Albendazole. Preliminary phytochemical screening of extract revealed the presence of Terpenoids, Phenylpropanes, Tannins, Saponins and mucilage.

The experimental evidence obtained in the laboratory model could provide a rationale for the traditional use of this plant as anthelmintic. Our findings confirm that the traditional therapeutic claims for this plant, in near future surely be able to replace the anthelmintic drugs to which there is increased incidence of drug interactions.

CONCLUSION:

The Ethanolic extract of *Ocimum basilicum* Linn., has shown significant anthelmintic activity. From the above results, it is concluded that *Ocimum basilicum* Linn.., used by common people traditionally to treat intestinal worm infections, is proved to have efficient anthelmintic activity.

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RESEARCH ARTICLE

Strategic design of potential siRNA molecules for *in vitro* Evaluation in ABCG2 resistant Breast cancer and *in vivo* toxicity Determination

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ABSTRACT:

Better understanding of breast cancer and drug resistance is possible today due to significant advancements in the field of cell and molecular biology. In this study, we provide a therapeutic approach (Ch-PLGA-siRNA NP) to downregulate the ABCG2 pump, highly expressed in drug resistant breast cancer. *Methods:* We designed an efficient siRNA, evaluated its binding and off targeting properties. Further, formulated and assessed for the in vitro potency and in vivo toxicity. *Results:* Three potential siRNA molecules, satisfying the important criteria with specificity towards ABCG2, were designed and validated. Further the siRNA molecules were delivered to the drug resistant breast cancer cells using the nanoparticles and observed for the levels of reduction in the expression of ABCG2. We then tested the formulation loaded with siRNA for acute toxicity in Swiss albino mice and found to be non toxic in nature. *Conclusion:* This study proved that the designed siRNA molecules as very potent moieties against ABCG2 resistant breast cancer with non toxic profile in vivo.

KEYWORDS: siRNA, ABCG2, breast cancer, resistance and toxicity.

INTRODUCTION:

Breast cancer is a problem of public health importance all over the globe, transcending the economic status of the nations[1]. The global prevalence of diagnosed breast cancer as per GLOBOCAN 2018 report is 2.089 million (11.6%), next only to lung cancer (2.094 million cases/ 11.6% of all new cases)[2]. Breast cancer is not only the number one cancers in women worldwide but also in India, with a high age-adjusted rate of 25.8 per one lakh women[3]. In India, one in every 28 women has a higher chance of developing breast cancer in her lifetime[4]. The incidence of breast cancer in India is found to increase at the age of thirties and is highest in the age group of 50-64 years of age[3][5,6]. Factors such as age at first full term pregnancy, smoking, alcohol, never breast fed, obesity, delayed menopause etc., are the key factors[7,8].

Drug resistant breast cancer consists a subpopulation of the cancer cells not responding to chemotherapeutic agents, either at the beginning of therapy or in due course of treatment, because of 'selective pressures on them by the chemotherapeutic agents'[9]. In spite of several therapeutic options available to treat breast cancer, the process to limit the disease has become extremely tedious off late due to drug resistance[10,11]. Administering chemotherapy with one drug is generally not preferred as it may lead to lethal side effects, its potency might decrease over time and might also lead to resistance to several drugs, and this phenomenon is called as multidrug resistance or cross-resistance. Hence, most often, a combination of two or three of the drugs is used[12]. In light of the above reasons, there is a need to design an alternative treatment modality, targeting the cancer cells and to circumvent these side effects.

ABCB1, ABCC1, and ABCG2 are the genes primarily responsible for breast cancer chemoresistance. Mechanism of action of ABC cassette proteins is ATP driven, in which ATP hydrolyzes to release the drug outside the cell and resort to resting phase[11][13]. Binding

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of these drugs with the overexpressed membrane proteins result in the expulsion of large amounts of drugs such as DOX through these efflux pumps. ABCG2 overexpression leads to resistance to doxorubicin[14]. Small interfering RNA (siRNA) controls the expression of the genes and blocks the translation process. This forms the basis for one of the robust approaches to inhibit the overexpression of ABC transporters[15][16]. Inhibiting the overexpressed pump prevents the efflux of chemotherapeutic drug thereby resensitizing the cells to the drug (Fig 1). In our study, we designed multiple siRNAs' targeting ABCG2 and screened for the potential siRNA candidate with strong knockdown efficiency. Toxicity studies in mice model were also carried out.

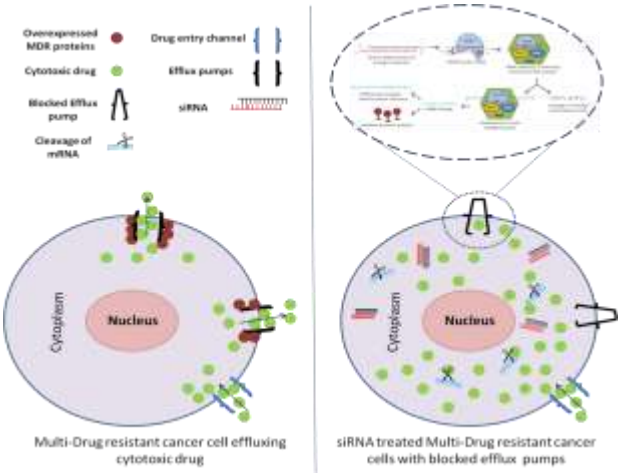

Fig 1: A comparative representation of drug efflux in untreated and siRNA treated drug resistant cancer cells.

MATERIALS AND METHODS:

Chemicals and reagents: Poly (D, L lactide co-glycolide), [Purasorb® PDLG 5002A (MW = 17 kDa), acid terminated, Lactic: Glycolic acid ratio L:G = 50:50] was provided as a gift sample from Purac Biomaterials (Gorinchem, Netherland). Polyvinylalcohol (PVA; 88% hydrolyzed, MW 22000), Dimethylsulfoxide (DMSO), Dichloromethane (DCM), isopropanol, glacial acetic acid, (Purchased from Merck), chitosan **(**140-220 kDa mol wt, ≥75% deacetylated), PBS (Purchased from Himedia), Doxorubicin (Purchased from Cayman chemicals).

Cell lines and culture media:

MCF-7, a human breast cancer cell line was purchased from National centre for cell sciences (Pune, India). Cell line was maintained in DMEM medium containing sodium pyruvate with 10% FBS and 50 µg/ml gentamycin sulfate and incubated at 37°C in 5% CO_2. Doxorubicin was used to induce resistance in the MCF7 cells. The resistant cells were selected by stepwise selection[17].

Design of siRNA:

siRNA sequence designing includes searching of various sequences of siRNA from the computational tools and selection of the most specific sequence following the rules for designing siRNA[18,19].

Prediction of secondary structure:

Secondary structure and free energy (ΔG) of folding for the ABCG2 dicer based siRNA was predicted at 310.15 K (equivalent to 37°C) using RNA structure web server [20].

Calculation of Target-siRNA binding and determination heat capacity:

RNA structure web server was used to determine the binding between target mRNA and siRNA (guide strand) in a thermodynamic manner. Determination of ensemble heat capacity of the designed siRNA as function of temperature with melting temperature Tm (Cp) was performed using the DINAMelt web Server provided by RNA Institute, University of Albany, USA.

Nanoformulation of siRNA:

30mg of PLGA was solubulized in Dichloromethane. 10 nM siRNA, previously solubulized in Tris EDTA buffer was added to the PLGA solution. Then the mixture was briefly sonicated to form a primary emulsion. Later, chitosan in 2% PVA was added and the whole mixture was sonicated to allow the formation of double emulsion. To the double emulsion, another 2ml of PVA was added and stirred overnight to evaporate DCM. The nanoparticles were collected by centrifuging at 12000 rpm at 4° C for 20 min and then the pellet was washed with RNAase free water. The resulting mixture was collected and freeze dried[21].

Cellular uptake/ transfection efficiency of siRNA:

Ch-PLGA nanoparticles loaded with TYE 563 labeled siRNA were prepared and analysed using fluorescent microscopy. Cells were seeded in a six-well plate at a density of 2 x 10^5 cells per well.siRNA containing formulations were incubated for 6 h with predetermined time intervals at 37°c in 5% CO_2. At each interval, the cells were washed with PBS, then fixed with methanol and observed under the fluorescent microscope[22].

Cytotoxicity study:

To evaluate dose dependant cytotoxicity of Doxorubicin, MCF-7 cells and MCF- 7 Dox resistant cells (10 x 10^3cells/well) were seeded in a 96-well plate in 100µl of DMEM medium and incubated for 24 h prior to the test. Drug dilutions of different concentrations were prepared and added to the 96well plate, and incubated for 48 h. Chemosensitivity was assessed using tetrazolium salt MTT assay. MTT was prepared in PBS pH 7.4 and added to each well. The plate was incubated for 4 h, and

then 100µl of DMSO was added to each well. The absorbance was read on a microplate reader at a wavelength of 570nm[23].

Gene expression study:

Total RNA of cells was extracted with the Trizol Reagent at 48 h after transfection. cDNA was generated by reverse transcription of 2µg of total RNA using Verso cDNA synthesis kit in a total reaction volume of 20µl according to the manufacturer's instructions. The primer sequences for ABCG2 gene (Forward) 5'-GGATGAGCCTACAACTGGCTT-3', (Reverse) 5' CTTCCTGAGGCCAATAAGGTG 3' and β2M (Forward) 5'GTCTTTCAGCAAGGACTGGTC 3', (Reverse) 5' CAAATGCGGCATCTTCAAACC 3' were used.Real-time quantitative PCR was performed using Quantstudio -5 Real Time System. A 10-fold dilution of each cDNA was amplified in a 20-µl volume, using the Power SYBR green with reference dye ROX. PCR cycle conditions were; hold stage 50° for 2 min and 95° for 10 min and at PCR stage 40 cycles of 95° C for 15 s and 60° for 60 s. For all the target genes, the mRNA levels were normalized to β2M mRNA levels[24].

In-vivo toxicity of the formulation:

Approval from Institutional animal ethical committee (IAEC) of Manipal College of Pharmaceutical Sciences, Manipal Academy of Higher Education (MAHE) and the permission from committee for the purpose of control and supervision of experiments on animals (CPCSEA) was obtained. *Sighting study:* siRNA nanoparticles were administered to single animal in a sequential manner from Dose FIRST to Dose LAST. The result of the study was to determine the starting dose, study completes if the initial dose for the main study was found. *Main study:* siRNA nanoparticles were administered in a single dose through I.V tail vein. Animals were fasted 3-4 h prior to dosing. After completion of the fasting, the animals were marked, grouped and then dosed. The last dose from sighting study was considered as the initial dose in the main study (0.75mg of siRNA/kg body weight of siRNA) and (Placebo: 150mg of polymeric NP/kg body weight). 24 h break was given between dosing each animal. If the animal survived, then the next animal receives the higher dose. If the animal dies or appears moribund (state of dying) then the next animal receives lower dose. Weights were recorded before initiating the study and after completion of the study. Then all the animals were caged and observed for 14 days for any kind of toxic effects. All the animals were euthanized after the completion of the study. Visceral organs were collected and evaluated for toxicological changes[25].

Statistical analysis:

The data expressed as Mean± SEM values. Statastical analysis was performed using GraphPad Prism Anova. A value of $P<0.05$ is considered as significant.

RESULTS:

Design of siRNA:

Dicer substrate siRNA was designed (27 mer RNA duplex) using IDT software. For the uptake of the guide strand by RISC, maintaining a less stable 5' end is critical. Asymmetric designing of siRNA by generating a 2 nt on 3' overhang for an antisense strand can be effective for dicing. While designing siRNA, the following rules were considered: GC content, thermodynamic parameters, usage of appropriate bases and avoidance of some bases at specific positions. Especially inclusion of U at 10th position of sense strand, absence of G at 13th position of sense strand, inclusion of U at 19th position of sense strand, maintenance of A/U richness in15-19 of sense strand similarly on 5' end of anti sense strand and satisfying the rule of GC content (44, 37 and 44%). BLAST analysis was done to ensure no off target binding. Fig 2 depicts the siRNA sequence generated and it was synthesized by IDT.

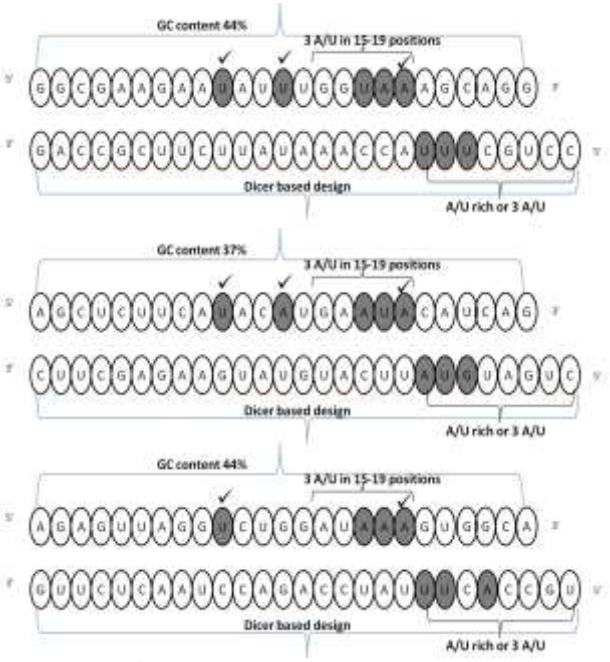

Fig: 2 Design of dicer substrate siRNA

Prediction of secondary structure:

Folding pattern of designed siRNA molecules yielded free energies as follows 2.3 kCal, 1.9 kCal and 2.3k Cal. The predicted secondary structure was depicted in Fig 3.

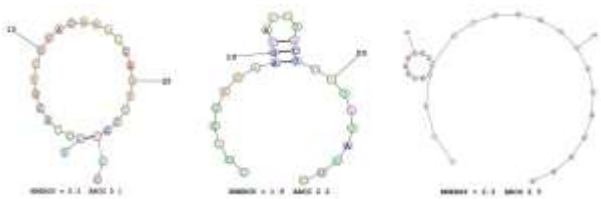

Fig 3: Predicted siRNA secondary structures with free energy for the sequences

Calculation of Target-siRNA binding and determination heat capacity:

Free energy binding of the predicted siRNA molecules with the target mRNA were represented in Fig 4. The binding energy was -40.6kCal, -44.4kCal and -45.3kCal. Heat capacity Tm (Cp), a function of temperature and concentration was observed to be 77.1°C, 80.2°C and 74.8°C.

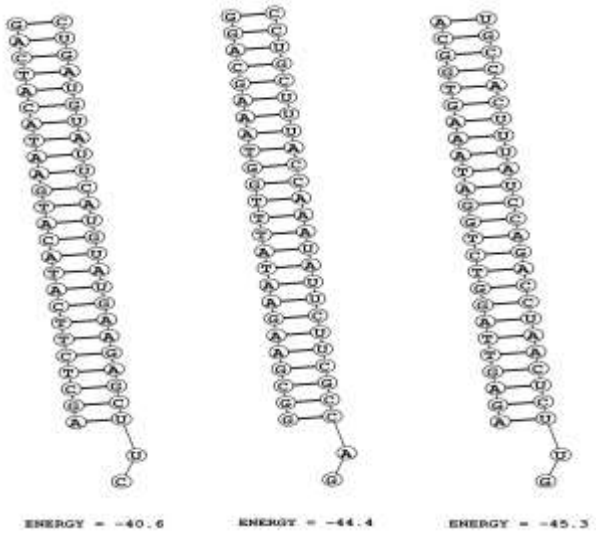

Fig 4 A: Predicted lowest free energy binding structure for the guide strand and target mRNA

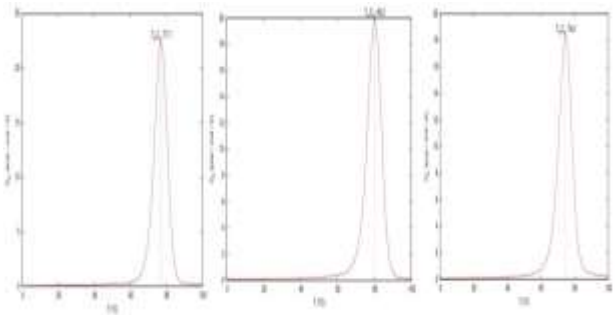

Fig 4 B: Determination of heat capacities of the sequence.

Zeta potential and size of the nanoparticles:

Chitosan tagged PLGA loaded ABCG2 siRNA nanoparticles were synthesized and characterized for size and zeta potential. Zeta potential of nanoparticles was +14.35±5.02 mV, PDI was 0.227±0.063. The particle size was observed using Dynamic light scattering technique and was found to be 215.85 nm±12.94 nm.

Cellular uptake study:

Cellular uptake of the formulation was evaluated on a qualitative basis using florescent microscopy. An hourly basis uptake of TYE 563 labeled siRNA formulation with a gradual increase in siRNA Florescence where maximum accumulation was observed by 6th hour was shown in Fig: 5.

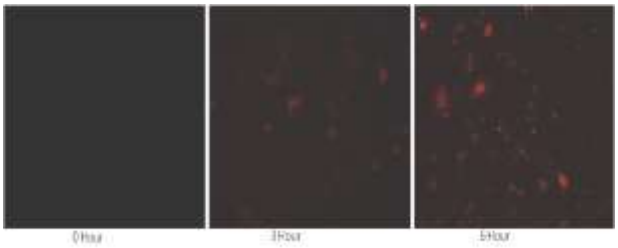

Fig 5: Florescent microscopic observations of cellular uptake of labeled siRNA

Cytotoxicity study:

$IC_{50}$of Doxorubicin alone was 7.04µmol,and in combination with our siRNA formulation, 2.77 µmol, a significant reduction.

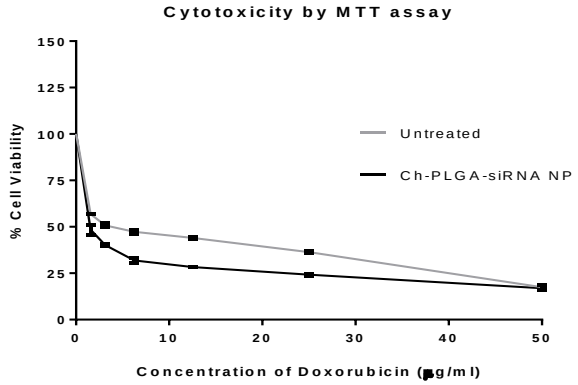

Fig 6: Graphical representation of cytotoxicities of control and treated group

Gene expression studies:

The expression profile of the gene ABCG2 was normalized with β2M (Housekeeping gene) in MCF-7 and Doxorubicin resistant MCF 7. It was observed that the expression levels of ABCG2 gene in resistant cells was 24.44 folds higher compared to the normal cells. But in the presence of siRNA, there was only < 1 fold expression is remaining, depicting >97% knockdown efficiency.

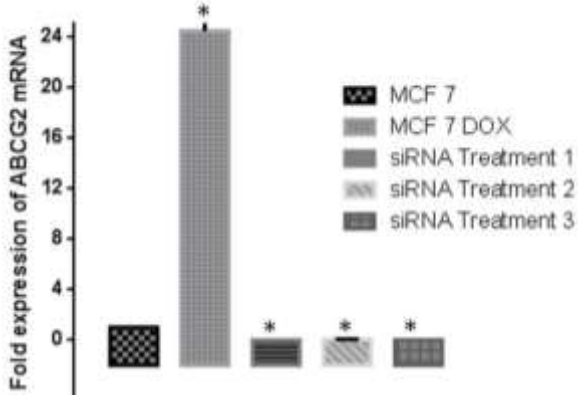

Fig 7: Fold expression of untreated and treated groups of ABCG2 mRNA after normalization with the housekeeping gene

In vivo acute toxicity study:

Nanoformulation was administered intravenously through the tail vein with siRNA and placebo. The maximum concentration 0.75mg/kg ABCG2 siRNA was used. Further increase in dose was not attempted, as this would be sufficient to attain therapeutic efficacy. According to the histopathological inferences and observations from the study the formulation was not having any signs or toxic symptoms or mortality even at a very high polymer concentration (300mg/kg polymeric NP).

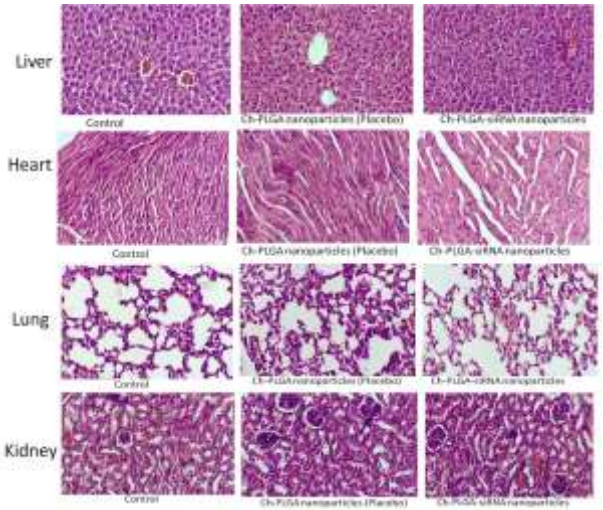

Fig 8: Histopathological findings of acute toxicity of the formulation

DISCUSSION:

To begin with, tumors are responsive to the drugs initially but gain resistance with time. About 80% breast cancer tumors are said to be chemo sensitive initially. They develop cross-resistance to structurally and functionally unrelated drugs. Many small molecules were developed to inhibit the drug resistance but were unsuccessful due to toxicity issues[26-28]. Elevated expression of pumps/transporters is the major cause for efflux of the drug, illustrated in Fig 1. It was proved in clinical trials that over expression of ABCG2 led to failure of chemotherapies and targeted approach (over expression ABCG2/BCRP actively transported Rucaparib, a PARP inhibitor)[29,30]. In the present study, we evaluated ABCG2 mediated resistance in doxorubicin resistant breast cancer (MCF7- DOX) and developed an approach to down regulate the over expressed ABCG2 pump using siRNA. We observed a strong knockdown of ABCG2 gene expression in our expression studies, supported by cytotoxicity study as well. Further, the siRNA loaded nanoformulation was evaluated for toxicity in animal studies. The siRNA loaded nano formulation was non toxic.

To design the siRNA, specifically targeting the desired gene of interest, we followed a strategic way of designing. Sufficient number of studies experimentally proved that the potency and functionality of siRNA were significantly improved with a dicer substrate siRNA[19][31]. Therefore, our study aimed in developing a dicer substrate siRNA (i.e 27 mer siRNA) that efficiently targets and produces a strong knock down potential with no off target effects. To design the dicer substrate siRNA targeting ABCG2 gene, mRNA sequence of the gene was retrieved from NCBI database and was used to generate sequence specific siRNA. We designed three siRNA molecules and validated for all the important criteria for improving gene silencing efficiency. We analyzed the knock down efficiency of siRNA molecules through gene expression studies and cytotoxicity studies as well. The GC content of our designed siRNA molecules were 37, 44 and 44%. We designed Dicer substrate siRNA- 27 nucleotides in the guide strand and 25 nucleotides in the passenger strand by allowing 2 nucleotides on 3' overhangs of antisense strand to make the siRNA further more potent, as this terminal end structure helps in proper loading of siRNA into RISC[32,33]. Presence of two nucleotides on 3' overhangs of antisense strand makes the site as an excellent site for the RISC processing and the blunt end gives asymmetry[18]. This approach also serves as an advantage in preventing the consideration of passenger strand as guide strand due to its thermodynamically unstable ends[34]. In this effort of producing a highly functional siRNA, features that facilitate the guide strand selection by the RISC along with an additional parameter to predict the secondary structure of siRNA was also employed, as these factors impact potency and stability [20]. In this regard, minimum free energy (ΔG) of the siRNA was the determinant of secondary structure, and the folding energies of the designed siRNA molecules were 2.3 kCal, 1.9 kCal and 2.3 kCalat 37.0°C. Off targeting is a major hurdle in generating a successful siRNA candidate. If the designed siRNA is not precise enough, it can cross match with multiple genes and may have higher chances to induce off target effects. Therefore, BLAST was performed to refrain cross match/homology match. Targeting inaccessible sites or secondary structures of mRNA can also compromise the efficacy. Using computational tools to evaluate the folding properties can enhance the design of the siRNA [35]. Knockdown, another determinant of efficacy, depends on the interaction of siRNA with target mRNA. Binding site plays an important role in determining the downregulating potential. A critical parameter to consider here is to choose the mRNA binding site in the coding sequence that is 50-100 nucleotides from start codon. In our study, all the siRNA molecules targeted on the coding region[36]. The binding locations on mRNA were in the range of (siRNA sequence 1) 2101-2125,

(siRNA sequence 2) 1900-1924 and siRNA sequence 3) 1040-1064 and acomputational tool (Bifold) was used to hybridize target mRNA and siRNA to determine the lowest binding free energy[20,37]. In our study, the free energies of the siRNA molecules were -40.6 kCal, -44.4 kCal and -45.3kCal, lower enough to bind with target mRNA, thereby preventing translation as depicted in Fig 3. Heat capacity (Cp), as a function of temperature and concentration is used to determine melting temperature (Tm) of the hybridized nucleic acids considering temperature[38]. siRNA with higher Tm can make it functional. In our study, the melting temperatures (Tm) were 77.1, 80.2 and 74.8°C.

Delivery of the siRNA with high specificity and keeping the siRNA intact by preventing the degradation mediated by nucleases are major obstacles. siRNA is highly polyanionic and will not diffuse in the cell membranes easily. In this study, we utilized biologically compatible polymers. PLGA, a polymer approved by FDA for its use in delivery[39,40] and another coating polymer, chitosan were used in this study. Chitosan coating improves the surface charge properties by modifying the overall charge of the particles. Usage of chitosan can also have a favorable impact in the incorporation of negatively charged siRNA[21][41]. Improved transfection efficiency is the major determinant of gene silencing potential. Through cellular uptake study, using a TYE 563 labeled siRNA, we observed that chitosan coated formulation had better transfection efficiency. This could be due to adsorptive endocytosis[42]. The size of the nanoparticles can influence the intracellular uptake[43]. Studies state that intracellular uptake of nanoparticles is influenced by the size of the nanoparticle; the lower the size of nanoparticles, the easier it is internalized, as the small particles have a larger surface area which enables them to be in contact with the biological membranes[22]. With our ABCG2 siRNA formulation the mean particle size of our formulation was observed to be 215.85 nm±12.94 nmand the zeta potential was observed to be +14.35±5.02 mV. Zeta potential of the coated formulation was positive which implies that chitosan was layered on the negatively charged PLGA core due to its electrostatic attraction[44]. Stability of the unprotected or unmodified siRNA delivered through bloodstream is prone to undergo degradation by nucleases. siRNA degradation studies conducted earlier proved that it was immediately degraded in serum but was stable at elevated temperatures and freeze-thaw cycles[45]. We also experienced similar pattern in our study where naked siRNA underwent instantaneous degradation but the nanoparticles resisted serum nuclease degradation. The knockdown potential of the designed dicer based siRNA finally depends on the ability to silence the expression of ABCG2. In our study, the dicer based siRNA produced a knockdown potential >97%. The 27nt siRNA serves as a substrate for the Dicer, where it integrates and introduces 27nt siRNA into RISC in proper orientation that is essential for strong knockdown potential[18]. Additionally presence of 2 nt on 3' overhang of guide strand produces homogenous dicer products rather than blunt 27nt siRNA which reduces the off targeting[46,47]. According to the OECD guideline, the acute toxicity studies of the formulation were conducted in Swiss Albino female mice. Observations such as changes in skin, fur, eyes or any behavioral changes like salivation, tremors, convulsions diarrhea were not found when compared to control group[48]. As per the physical and histopathological observations the formulations did not producetoxic symptoms. Maximum tolerable dose of the formulation could be very much higher than 0.75mg siRNA/kg body weight and 300mg polymer NP/kg body weight (placebo nanoparticles)[25].

Strategic designing of siRNA molecules, satisfying the essential designing criteria, serves as good approach for down regulation of the over expressed genes with a strong knockdown efficiency. Specificity, safety and potency are the major factors to be considered for formulating siRNA nanoparticles. In our study, three siRNA molecules targeting ABCG2 gene, were designed and delivered using Chitosan coated PLGA nanoparticles. The carrier we used for siRNA delivery was tested for toxicity and found to have no apparent toxic effects. We also observed that our designed siRNA had a better binding ability with the target gene and produced a strong knockdown efficiency.

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RESEARCH ARTICLE

Development and Validation of Spectrophotometric Estimation of Vildagliptin through Oxidative Coupling Reaction using MBTH Reagent in Pharmaceutical Dosage Form

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ABSTRACT:

Scientifically estimation of Vildagliptin in bulk and pharmaceutical dosage forms by 3-methyl-2-bezothiazoline hydrazone reagent using longer wavelength give strong absorbance. The purpose of this analytical validation procedure is to validate using ICH guideline.3-methyl-2-bezothiazoline hydrazone reacts with ferric chloride in the presence of amine group, Resulting green coloured species scanned in uv-visible spectrophotometric with the range 400-800nm. Linearity concentration ranges10 to 50µg/ml and it given absorbance at 620nm, Correlation coefficient value 0.998, Recovery studies obtain within range of 98.41 to 99.75% at maximum absorbance 620nm. This method can be successfully applied for the estimation of drug content in pharmaceutical formulations. Resulting Analytical Validate method suitable for its intended use.

KEYWORDS: 3-methyl-2-benzothiazoline hydrazone, Vildagliptin, Ferric chloridespectrophotometric determination, ICH guidelines.

INTRODUCTION:

Vildagliptin is an oral Anti hyperglycemic agent that enhance pancreatic islet cell.[1] known as dipeptidyl peptidase-IV inhibitors (DPP-IV), inhibits the inactivation of GLP-1 by DPP-IV, allowing GLP-1 to potentiate the secretion of insulin in the beta cells, It has a chemical name of (2S)-{[(3-hydroxyadamantan-1-yl) amino]acetyl}pyrrolidine-2-carbonitrile reported in figure1. Half life 1.5 - 4.5 hr. Vildagliptin is acyanopyrrolidine-based, coupling reaction mechanism of MBTH with vildagliptin reported in figure 2.[2] Vildagliptin cyano moiety undergoes hydrolysis, execrated from kidney, minimum metabolized by the major Cytochrome p450 enzymes. Vildagliptin molecular Formula of $C_{17}H_{25}N_3O_2$, molecular weight of 303.406g/mol, white Amorphous with a soluble in distilled water, freely soluble in methanol. Absorption maximum at 620 nm and Beer's law is obeyed in the concentration range of 10-50µg/ml.

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Vildagliptin react with MBTH losses two electrons and one proton it form an electrophlic intermediate and it is highly coloured species, then couples with Vildagliptin to form a green coloured chromogen which measured at 620 nm.

The purpose of development and validation of analytical methods is to demonstrate that it is suitable for its intended purpose. Vildagliptin react with 3-methyl-2-benzothiazolinone hydrazone hydrochloride reagent and ferric chloride to form green colored chromogen.[3]

MBTH react with aldehyde first to form an azine, remaining MBTH, it is oxidized to another species which combines with the azine to form formazan. so, if there is enough aldehyde, all the MBTH is converted to azine and there is no formation of blue color. Thus, by using the limiting agent MBTH to test the amount of aldehyde around the point of interest, then less aldehyde would produce more blue color and more aldehyde would produce less blue color. This method is especially useful in the quality control of aldehyde products. The end color may be different depending upon the order of addition of the reactants. For example, if an oxidizing

agent and MBTH are mixed before adding the aldehyde, a light green to green color results.

There is no visible spectrophotometric oxidative coupling reaction method has been reported for vildagliptin in tablet dosage form.[4-12]

Main Objective is to develop and validate visible spectrophotometric estimation by oxidative coupling reaction using MBTH reagent.

Vildagliptin give weak UV absorbance at maximum wavelength 204nm.Therefore reported analytical method has low selectivity and sensitivity, longer wavelength give strong absorbance

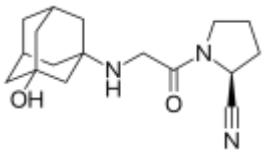

Figure 1: Chemical Structure of Vildagliptin

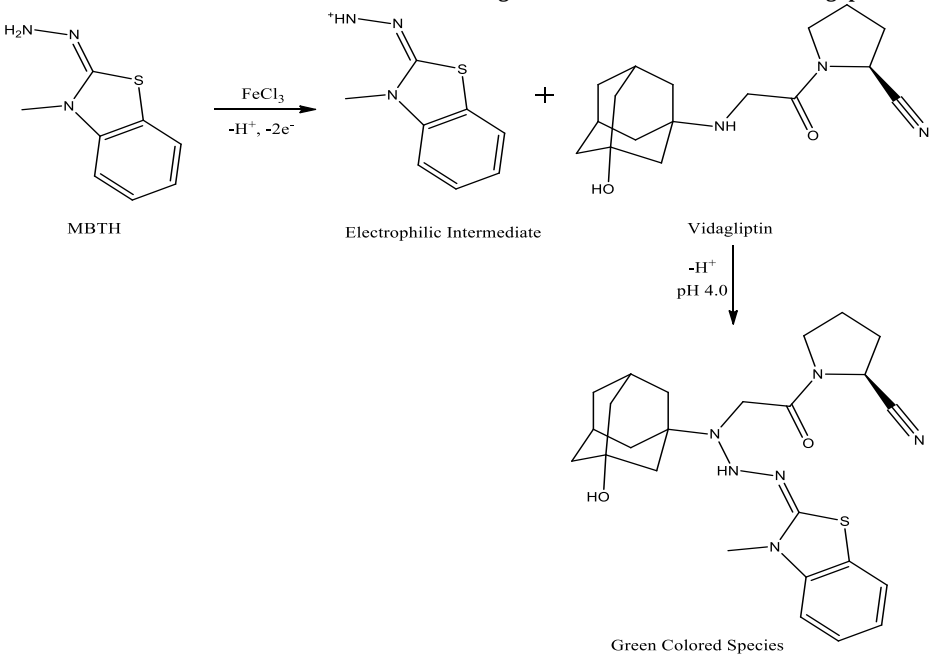

Figure 2: Coupling reaction mechanism of MBTH with Vildagliptin

MATERIALS AND METHODS:

Chemicals and reagents:

Vildagliptin sample was obtained from Cadila Pharamceuticals Pvt. Ltd. Dholka, Gujarat.

MBTH reagent obtained from Sisco Research laboratories pvt.ltd, Ferric chloride anhydrous obtained from Dev enterprise, Hydrochloric acid obtained from Umiya chemical industry. and all other chemicals were of analytical grade.

Jalra (Vildagliptin 50mg), tablets were manufactured by NOVARTIS, and were purchased from local market.

Instruments:

UV-VISIBLE Spectrophotometer (SCHIMADZU 1800), Digital balance (Reptech RA 123).

Preparation of MBTH (0.2%w/v):

Weigh accurately 200mg of (MBTH), dissolved in 10ml distilled water.

Preparation of 0.7% ferric chloride:

Weigh precisely 0.7g of Ferric chloride anhydrous dissolved in 100ml 0.1N hydrochloric acid.

Preparation of stock solutions:

Weigh precisely 100mg of Vildagliptin than it dissolved in 40ml of methanol in 100ml volumetric flask and make up the volume up to the mark with methanol. i.e. 1000µg/ml (Stock solution). 10ml of withdraw out from above stock solution and add into 100ml volumetric flask and make up the volume up to the mark with methanol to get the final concentration of 100µg/ml. The absorbance of resulting coloured solution was measured against respective blank solution in visible region, i.e., 400-800nm which showed a maximum absorbance at 620nm.

Calibration curve of vildagliptin:

From final stock solution, pipette out fresh aliquots from 1 to 5ml were transferred into a series of 10ml volumetric flasks to make up final concentration which ranges from 10 to 50µg/ml. To each flask, add 1.5ml of (0.2%) MBTH reagent, than add 2ml of (0.7%) Ferric

chloride solution and resulting solution was heated for 15 min and add finally 1ml (0.5N) HCl solution. Cool the solution at room temperature and make up the volume up to mark with distilled water. The absorbance of Green coloured chromogen was measured at 620nm against the reagent blank. The color species observation stability for 24 hr. Concntration of Vildagliptin present in the sample solution was calculated from its calibration curve reported in Figure 4.

METHOD VALIDATION[4]:

Linearity is the ability within a given range to obtain test results which are directly proportional to the concentration of analyte in the sample. Correlation coefficient, y-intercept, slop of the regression line and residual sum of squares should be submitted. The response of the drug was found to be linear in the investigational concentration range 10-50µg/ml. The calibration curve was found to be linear with an r^2 value 0.998 and regression equation was y=0.004x-0.065.

Detection limit of an individual analyte procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantify.

Quantitation limit of an individual analyte procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy.

$LOD=3.3\sigma/S$

$LOQ=10\sigma/S$

Where, σ = standard deviation of the response (intercept) S = slope of the calibration curve The mean of the slope and standard deviation of response were obtained after plotting three calibration curves. The optimum conditions such as Beer's law limits, molar absorptivity and other regression characteristics like slope (m), intercept (c), correlation coefficient were calculated.

Accuracy of analytical method is the closeness of test results obtained by that method to the true value. Accuracy for drug substance was determined at varying concentration levels in the range of (50-150 %) 25µg/ml, 50µg/ml, 75µg/ml by analyzing three replicates of each sample as a batch in a single assay. %RSD was calculated and reported in Table 3. To study of accuracy 10 tablets (Vildagliptin 50mg) were weighed, powdered and estimation was carried out. Recovery studies were carried out by adding known amount of standard drug solution (25, 50, 75µg/ml) to the sample solution (50µg/mL). The % recovery was calculated and reported in the Table 4. The obtained values of recovery studies were in the range of 98.41 to 99.75% which indicates that the proposed method is accurate. ICH demands that 'a good accuracy value' should be in the range of 98-102% of the true value.

Precision of an analytical method is the degree of agreement among individual test results, when the method is applied repeatedly to multiple samplings of homogeneous sample. Indication of random errors results and Relative Standard Deviation (%RSD). Repeatability performed by analyzing six replicates of single concentration that is 30µg/ml. The % relative standard deviation (RSD) was calculated and reported. Variations of results within the same day (intraday) and variation of results between days (inter day) were analyzed and reported the value. The intra-assay precision performed of drug solutions at varying concentration levels (10µg/ml, 30µg/mL, and 50µg/mL) by analyzing three replicates of each sample as a batch in a single run at 620nm. The inter-assay precision was determined by analyzing the same samples (10µg/ml, 30µg/ml, and 50µg/ml) in three consecutive days at 620nm. The %RSD was calculated.

Estimation of Vildagliptin in tablets using MBTH Reagent:

Take an average weighed of twenty tablets containing Vildagliptin. An accurately weighed of powder equivalent to 100mg of Vildagliptin was dissolved in a 100ml of methanol and mixed for about 5 min and then filtered. 10ml of aliquots was pipetted out into 100ml volumetric flask and the volume was made up to the mark with methanol to obtain the final concentration of 100µg/ml (Stock solution). Subsequent dilutions of this solution were made with methanol to get concentration of 10 to 60µg/ml and were prepared as above and analyzed at the selected wavelength, 620nm and the results were statistically validated.

RESULT:

Linearity study for Vildagliptin:

From stock solution B, pipette out fresh aliquots of Vildagliptin ranging from 1 to 5ml were transferred into a series of 10ml volumetric flasks to make up final concentration which ranges from 10 to 50µg/ml. To each flask, add 1.5ml of (0.2%) MBTH solution after than add 2ml of (0.7%) Ferric chloride solution and resulting solution was heated for 15 min and add finally 1ml (0.5 N) HCl solution. Cool the solution at room temperature and make up the volume up to mark with distilled water. The absorbance of Green coloured chromogen was measured at 620nm against the reagent blank. The color species was stable for 24 h. The quantity of Vildagliptin present in the sample solution was calculated from its calibration curve reported in Figure 3.

Table 1: Linearity and range data for Vildagliptin

Parameters	Result
Linearity-range	10-50 µg/ml
Regression Equation	y=0.004x + 0.065
Correlation coefficient	0.998

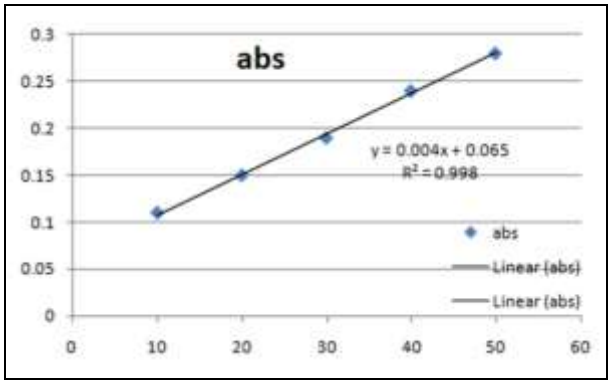

Figure 3: Calibration curve of Vildagliptin with MBTH Reagent.

Accuracy [Recovery Study]:

Vildagliptin which was dissolved in methanol than react with 3-methyl-2-bezothiazoline hydrazone (MBTH) reacts with the amine group in the presence of oxidizing agent, ferric chloride. The resulting apple green coloured chromogen was measured spectrophotometrically in visible region (i.e., 400-800nm). Absorbance of the sample was scan at 620 nm. Concentration levels in the range of 50-%-150% (25µg/ml, 50µg/ml, and 75µg/ml) by analyzing three replicates of each sample as a batch in a single assay. The %RSD was calculated and reported in Table 2.

Precision:

Precision was determined as intra-day and inter-day variations. Intra-day precision was determined by analyzing Vildagliptin (10, 30, and 50µg/mL) for three times on the same day. Inter-day precision was determined by analyzing the same concentration of solutions for three different days over a period of week. The results are reported in (Table 3 and 4.)

Table: 2 Recovery Data of Vildagliptin

Level	Sample conc. (µg/ml)	Amount of Drug added (µg/ml)	Total conc. (µg/ml)	Amount of drug recovered ((µg/ml)	% Recovery	Mean ± S.D.	% RSD
50%	50	25	75	74.98	99.97	99.96333±0.020817	0.02
	50	25	75	74.96	99.94		
	50	25	75	74.99	99.98		
100%	50	50	100	100	100	99.48±0.695485	0.69
	50	50	100	98.69	98.69		
	50	50	100	99.75	99.75		
150%	50	75	120	118.20	98.5	98.83±0.651076	0.65
	50	75	120	118.10	98.41		
	50	75	120	119.15	99.58		

Table 3: Intraday Precision Data for Vildagliptin

Concentration (µg/ml)	Mean abs ± S.D. (n=3)	%RSD
10	0.99	0.92
30	0.78	0.70
50	0.54	0.52

Table 4: Interday Precision Data for Vildagliptin

Concentration (µg/ml)	Mean abs ± S.D. (n=3)	% RSD
10	0.89	0.08
30	0.53	0.04
50	0.67	0.02

Repeatability:

Repeatability assessment of an analytical method is performed by analyzing six replicates of single concentration that is 30µg/mL. Absorbance of samples is recorded at 620nm.The % relative standard deviation (RSD) was calculated and reported.

Table: 5 Repeatability data for Vildagliptin

Sr. No.	Concentration(µg/ml)	Absorbance
1	30	023
2	30	0.23
3	30	0.23
4	30	0.23
5	30	0.23
6	30	0.23
Mean		0.228333
SD		0.004082
%RSD		1.8

Table: 6 Summaries of Vildagliptin Parameters

Parameters	Vildagliptin
Linearity Range (µg/ml)	10 to 50 µg/ml
Correlation Coefficient (r^2)	0.998
Slope	0.004
Intercept	0.065
Repeatability	0.22833±0.004
Accuracy (% Recovery)	98.41-99.75%
Interday precision (% RSD)	0.02-.0.08%
Intraday precision (% RSD)	0.52-0.92%
LOD(µg/ml)	7.5 µg/ml
LOQ(µg/ml)	23 µg/ml

RESULT AND DISCUSSION:

Vildagliptin which was dissolved in methanol than react with 3-methyl-2-bezothiazoline hydrazone (MBTH) reacts with the amine group in the presence of oxidizing agent, ferric chloride. The resulting apple green coloured chromogen was measured spectrophotometrically in visible region (i.e., 400-800nm). Absorbance of the sample was scan at 620nm. concentration levels in the range of 50-%-150% (25µg/ml, 50µg/ml, and 75µg/ml) by analyzing three replicates of each sample as a batch in a single assay. The %RSD was calculated and reported in table 2. To study the accuracy of drug product, 10 tablets (Vildagliptin 50mg) were weighed, powdered and estimation was carried out. The obtained values of recovery studies were in the range of 98.41 to 99.75%

which indicates that the proposed method is accurate. The response of the drug was found to be linear in the investigational concentration range 10-50µg/ml. The calibration curve was found to be linear with an r^2 (correlation coefficient) value 0.998 and regression equation was y=0.004x-0.065 reported in Table 1. Repeatability assessment of an analytical method is performed by analyzing six replicates of single concentration that is 30µg/ml. Absorbance of samples is recorded at 620nm. The % relative standard deviation (RSD) not more than 2 reported in Table 1. The intra-assay precision of the proposed method was determined on samples of drug solutions at varying concentration levels (10µg/ml, 30µg/ml, and 50µg/ml) by analyzing three replicates of each sample as a batch in a single assay run at 620nm. The inter-assay precision was determined by analyzing the same samples (10µg/ml, 30 µg/mL, and 50µg/mL) in three consecutive days at 620nm in Table 4 and Table 4. %RSD was calculated. LOD Was found out to be 7.59µg/ml. LOQ was found out to be 23µg/ml in Table 6. An accurate, precise, repeatable and specific Oxidative coupling reaction method was developed by visible spectrophotometric determination and r^2 =0.998 was found from Calibration curve, Recovery studies from validation parameter was close to 100% and %RSD was not more than 2%.

There is no any interference from the from tablet dosage form.

The proposed method can be applied successfully for analysis of vildagliptin for quality Assurance and analysis of single dosage form.

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RESEARCH ARTICLE

A Novel Method of CFIA / Merging zones technique for assay of Doxycycline in Bulk and Pharmaceutical preparation depending on Azo dye Formation

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ABSTRACT:
A simple, automated and sensitive flow-injection analysis/merging zones technique (MZ-FIA) method was developed for the determination of doxycycline hyclate (DOX) in pure and pharmaceutical formulations. This method Involved the reaction of AzoAnthranilic Acid with DOX to produce an orange - yellowish dissolved complex which has maximum absorbance at 468nm. The method was sensitive, precise and accurate were the limit of detection was less than (0.3µg/ml) and the RSD% was less than 1% and Recovery was 98-100%. Various chemical and physical conditions that affected the reaction have been studied. The calibration curve was rectilinear within the concentration range 5-125µg/mL with a sample through put of 80 sample/hour. The proposed procedure was applied successfully for the estimation of DOX and the results obtained were favorably compared with those given by a reference method of British Pharmacopeia, and there was no significant difference between the obtained results, regarding accuracy and precision at the 95% confidence level.

KEYWORDS: Azo dye, CFIA, Spectrophotometric detection, Doxycycline.H_2O, Anthranilic Acid.

INTRODUCTION:
Doxycycline ($C_{22}H_{24}N_2O_8 \cdot H_2O$), M.wt= 462.5 g mol^{-1}, CAS number: 17086-28-1), is the monohydrate of (4S,4aR,5S,5aR,6R,12aS)-4- (dimethylamino)-1,4,4a,5,5a,6,11,12a- octahydro-3,5,10,12,12a-pentahydroxy-6-methyl-1,11--dioxonaphthacene-2-carboxamide, a substance gains from oxytetracycline or metacycline or by other abject[1]. It is an extensive specter anti-bacterial tetracycline derivative with a broad range of activity against gram positive and gram negative organisms, intercept Spirochetes, Actinomyces sp., and Mycoplasma[2]. It is the physic of option in the treat of sexually transmitted diseases[3].

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Doxycycline is promoting to other tetracyclines in the management of limited infections forasmuch as of its clearly certain absorption and its extended half-life, which permits less frequent dosage[4]. Doxycycline hyclate ($C_{22}H_{24}N_2O_8 \cdot HCl \cdot 0.5C_2H_5OH \cdot 0.5\ H_2O$, molecular mass 512.94 g mol^{-1}, CAS number: 24390-14-5) is the hydrochloride hemiethanol hemihydrate of doxycycline. The synonym for doxycycline hyclate is doxycycline hydrochloride. Doxycycline hyclate is much more dissolvable than doxycycline monohydrate, which is one of the chief purpose for it more frequent employment in pharmaceutical samples[2].

The drug is official in the British Pharmacopoeia (BP)[1] and the United States Pharmacopoeia (USP)[5], which describes HPLC methods for the determination of DOX either in raw material or in pharmaceutical formulations. Also DOX is official in Indian pharmacopeia[6] European pharmacopeia[7] and Japanese pharmacopeia[8]. Several methods have been reported for the determination of DOX in pharmaceutical dosage forms including cloud point extraction[9], liquid chromatography[10] sequential injection chromatography[11], and capillary electrophoresis[12]. spectrophotometric methods[13] FIA-spectrophotometry with copper carbonate[14], FIA-Chloramine-T[15] and FIA- *o*-nitro phenol[16] and also based

on Spectrophotometric methods contains: color reactions with thorium (IV)[17], sodium cobalt nitrite[18] and uranyl acetate[19]. Besides, kinetic spectrophotometry using Cu(II)/H_2O_2[20] and multivariate calibration method[21].

The chromatographic techniques[22-24] and spectrophotometric methods are most widely used. Although the procedures are specific, most of the described methods are time consuming and require multistage extraction procedures. On the other hand, the reported spectrophotometric methods suffer from one or the other disadvantage such as poor sensitivity, use of organic solvent, the problems of extraction scrupulous control of experimental variables and special equipment, or small ranges.

The proposed method of Flow Injection analysis (MZ-FIA) is simple, accurate and do not need for farther treatment for the samples as well as it is not costly and do not required an expensive or toxic reagents, beside short time required for the analysis with highly sampling per hour.

Experimental Apparatus and Manifold:

All of spectral absorbance quantifications were applied on an Optima VIS 9200, digital single beam that record spectrophotometer with (1cm quartz) cell. The Flow cell (quartz silica, 1cm) with 100µL internal volume is inside the detection unit and (1cm) an optical path length using for the absorbance measurements as peak height expressed in mV(n=3).

A one channel manifold Flow Injection Analysis - merging zones for spectrophotometer estimation of DOX. A power supply (Yaxun, 1501AD, China) with Peristaltic pump (Master flexC/L, USA) that using for pump a carrier stream (distilled water) and solutions were passed through the injection valve that (homemade); five-three-way injection valve that contain towloops were made of Teflon. chemicals and reagents solutions which based on merging zones version. The injection valve that used to supplied suitable volumes that were injected of standard solutions and samples. The tubes were made of flexible vinyl with 0.22mm (I.D) using for the peristaltic pump; mixing coil that was manufactured from glass with 2mm (I.D). All of parts of the CIFA as shown as in (Fig.1) with details.

A carrier stream was distilled water that was joined with injected sample (DOX in NaOH solution in L1) and merged with the reagents (Azo-Anthranlic Acid in L2). Then mixed it in mixing coil that it has length of 50cm, flow rate of distilled water (carrier) 3.2mL.min^{-1}. The maximum absorption was found under 468nm for Azo Anthranilic-DOX as peak height in (mV).

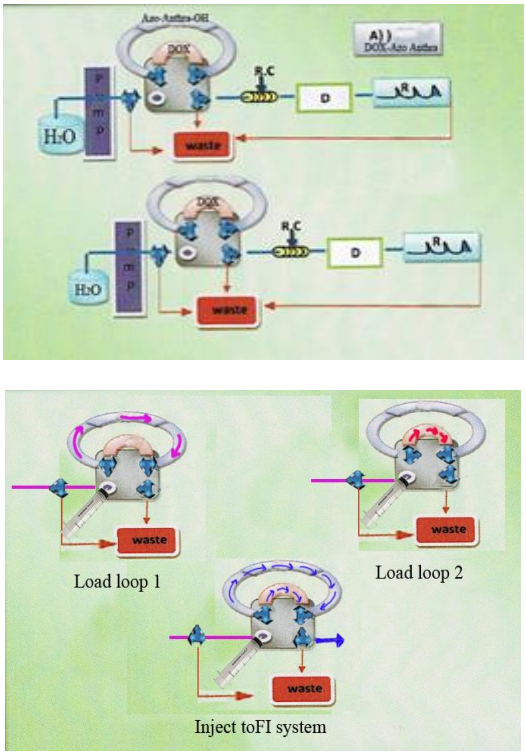

Fig.1: Developed FI system and valves system

Chemicals and reagents:

All the chemical materials and reagents employed were of analytical class and all the solutions preparing always used.

DOX stock solution (M.wt=512.94 g.mole^{-1}, Small Molecule Ltd, India): (1000 µg. mL^{-1} =19.5x10^{-4}M): A 0.1g amount of pure DOX was dissolving in distilled water then be consummated to 100mL in standard flask with distilled water. More the diluted solutions preparing by adequate diluting of the stock standard solution with distilled water.

Anthranilic Acid (M.wt=137.138 g.mol^{-1}, Merck) (7x10^{-3}M) :

A 0.1g amount of Anthranilic Acid was dissolved in smallest amount of water then add 2ml of 0.5% $NaNO_2$ and 1ml of 0.1% HCl in ice bathe (0-5C^0) for Azotizing the Anthranilic Acid, after 5-10min the Azotizing complete and 2ml of 2% sulphamic Acid was add to react with the residual of $NaNO_2$/HCl than the volume was made to 100mL in standard flask and farther dilution to these solutions to obtain desired concentrations.

NaOH (M.wt=40 g.mol^{-1},BDH) (0.25 M):

1% of NaOH solution was prepared by dissolving 1gm of it in 100ml of water. More the diluted solutions preparing by adequate diluting of the 1% solution with distilled water.

Batch method:
A 1mL of AzoAnthranilic Acid (1.5 x 10^{-4}M) was transferred into a set of 10mL standard flask; after that were added 1mL of NaOH (0.1 M), Then an increasing concentration (0.1-100) µg.mL^{-1}DOX were prepared in a set of 10mL standard flask and consummate the volume of the solutions to the mark with distilled water. Stand for 5min; the maximum absorption of the orange - yellow complex was found at λmax 468 nm opposition reagent blank.

Pharmaceutical preparations of DOX (1000 µg.mL^{-1})
Pharmaceutical formulations were gained from trading sources obtainable capsules by choosing 20 capsules from four kinds companies were assayed by the proposed procedures. The various providers from different companies were containing:

1. Tabocine (100mg) Tabuk Pharmaceutical Mfg. Co., Tabuk, Saudi Arabia.
2. DuraDox (100mg) Julphar, Gulf Pharmaceutical Industries, Ras Al Khaimah, U.A.E.
3. Doxycycline Capsules (100mg) Actavis, New Delhi, India
4. Medomycin (100mg) Medochemie Ltd –Cyprus.

Each 20 capsules from each sours were weighed exactly and mixed, thane a weigh of average one capsule was taken which contain of 100mg of DOX. Each weigh that taken was treated as pure material in the procedure mentioned above.

Further solutions were diluted to preparing allot to the concentration inside of the linearity of the calibration graph.

Recovery experiment was performed by applying the standard-addition technique. The recovery was assessed by determining the agreement between the measured concentration and the final known concentration to the sample. Each test was repeated three times.

The limits of detection (LOD)and quantification (LOQ) were calculated according to ICH guidelines[25] using the formulae:

LOD = 3.3 SD/b and LOQ = 10 SD/b,

where SD is the standard deviation of blank absorbance values (n = 3), and $b$is the slope of the calibration line.

RESULT AND DISCUSSION:

Absorption spectra:
A final concentration of 50µg/ml (of DOX) equal to (9.7x10^{-5} M) was reacted with 50µg/ml equal to (3.6x10^{-3}M) of AzoAnthranilic Acid in basic medium (0.1 M NaOH) to give the colored product complex which was examined under visible spectrum (from 350-800) in order to determine the maximum absorbance for the complex and it was clear that the λmax was 468nm for AzoAnthranilic-DOX complex.

Study of stoichiometry complex of (Azo Anthranilic) to Drug (DOX):
In order to know the ratio of reaction that occur between the reagent and the drug two important way were proceed which is mole ratio method and continues variation method (Job's method) and the result shown that DOX produced a 1:1 complex with AzoAnthranilic Acid as shown in (Fig. 2):

1. Mole ratio:(0.0002 M of each DOX and AzoAnthranilic Acid mix in order to the procedure of mole ratio and complete to 10 ml with distal water.
2. Job's method:(0.0002 M of each DOX and AzoAnthranilic Acid mix in order of Job's method and complete to 5 ml with distal water

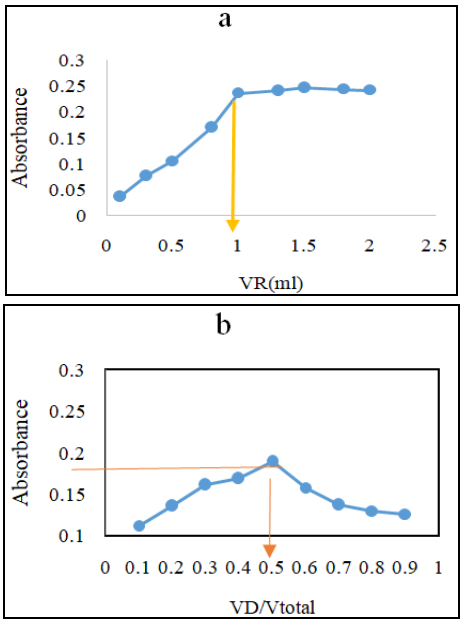

Fig. 2: The complexation ratio between a reagent with drug,
a-mole ratio for AzoAnthranilicAcid - DOX
b- job's method for AzoAnthranilicAcid - DOX

The proposed mechanism of reaction:

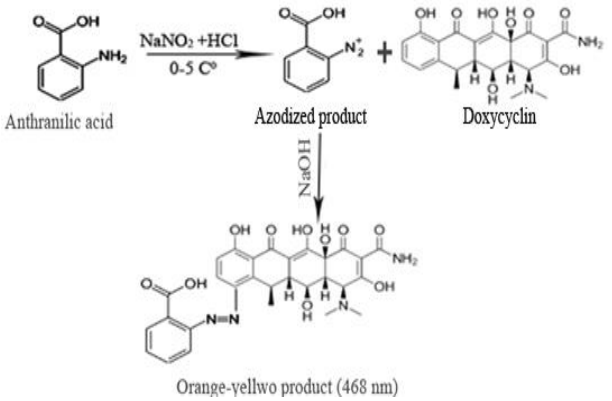

Fig. 3: The proposed mechanism for Azo Anthranilic –DOX complex

Depending on the results that collected from the mole ratio and Job's methods it is clear that the Azo Anthranilic – DOX complex associate in 1:1 ratio so the proposed below mechanism is likely to suggest:

Preliminary investigation:

Effect of AzoAnthranilicAcid:

The effect of AzoAnthranilic Acid concentration was examined. It has been monitored that the absorbance increase with increase of AzoAnthranilic Acid concentration till ($1.5x10^{-4}$). The more than enough of AzoAnthranilic Acid can inhibiting the favored quantitative composition AzoAnthranilic Acid-DOX complex thereby inhibiting the absorbance. The $1.5x10^{-4}$ M of AzoAnthranilic Acid was selected for subsequent experiences, as shown in (Fig.4-a).

Effect of medium:

The influence of medium was studied carefully due to it was directly effect on the coupling of AzoAnthranilic Acid with DOX. A series of final concentration (0.05-1 M) of NaOH solution were used for the experiment in the presence of final concentration of AzoAnthranilic Acid ($1.5x10^{-4}$M), and suitable Final concentration of DOX (50µg/ml). The absorbance increase with observed up to 0.1 M than decrease until 0.4 M and remains stable to 1 M. A 0.1M was chosen the optimum NaOH solution as a suitable medium for the formation of colored product, as shown in (Fig.4- b).

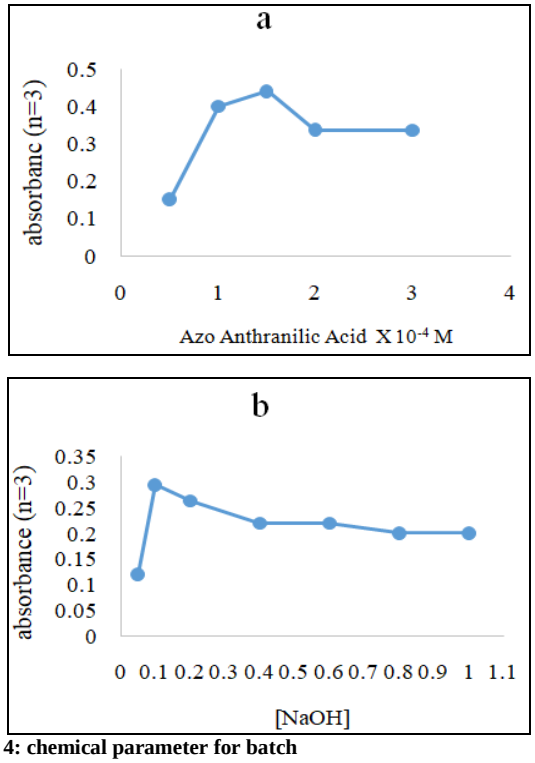

Fig. 4: chemical parameter for batch
a- concentration of Azo –Anthranilic Acid
b-effect of concentration of NaOH

Calibration curve of classical method:

Transfer a series of volumetric flask (10mL) containing 1mL of AzoAnthranilic Acid ($15x10^{-4}$M), then added 1mL of NaOH (1M). Then an increasing volume (0.1 – 7 ml) standard solutions of DOX ($100\mu g.ml^{-1}$). The solutions had been diluted to the marked with distilled water. Then the reaction mixture to stand for 5min and measure the maximum absorption of the colored product at 468nm against reagent blank prepared in same way without DOX. Each measurement repeated three times. The standard curve was constructed and linear range (1-70)µg. mL^{-1} for the estimation of DOX, as shown in (Fig.5)

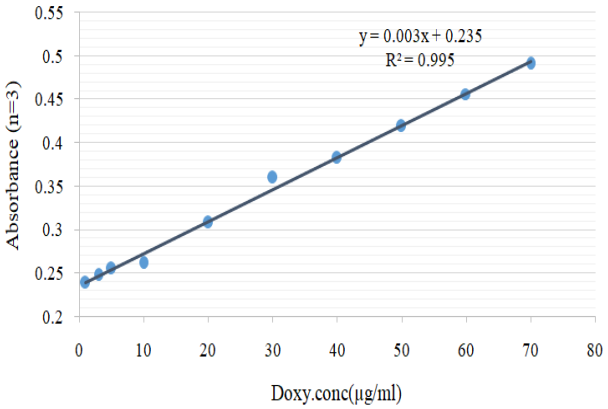

Fig. 5: Linear calibration carve for AzoAnthranilic Acid –DOX complex using spectrophotometric method

Accuracy and precision:

Under the ideal conditions described in established method, accuracy and precision was studied through measuring three different concentrations of DOX, and according to the results that have been reached as shown in (Table1) show that the classical method have good with high accuracy and precision; each measurement is repeated for three times.

Table 1: accuracy and precision

DOX conc. µg/ml		*Rec%	E%	*repeatability RSD%
present	found			
20	20	100	0	1%
50	49.8	99.6	-0.4	0.82%
70	69	98.5	-1.5	0.81%

*Average of three determinations
Rec % (the recovery) = (found/present) x100%
E% = [(found-present) / present] x100
RSD % (relative standard deviation)

Calculations of stability constant:

Calculated static stability[26] for the proposed interaction (AzoAnthranilic Acid.: DOX) was calculated depending on the two groups of solutions were prepared; first group of solutions were placed to include stoichiometric lot of DOX to reagent AzoAnthranilic Acid, while the second group were placed to include two-fold excess of AzoAnthranilic Acid. According to the proposed mechanism and stoichiometry ratio between reagent and

drug (1:1**)**. The stability constant can be writing as follows:

$K = 1-\alpha/4\alpha^3C^2$

While (α) (degree of dissociation) can be wrote as follows:

$\alpha = Am - As /Am$

Where Am; As are the values of absorbance of the aqueous solution including a more than enough and stoichiometric amount of the reagent (AzoAnthranilic Acid) where 0.242 and 0.236 respectively and alpha is 0.024, where the concentration is $1.5x10^{-4}$, K equal to $7.1x10^5$ $(L/mol)^2$

Flow injection/ Merging zones spectrophotometric determination

After selecting the optimum conditions of reaction of DOX with AzoAnthranilic Acid in the classical spectrophotometric method. The spectrophotometric reaction was automated with flow injection-merging zones technique to study the best practical parameters and to obtain spectral automated with fast way to estimate DOX. So the batch procedure for estimation of DOX was employed as a basis to develop flow injection analysis method.

Manifold of flow injection system

After installing the system and linked portions, been the study of optimal design of system. The developed system shown in (Fig. 1) is composed of one line supplies the carrier is distilled water leading to the injection valve; which contain two loops (different loop length with 0.5mm I.D.) that fills by the sample and reagents according to the order (DOX with appropriate concentration of NaOH in L1), (AzoAnthranilic Acid in L2).

Flow injection procedure:

Optimization of the FIA system conditions:

Initial studies were directed towards the optimization of the experimental conditions for MZ-FIA system.

Effect of the chemicals and physical variables:

Effect of chemical variables (concentration of reagent and base) and the physical parameters like the flow rate, length of reaction coil and the injected volume of reagent and sample were studied.

Optimum concentration of the reagent (Azo Anthranilic Acid) was studied by injecting different concentrations ($0.5x10^{-4}$- $3x10^{-4}$ M) using a homemade injection valve loading in (L2). The results in (Fig.6) indicated that the $1x10^{-4}$M gave the highest value of absorbance expressed as peak height in mV (n=3) with high repeatability. The best base concentration was 0.1 M.

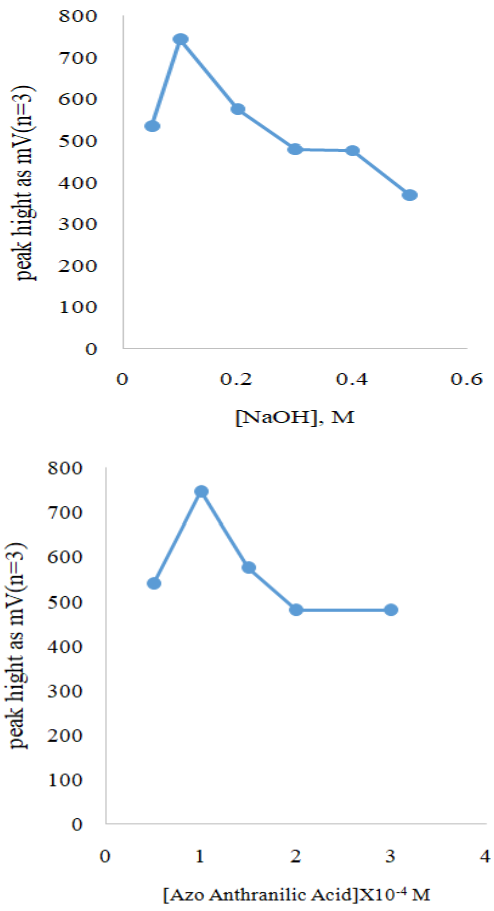

Fig.6: Influence of concentration of Azo-Anthranilic Acid and NaOH (DOX=100µg/ml) for the first system FIA

Effect of injected volume and reaction coil

For Azo Anthranilic Acid – DOX reaction the best loops for drug to reagent was (30-20µl) and the best reaction coil length was 50 cm.

(Tables 2) show the important parameters for the best loops that used for determination of DOX for Azo Anthranilic Acid system.

Table 2: DOX-AzoAnthranilic Acid –OH parameters

DOX-AzoAnthranilic Acid –OH							
Drug: Reagent	**Peak heigh as mV**			**mean**	**SD**	**RSD%**	**$y\pm t(SD/\sqrt{n})$**
30-30	559	561	560	560	1.00	0.18	560±0.01
30-20	722	722	716	720	3.46	0.48	720±4.71
30-40	604	610	608	608	3.06	0.50	608±4.15
30-60	628	630	632	632	2.00	0.32	632±2.72
60-20	693	690	693	392	1.73	0.44	392±2.35
40-20	691	690	695	592	2.65	0.45	592±3.59

Effect of optimum total flow rate and sample through-put:

All available flow rates were studied for the system and that shows the best flow rate for (AzoAnthranilic Acid – DOX-OH$^-$) FIA system was 3.2ml/min with sample through-put about 80 sample /hour the sampling rate was calculated depending on time required for loading the drug and the reagent to loops of the five three-way valve plus the time required to maximum peak height appear(this time was found to be 30 s) in addition to 15 s required for loading reagent and drug, so the sampling rate was 80 sample/ hour for this method.

Dispersion of zone:

Dispersion is a physical phenomenon that occurs in flow injection technique as a produce of the confluence of different concentration solutions, the sample mixed with carrier stream and then spread the sample in the solution. Success of the analysis process by FIA on three principles[27]:

1 Reproducible injection time.
2 Reproducible sample injection volume.
3 Control on the dispersion of sample zone.

The dispersion of Azo Anthranilic Acid – DOX reaction was 1.2. The dispersion was calculated according to the law (D.=Co/Cmax).

Calibration carve:

After verbal and verification all optimum condition, a series of DOX concentration (from 0.5 to 150µg/ml) were prepared and inject to FIA system with Azo Anthranilic Acid in order to know the optimum range of DOX concentration which can be applicable for this method and it shows that the best concentration range extend from 5µg/ml to 125µg/ml as shown in (Fig. 7) and (Table 3):

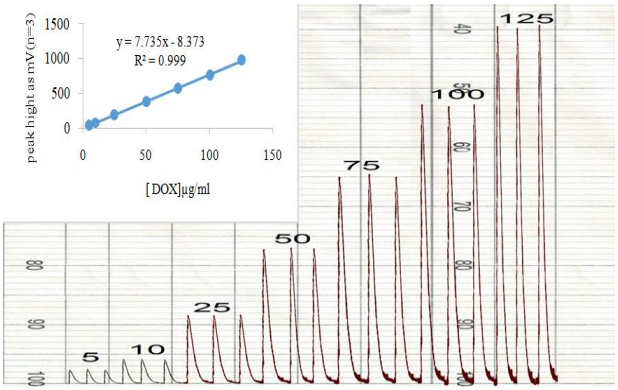

Fig. 7: the dynamic range for Azo Anthranilic Acid – DOX reaction

Table 3: Calibration table as S.E.M for DOX-Azo Anthranilic Acid - OH

Conc.	Peak high as mV			mean	SD	RSD%	y±t (SD/$\sqrt{n}$)
5	34	34	38	36	2.31	6.42%	36±3.14
10	71.4	69.4	70.4	70.4	1.00	1.42%	70.4±1.36
25	184	187	185.6	185.6	1.50	0.81%	185.6±2.04
50	376	378	374	376	2.00	0.53%	376±2.72
75	560	566	566	564	3.46	0.61%	564±4.71
100	750	751	753	752	1.53	0.20%	752±2.07
125	974	974.8	974.4	974.4	0.40	0.04%	974.4±0.54

Table 4: Analysis of variation for developed method

Source of Variation	sum. of squares (SS)	df	mean of squares (MS)	F(S_1^2/S_2^2)	F crit
Between Groups (Error)	21.53981	1	21.53981	6049.9	4.60011
Within Groups (Regression)	1824396	14	130314		
Total	1824417	15			

F crit. (4.6) <<F (6049.9) so it may be complete which there is an important relation between the concentration of DOX and the signal got.

Analysis of variation (ANOVA)[28,29] of linear equation.

Calculate sum of squares of the difference of values yi (response) from ŷi (appraiser response), (imply error) and called (about regression) to obtain $\Sigma (yi - \hat{y}i)^2$ for (n-2) freedom degrees to get sum of squares $(S_o)^2$. Calculate the sum of squares of the variance of values yi from average value ȳ (due to regression) to obtain $\Sigma (\hat{y}i - \bar{y})^2$ and for (1) of degrees of freedom to obtain sum of squares $(S1)^2$, when dividing the $(S1)^2$ on $(S_0)^2$ get the value(F) as showed in the (Table 4)

Methods validation:

The analytical characteristics just as correlation coefficient (r), detection limit, linear range and relative standard deviation of each procedure were estimated[30,31] at the improved conditions; as shown in the (Table 5). A calibration curve was constructed (Fig. 7) for a set of DOX standard solution and the basic analytical figure of deserts of the proposed method. Statistical assessment of regression line presented result of standard deviation for residuals (Sy/x); intercept (Sa) and slope (Sb) under 95% confidence limits for (n-2) freedom degrees were clarified in the table. The small subjects were showed the high repeatability reproducibility of the proposed flow injection analysis compared with the batch method. The flow injection analysis / merging zones was easier than first procedure because that was rapid (sample throughput of 80 sample/h); large linear scale of calibration curves was gotten.

Table 5: Analytical characteristic of calibration carve for Azo Anthranilic Acid –DOX parameters

Parameters	FIA method	Batch method
λmax (nm)	468	468
Regression equation	y = 7.7359x - 8.3732	y = 0.0037x + 0.235
Dynamic range (µg/mL)	5-125	1-70
Best recovery %	98.25	99.4
Error E%	-1.75	-0.6
RSD%	0.4	0.008
Slope, b (mL/µg)	7.7359	0.0037
Intercept, a	8.3732	0.235
Correlation coefficient (R^2)	0.9993	0.9957
Standard deviation of slope (Sb)	0.000579272	$4x10^{-5}$
Standard deviation of intercept (Sa)	0.000748	0.000231
LOD* (µg/mL)	0.163725	0.03
LOQ** (µg/mL)	0.545749	0.1
Molar absorptivity (ε) (L / mole.cm) ε = b × M × 1000	-	4298.5
Sandell's sensitivity (S) (µg. cm^{-2}) S =M / ε; M=M. wt of drug	-	0.12
Sample throughput (samp le/h)	80	8
*LOD = 3×SD /b **LOQ = 10×SD /b.		

Study of interferences:

In order to examine the selectivity of the method, the interference likely to be introduced from excipients and for capsules weigh adjustments (as starch, sucrose, fructose, glucose and sodium citrate) was studied. A sample of pure DOX spiked with half, equal and double fold excess concentration of selected interferences three excipients were analyzed. The acceptable recovery values (95-102%) demonstrated that, there were no interferences during the determination of DOX using proposed MZ-FIA methods, the results summarized in (Table 6)

Table 6: interferences effect on AzoAnthranilic Acid –DOX (50µg/ml)

Interferences	conc.(µg/ml)	Peak high	mV	Rec%
Standard	100	47	752	100%
Starch	50	46.5	744	98.93
	100	45	720	95.74
	200	43	688	91.48
Sucrose	50	45.5	728	96.80
	100	45.25	724	96.27
	200	45	720	95.74
Glucose	50	45.5	728	96.80
	100	47.25	756	100.53
	200	46	736	97.87
Fructose	50	47	752	100
	100	46.25	740	98.40
	200	46	736	97.87
Sodium citrate	50	46.5	744	98.93
	100	44	704	93.61
	200	41	656	87.23

Assessment of suggested method:

To assessment the success and efficiency of proposed method, four types of capsules containing DOX were analyzed under proposed method which is come from different origins (KSA, UAE, INDIA and CYPRUS).

The capsules were prepared as mentioned procedure shown in practical paragraph.

After preparing the solution of these capsules, the proposed MZ-FIA method was successfully applied for estimation DOX in capsules and the results are listed in (Tables 7). In the direction of assessing the proficiency of the developed method, the obtained results were compared with those obtained upon applying standard (BP) method HPLC method (Table 7). The statistical comparison between proposed and official methods using the student t- and F-test[25] indicated that the calculated values were less than the theoretical one, which referred to insignificant difference between both methods regarding accuracy and repeatability.

Table 7: Comparison of the proposed method with official method

Dosage form	Proposed FIA method						BP official method (theoretical)
	Taken conc. (µg/ml)	Pure added conc. (µg/ml)	Final taken conc. (µg/ml)	Found conc. (µg/ml)	REC%	RSD %	REC%
Tabucen (100mg) KSA	50	50	100	97.25	98.51	0.39	95%
Dura Dox (100mg) UAE	50	50	100	96.63	97.87	0.81	96%
Dox (100mg) India	50	50	100	92.94	95.10	0.45	97%
Medomycin (100mg) Cyprus	50	50	100	92.18	93.61	0.38	98%
							99%
F calculated		1.123					100%
t calculated		1.5					101%
							102%

t crit. =2.23 for (n1=8) (n2=4) so(n1+n2-2=10)
F crit. = 8.88 where (n1-1=7) (n2-1=3) at 95% confidence

CONCLUSION:

A few FIA methods were describe for determination of DOX, and most of these methods, were either not enough precise or complicated and want costly means. From a simple compare between the design method and reported flow injection methods it is clear that the proposed method is characterized by a wider calibration range, high sampling rate. Moreover, it was conduct out in watery intermediate and did not demand any sample pretreatment or transmutation. The design homemade CFI /Merging zones analytical process are strong, purchase and compassionate for the spectrophotometric of DOX. in elegant system and pharmaceutical formulations. These methods can be employment for the regard of $\mu g.ml^{-1}$amount of DOX. without indigence for prior divorce action, temperature or pretreatment of specimen and solid phase extraction. The capital benefit of the methods are its huge workings range; suitable sensitivity and its proper for appropriate in routine examination in pharmaceutics specify control laboratories due to their expertness and their result in decrease reagents waste and toxicity of organic reagents when comparison with batch methods and official HPLC (BP) method. The procedures have fit linearity, exalted analytical frequency with throughput 80 $sample.h^{-1}$. In addition, the broad applicability of improved progress for analyzation the search into of DOX at major of microgram even ($\mu g.ml^{-1}$) in pharmaceutical preparations samples.

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RESEARCH ARTICLE

Analytical Method Development and Validation of Teneligliptin by UV Spectroscopy

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ABSTRACT:

The measurement of teneligliptin in a drug pure in compliance with the ICH Guidelines, an easy and extraction free UV spectrophotometric method, was developed and validated. At 246nm and linearity of concentration of 10-50µg/ml, the drug shows strong absorption. The process for relaying the drug reaction to MeOH was calculated by its absorption maximum (λmax) 246nm in order to achieve a colorless solution. In the 10-50µg/ml concentration range, the rule on beer complied. The calibration chart was found to be linear, and the coefficient of correlation (r2) was found to be 0.9952%. The experimental value LOD and LOQ parameters for the proposed procedure are measured, respectively, like 2.25 and 6.83µg/mL. Teneligliptin recovery was reported as 101.58%. To maximize the reaction condition, all of the parameters were analyzed.

KEYWORDS: Teneligliptin, MeOH, Validation, ICH guidelines.

INTRODUCTION:

Teneligliptin is a Dipeptidyl peptidase-4 (DPP-4) inhibitor that has lately appeared as a new antidiabetic class that shows positive outcomes in improved glycaemic control.DPP-4 inhibitors boost effective GLP-1 (Glucagon-like Peptide 1 Hormone) and GIP (Glucose-dependent insulin tropic peptide) concentrations by inhibiting DPP-4 enzymatic activity, thus improving glucose-dependent hyperglycemia in patients with diabetes by raising serum insulin levels and lowering serum glucagon levels. Incretin-related agents such as DPP-4 inhibitors are therefore promising drugs that can reduce glucose fluctuations in diabetic patients and have emerged as a fresh antidiabetic class.[1-2]

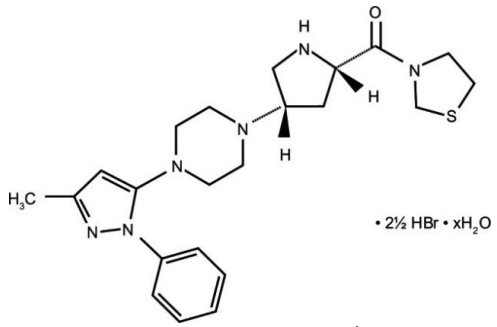

Figure 1. Chemical structure of Teneligliptin[4]

MATERIALS AND METHODS:

Instrumentation:

For the current study, UV Spectrophotometer 1700AD a make of Shimadzu was used.

Chemicals and reagents:

Teneligliptin standard was procured from Micro labs, Bengaluru. Teneligliptin marketed formulation manufactured by Well Care Pharmaceuticals was procured from local market. All chemicals used were

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analytical grade purchased from Merck pharmaceuticals. HPLC grade MeOH was used as the diluent for preparation of the solutions.[6-8]

Method Development:

Selection of wavelength:

The λ max of Teneligliptin was determined by using UV-visible spectrophotometer 1800. UV spectrum for Teneligliptin is shown in Figure 2.

Preparation of standard stock:

In a volumetric flask of 100ml, teneligliptin 100mg was collected. Dissolved with 50ml MeOH, make it up to label (1000µg/ml), and sonicated. In a 10ml volumetric flask, pipette 1ml of the above solution and use the diluent (100µg/ml) to appropriate volume. Linear alternatives are established by diluting the stock solution diluent. Conc of 10, 20, 30, 40, 50µg/mL of teneligliptin are prepared in 10ml volumetric flasks. pipette 1, 2, 3, 4, 5ml, and dissolved in the diluent to obtain these concentrations dimension of 10-50µg/mL.[3]

Preparation of Calibration Curve:

Aliquots of teneligliptin are made from the stock solution (100µg/mL) in a 10ml volumetric flask sequence. The HPLC grade diluent has been used to dilute the label to achieve a concentration range of 10-50 µg/mL. Solutions were observed for the absorbance and reported. The calibration curve has been set. The law on beer applies to 10-50µg/mL range.

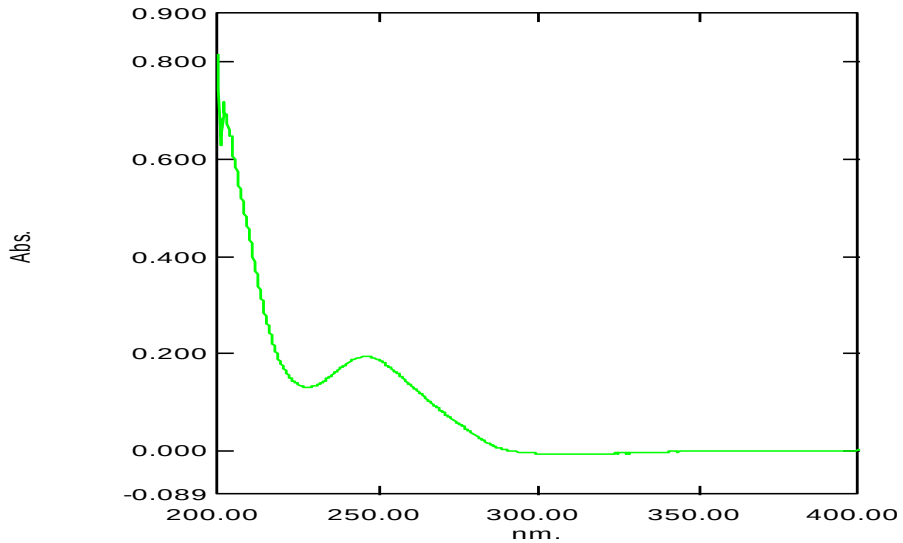

Figure 2: UV spectrum

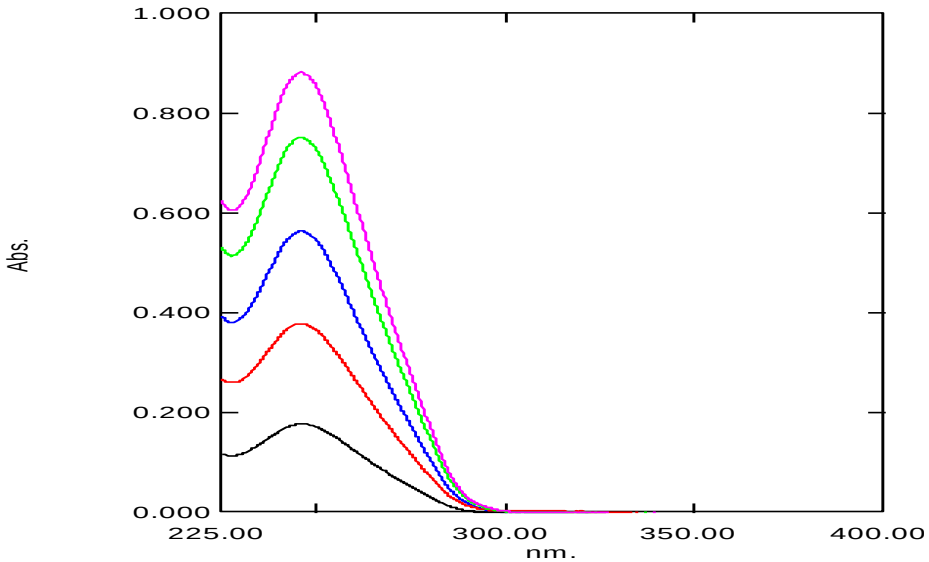

Figure 3: Overlay Complexity of Teneligliptin

METHOD VALIDATION [5,9-13]

Calibration curve:

It consisted of matrix sample processed without analyte and matrix sample with calibration standards. It is showing good linearity over the range of 10 to 50µg/ml with coefficient of correlation 0.9952

Table 1: Calibration data of Teneligliptin

Sl. No	Conc. (µg/ml)	ABS Of Teneligliptin
1	0	0
2	10	0.352
3	20	0.442
4	30	0.56
5	40	0.701
6	50	0.809

Linearity

The linearity was observed for Teneligliptin at 10-50µg/ml with r2=0.9952 and in the linear equation at Y=0.0117x+0.2209.

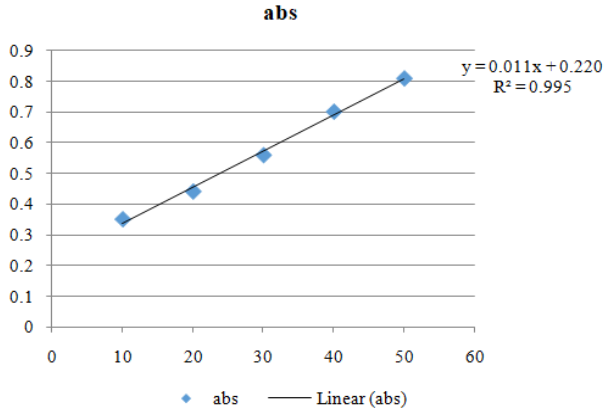

Figure 4: Linearity graph of Teneligliptin.

Accuracy:

At 30 percent, 60 percent, and 90 percent generic solution rates, the accuracy of Teneligliptin was calculated. Absorption was estimated at 246nm, and percent recovery results were reported

Table 2: Results for Accuracy of Teneligliptin by UV spectroscopy method

Sl. No	Level of % recovery	Amount of tablet sample (ml)	Amount of standard drug added (µg/ml)	Amount added µg	Amount found (µg/ml)	% recovery
1	0	2	0	0	0	
2	30	2	1.6	16	16.92	101.75%
3	60	2	2	20	21.38	102%
4	90	2	2.4	24	25.84	101%

Precision:

Excelling replicability with the present relative standard deviation (RSD percentage) under 2.0 percent was the precision (intra-day measurements, interday, repetitiveness) results.

Table 3: Results for Intraday precision of Teneligliptin by UV Spectroscopy Intraday morning precision

Sl. No	Conc (µg/ml)	ABS	
1	30	0.515	**STDV** = 0.008461678 **% RSD** = 1.63986014
2	30	0.522	
3	30	0.514	
4	30	0.502	
5	30	0.527	
	MEAN	**0.516**	

Intraday evening precision

Sl. No	Conc (µg/ml)	ABS	
1	30	0.523	**STDV** = 0.007939773 **% RSD** = 1.507742745
2	30	0.534	
3	30	0.538	
4	30	0.520	
5	30	0.518	
	MEAN	**0.5266**	

Table 4: Results for Interday Precision of Teneligliptin by UV Spectroscopy Interday morning precision

Sl. No	Conc (µg/ml)	ABS	
1	30	0.522	**STDV** = 0.009715966 **% RSD** = 1.840145121
2	30	0.536	
3	30	0.512	
4	30	0.532	
5	30	0.538	
	MEAN	**0.528**	

Interday evening precision

Sl. No	Conc (µg/ml)	ABS	
1	30	0.528	**STDV** = 0.008059777 **% RSD** = 1.55955431
2	30	0.506	
3	30	0.524	
4	30	0.514	
5	30	0.512	
	MEAN	**0.5168**	

Table 5: Results for Repeatability of Teneligliptin by UV Spectroscopy

Sl. No	Conc (µg/ml)	ABS	
1	30	0.539	**STDV** = 0.009495262 **% RSD** = 1.812764791
2	30	0.514	
3	30	0.530	
4	30	0.521	
5	30	0.515	
	MEA	**0.5238**	

Table 6: Results for LOD & LOQ of Teneligliptin by UV Spectroscopy

LOD (µg/ml)	2.25 µg/ml
LOQ (µg/ml)	6.83 µg/ml

Robustness:

The robustness of a method is a measurement of its ability to remain uninfluenced by limited, but deliberate, system parameter variations and offers a performance indicator during routine use.

Table 7: Results for Robustness of Teneligliptin UV Spectroscopy

Sl. No	Wavelength (nm)	Absorbance	STDV	% RSD
1	240	0.891	0.078201279	10.08268164
2	241	0.873		
3	242	0.835		
4	243	0.811		
5	244	0.789		
6	245	0.762		
7	246	0.751		
8	247	0.736		
9	248	0.681		
10	249	0.627		
	MEAN=	**0.7756**		

Ruggedness:

Two distinct analysts determined roughness, and the corresponding absorption was noted, and the outcomes were stated as RSD percentage for ruggedness.

Table 8: Results for Ruggedness of Teneligliptin by UV Spectroscopy

Analyst 1		
Conc (µg/ml)	**ABS**	**Statistical analysis**
30	0.562	**STDV** = 0.001258306 **%RSD** = 0.224097193
30	0.560	
30	0.563	
30	0.560	
30	0.563	
30	0.561	
AVG	0.5615	
Analyst 2		
30	0.564	**STDV** = 0.004307616 **%RSD** = 0.762859975
30	0.571	
30	0.569	
30	0.564	
30	0.562	
30	0.558	
AVG	0.564666667	

CONCLUSION:

While different UV-visible methods for developing and validating Teneligliptin were noted, attempts were undertaken with the use of UV-Visible spectrophotometric methods to develop simple and precise methods for developing and validating Teneligliptin. The work includes the design and testing through UV spectral photometric engineering of pure drug Teneligliptin. This technique has proven to be linear, precise, specific, and sensitive, safe, and effective for the analysis of pure drugs.

CONFLICT OF INTEREST:

The authors have no conflict of interest.

ACKNOWLEDGEMENT:

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RESEARCH ARTICLE

Preparation, Diagnoses of novel hetero atom compounds and Evaluation the Antibacterial Activity of them

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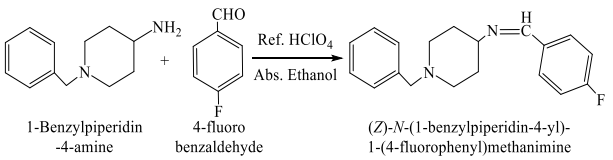
ABSTRACT:
This work included prepare of hetero atom compounds Ra_1-$Ra_5$from the interaction of prepared azomethines with anhydride compound. Azomethine compounds were prepared by condensation reaction. A number of new hetero atom compounds were prepared by acid-catalyzed Cycloaddition - reaction in anhydrous THF under reflux conditions. The formation of hetero ring has been achieved by Cycloaddition. The melting point, ^{1}H-NMR, FT-IR and ^{13}C-NMRspectroscopies technique were used to identified the final products. Biological activity of the prepared hetero compoundsRa_1-Ra_5evaluated on *E. Coli* and *S. aureus*.

KEYWORDS: Azomethine, 1,3-oxazepin, condensation reaction, cycloaddition mechanism.

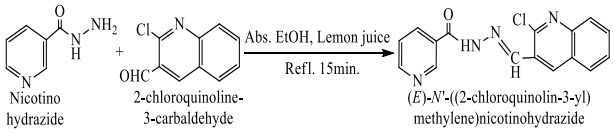
1. INTRODUCTION:
The azomethines are prepared by condensation of (-NH_2) group with (C=O) group [1]. They are versatile precursors in prepare of industrial compounds via ring closure, and they exhibit a wide range of pharmacological applications [2-4]. The reaction of 4-fluorobenzaldehyde with 1-benzylpiperidin-4-amine in presence of per chloric acid gives efficiently an imine product [5]. (Scheme 1)



1-Benzylpiperidin -4-amine | 4-fluoro benzaldehyde | (*Z*)-*N*-(1-benzylpiperidin-4-yl)-1-(4-fluorophenyl)methanimine

Scheme 1. Using $HClO_4$ as a catalyst to prepare the azomethine compound

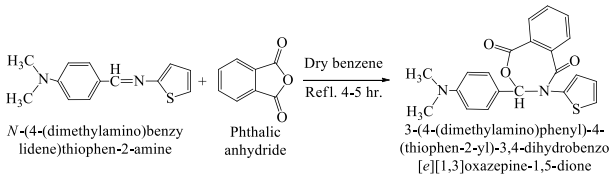
The reaction of nicotinohydrazide with 2-chloroquinoline-3-carbaldehyde lead to a good yield of the azomethine compound [6]. (Scheme 2)



Nicotino hydrazide | 2-chloroquinoline-3-carbaldehyde | (*E*)-*N*'-((2-chloroquinolin-3-yl) methylene)nicotinohydrazide

Scheme 2. Using lemon juice as a catalyst to prepare the azomethine compound

1,3-Oxazepine is unsaturated seven-membered heterocyclic consist of oxygen atom in location 1 and nitrogen atom in location 3 beside five carbon atoms [7]. Oxazepines are used as antibiotics and enzyme inhibitors. There are many studies on oxazepine in pharmacological applications [8,9]. Oxazepines have been prepared mainly by dipolar cycloaddition reaction of azomethine compoundswith five atoms cyclic anhydride [10-13], such as phthalic, succinic, maleic, and pyromellitic [14-17]. The reaction of phthalic anhydride with N-(4-(dimethylamino) benzylidene) thiophen-2-amine in dry benzene gives an 1,3-oxazepinederivatives [18]. (Scheme 3)



N-(4-(dimethylamino)benzylidene)thiophen-2-amine | Phthalic anhydride | 3-(4-(dimethylamino)phenyl)-4-(thiophen-2-yl)-3,4-dihydrobenzo[*e*][1,3]oxazepine-1,5-dione

Scheme 3. Prepared 1,3-oxazepinecompound with dry benzene as solvent

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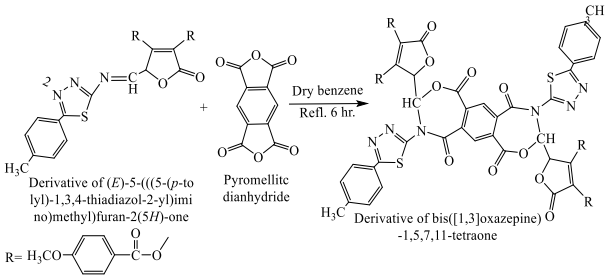
The product of the reaction between pyromellitic anhydride and derivative is showed in (Scheme 4) [16].



Scheme 4. Prepared 1,3-oxazepine derivatives with double rings

This work aims to prepare azomethine compounds from the reaction between aromatic aldehyde and aromatic primary amines to produced azomethine compounds, azomethine compounds reacted with anhydride compound, this reaction produced hetero atom compounds, these derivatives are very important in the pharmaceutical and medical fields.

2. EXPERIMENTAL PART:

2. 1. Prepare of hetero atom compounds Ra_1-Ra_5

A mixture of amine (0.01mol) and aldehyde (0.02mol) with trace of glacial acetic acid dissolved in 10mL absolute ethanol was placed in a 50-mL round-bottom. The reactions are refluxing for 4 hours, the purity of the compounds were proved with Thin Lear Chromatography. The solid products were recrystallized from ethanol [19,20], after that a mixture of (0.004mol) of prepared azomethine compounds with (0.008mol) of anhydride compounds in 15mL of benzene, was refluxed for 3h, the purity of the compounds were proved with Thin Lear Chromatography. The solid products were recrystallized from ethanol. Table 1 showed the properties of the Ra_1-Ra_5 [21,22].

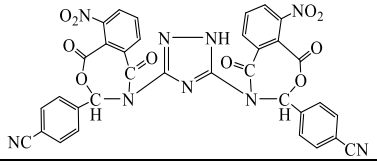
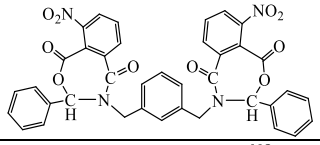
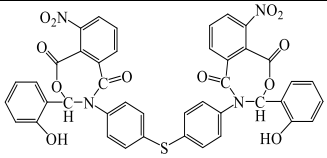
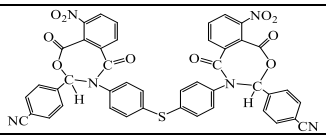
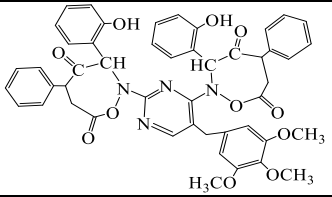
2.2. Antibacterial activity of prepared hetero atom compounds Ra_1-Ra_5

Antibacterial activity of the chemicals prepared hetero atom compounds Ra_1-Ra_5 against *E. coli* and *S. aureus*.6 µg $well^{-1}$ of hetero atom compounds. To measure the inhibition diameter a plate method was used [23].

3. DISCUSSION AND RESULTS:

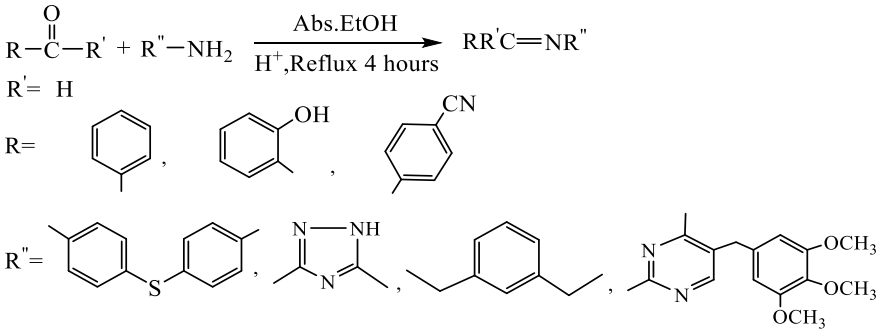
Physical properties of all prepared Ra_1-Ra_5 showed in 1 table. The higher m. p. of the prepared hetero atom compounds was for (Ra_1). The higher yield of hetero atom compounds was for Ra_4(91%).

Table 1. Physical properties of Ra_1-Ra_5

Comp.	Structural formula	Yield%	m.p. °C	Color
Ra_1		83	280-282	Red
Ra_2		81	210-212	Dark Yellow
Ra_3		77	205-207	Off White
Ra_4		91	170-171	Light Yellow
Ra_5		89	80-82	Orange

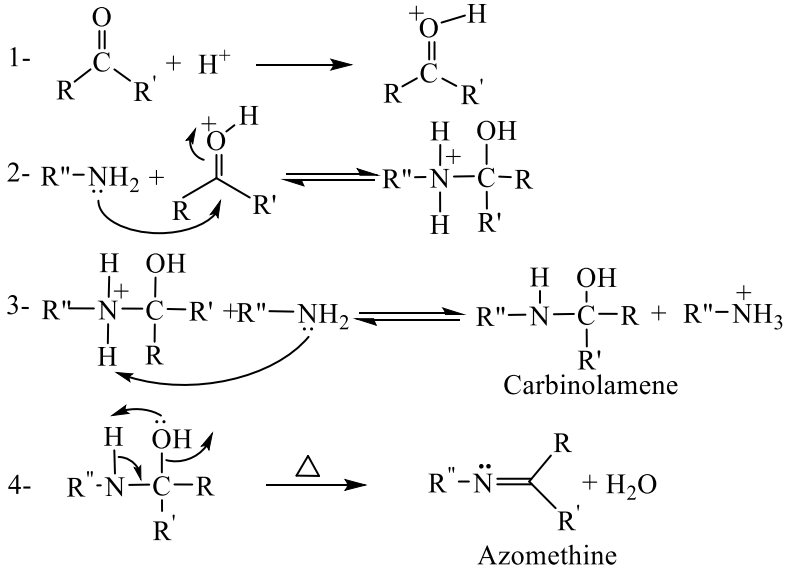
3.1. Diagnoses of prepared hetero atom compounds Ra_1-Ra_5

The general equation of prepared azomethines showed in scheme 5.



Scheme 5. The prepared azomethines

The reaction mechanism is believed to occur in (Scheme 6) [24].



Scheme 6. The azomethines formation mechanism

The FT-IR spectra was appeared the absorption at 1614-1702cm^{-1} of N-C=O, at 1718-1745cm^{-1} of O-C=O, at 1240-1282cm^{-1} of -C-O, at (1355-1381)-(1527-1584) cm^{-1} indicative of NO_2[25-27], See table 2for other absorbtion, seefigure1 and figure 2.

Table 2. IR of prepared hetero atom compounds Ra_1-Ra_5

IR, ν(cm^{-1})										
Comp.	**C-N**	**C-O**	**C=O Lactam**	**C=O Lacton**	**C=C Arom.**	**C-H Arom.**	**C≡N**	**C-H Aliph. Asym.**	**C-H Aliph. Sym.**	**Others**
Ra_1	1145	1274	1703	1745	1602	3095	22 31	---	2825	NO_2:1357, 1541 N-H: 3275
Ra_2	1149	1298	1614	1718	1568	3055	---	2975	2879	NO_2:1377, 1527
Ra_3	1153	1282	1614	1730	1587	3045	---	---	2991	C-S: 700 NO_2:1381, 1584 O-H:3500b
Ra_4	1168	1278	1620	1730	1579	3062	22 23	---	2989	C-S: 688 NO_2:1355, 1560
Ra_5	1172	1240	1650	1728	1593	3064	---	2981	2939	O-H:3355b

Table 3. The ^{1}H-NMR Spectrum of prepared hetero atom compounds (Ra_1-Ra_5) in Di Methyl Sulphoxide (DMSO)

Compound	Chemical Shift δ ppm
Ra_1	Singlet in location 4.40ppmfor N<u>H</u>, singlet in location 10.10 ppm for 2C<u>H</u>, multiplet in location 7.85-8.40 ppm for aromatic protons (14H).
Ra_2	Singlet in location 4.04 ppm for 2C<u>H</u>$_2$, singlet in location8.40 ppm for 2N-C<u>H</u>, multiplet in location7.10-7.30 ppm for aromatic protons (20H).
Ra_3	Singlet in location 8.63 ppm for2N-C<u>H</u>, singlet in location 13.22 ppm for (2O<u>H</u>), multiplet in location 6.69-7.22 ppm for aromatic protons (22H).
Ra_4	Singlet in location 10.10 ppm for 2N-C<u>H</u>, multiplet in location 6.69-8.51 ppm for aromatic protons (22H).
Ra_5	Singlet in location1.33 ppm for meta of 2O-C<u>H</u>$_3$, singlet in location2.67 ppm for para of O-C<u>H</u>$_3$, singlet in location 3.54 ppm for C<u>H</u>$_2$-Ph, doublet in location3.82 ppm for 2C<u>H</u>$_2$-C=O, triplet in 4.02 ppm for 2C<u>H</u>-C=O, singlet in location 7.90 ppm for 2N-<u>CH</u>, singlet in location9.90 ppm for 2O<u>H</u>, multiplet in location 6.33-7.40 ppm for aromatic protons and 1H of pyrimidinering (20H).

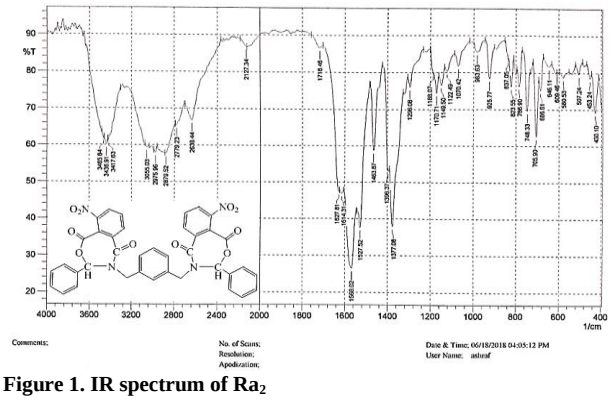


Figure 1. IR spectrum of Ra_2

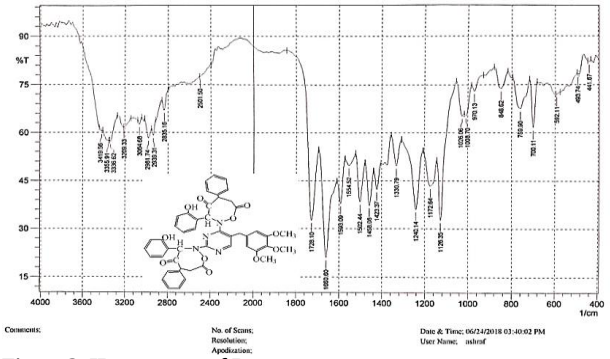


Figure 2. IR spectrum of Ra_5

^{1}H-NMR spectrum appeared DMSOthe chemical shiftsof Ra_1: Singlet in (4.40, 1H) of N<u>H</u>, singlet in (10.10, 2H) of (2C<u>H</u>), multiplet in (7.85-8.40,14H of aromatic protons)[30-34].^{1}H-NMR of Ra_1-Ra_5 showed in table 3. See figure3 and figure4.

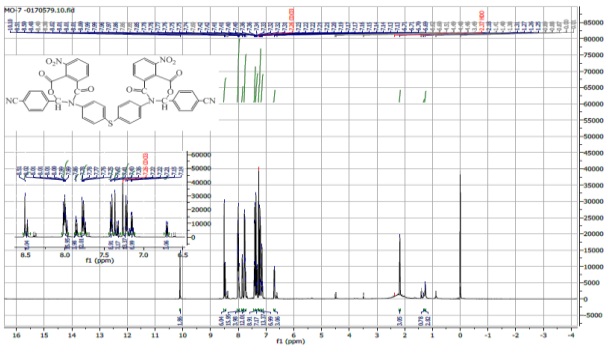


Figure3. The^{1}H-NMR spectrum ofRa_4

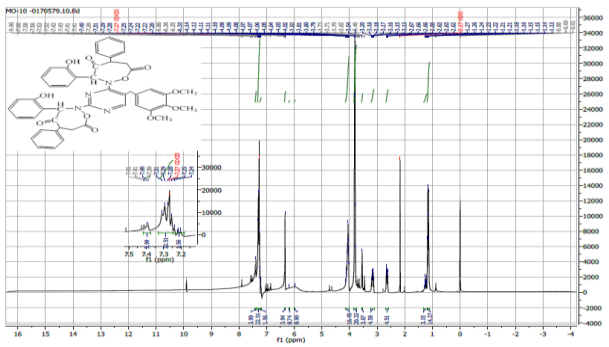


Figure 4. The^{1}H-NMR spectrum ofRa_5

The resonance spectra of ^{13}C-NMR was taken for three derivatives (Ra_3-Ra_5), Ra_3 showed the signals: 147 indicated to presence 2 groups of N-<u>C</u>H, 160 indicated to presence 2 lactam groups of N-<u>C</u>=O, 163 indicated to presence 2 lactone groups of O-<u>C</u>=O, 116-133 indicated to presence aromatic carbons [35-41]. See table 4, figure5 and figure 6.

Table 4. The ^{13}C-NMR Spectra of 1,3-oxazepin-5(1*H*)-one derivatives (Ra_3-Ra_5) in Di Methyl Sulphoxide (DMSO)

Compound	Chemical Shift δ ppm
Ra_3	147ppmindicated to presence 2 lactone groups of N-<u>C</u>H, 160 ppm indicated to presence 2 lactone groups of N-<u>C</u>=O, 163 ppm indicated to presence 2 lactone groups of O-<u>C</u>=O, 116-133 ppm for aromatic carbons.
Ra_4	150 ppm indicated to presence 2 lactone groups of N-<u>C</u>H, 156 ppm indicated to presence 2 lactone groups of N-<u>C</u>=O, 157 ppm indicated to presence 2 lactone groups of O-<u>C</u>=O, 190 ppm indicated to presence 2 lactone groups of cyanide <u>C</u>N, 114-136 ppm for aromatic carbons.
Ra_5	14 **ppm** for two meta (O-C<u>H</u>$_3$) group, 38 ppm for one para (O-C<u>H</u>$_3$)group, 56 ppm indicated to presence 2 lactone groups of <u>C</u>H$_2$-Ph, 61 ppm for (<u>C</u>H$_2$-C=O), 172 ppm indicated to presence 2 lactone groups of <u>C</u>H-C=O, 173 ppm indicated to presence 2 lactone groups of 2N-<u>C</u>H), 176 ppm indicated to presence 2 lactone groups of N-<u>C</u>=O, 178 ppm indicated to presence 2 lactone groups of O-<u>C</u>=O, 117-153 ppm for aromatic carbons pyrimidine ring carbons.

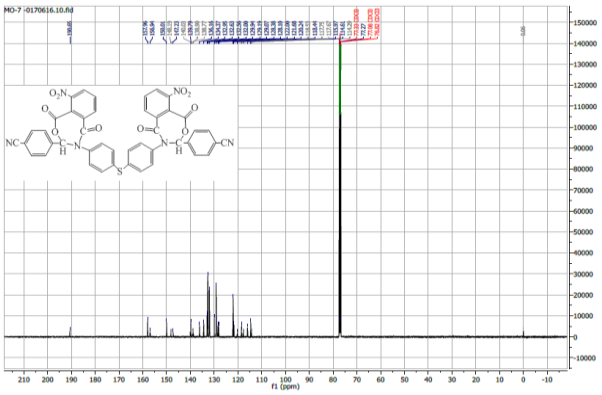


Figure5. ^{13}**C-NMR spectrum of**Ra_4

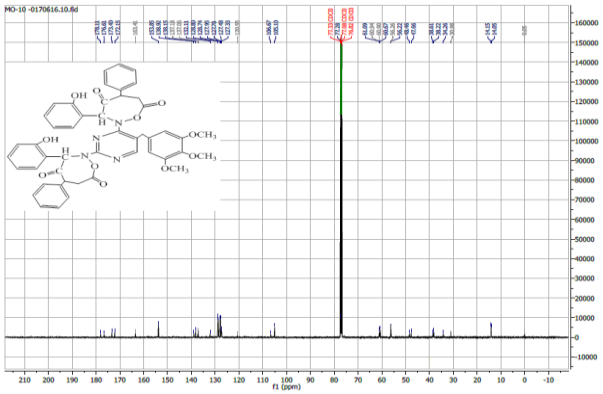
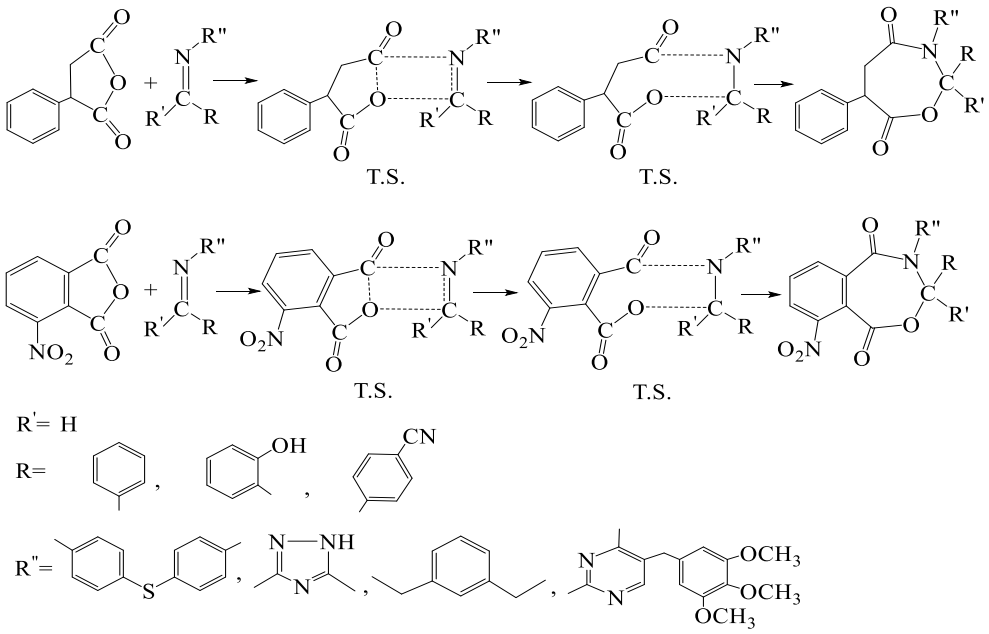


Figure6. ^{13}**C-NMR spectrum of** Ra_5

The reaction included many transition state formed from the bond of O-C=O of the anhydride with the C=N of Azomethine [27-30]. See (Scheme 7)



Scheme 7. Mechanism of prepared hetero atom compounds formation

3.2. The biological activityof prepared hetero atom compoundsRa_1-Ra_5

Tables 5 and 6 appeared the comparison between the drug in table 5 and the prepared hetero atom compounds in table 6.

Table 8 appeared that the best result of inhibition was 20mm for (45%) concentration, 25mm (50%), 28mm (75%) concentration and 32mm (100%) concentration against ***S. aureus*** by Ra_5compounds.Maybe the composite of the prepared hetero atom compounds destroyed the wall of microbes [41,42].

Table 5. The zoneinhibition of the drug and Di Methyl Sulphoxide (DMSO)

Type of bacteria	**Inhibition** (mm)	
	The drug (Gentamycin) 50 µg/well	**DMSO** 50 µg/well
E. coli	24	0
S. aureus	28	0

Table 6. Biological activity (mm) of the prepared hetero atom compounds Ra_1-Ra_5

Compound	Isolated	5%	10%	15%	20%	25%	30%	35%	40%	45%	50%	75%	100%
Ra_1	*E. coli*	-ve	-ve	ve	-ve	ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Ra_1	*S.aureus*	-ve	-ve	ve	-ve	ve	-ve	5mm	8mm	8mm	10mm	11mm	12mm
Ra_2	*E. coli*	-ve	-ve	ve	-ve	ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Ra_2	*S.aureus*	-ve	-ve	ve	-ve	ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Ra_3	*E. coli*	-ve	-ve	ve	-ve	ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Ra_3	*S.aureus*	-ve	-ve	ve	-ve	ve	-ve	-ve	-ve	-ve	7mm	8mm	10mm
Ra_4	*E. coli*	-ve	-ve	ve	-ve	ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Ra_4	*S.aureus*	-ve	-ve	ve	-ve	ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Ra_5	*E. coli*	-ve	-ve	ve	-ve	ve	-ve	7mm	7mm	9mm	11mm	11mm	13mm
Ra_5	*S.aureus*	-ve	-ve	7mm	7mm	10mm	13mm	15mm	17mm	20mm	25mm	28mm	32mm

4. CONCLUSIONS:

DerivativeRa_5showed the best zone of inhibition against *S. aureus*, this result helpful the other researchers to applied the concentration of the active prepared hetero atom compounds against pathogenic bacteria *in vivo* such a rats or rabbits.

5. ACKNOWLEDGEMENTS:

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RESEARCH ARTICLE

Cell Proliferative Potential of Ethnomedicinal plants for the management of Osteoporosis

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ABSTRACT:

Osteoporosis is a silent disease leading to complications with the progression in the disease. Osteoporosis is characterized by a decrease in bone mass and microarchitectural deterioration of bone tissue, leading to enhanced bone fragility and a consequent increase in fracture risk. During the early menopausal stage in women, there is reduction in circulating estrogen which leads to an increase in the rate of bone resorption. Hence, there is an imbalance between bone formation and resorption which lead to osteoporosis. Many traditional plants and phytoconstituents have been used as potential therapy for the treatment of the osteoporosis. However, the scientific approach for the validation of the use of phytoconstituents or herbs in the treatment of osteoporosis is the need of an hour. In present work five ethnomedicinal plants, *Asparagus racemosus*, *Hemidesmus indicus*, *Berberis aristata*, *Emblica officinalis*, and *Nigella sativa* were selected on the basis of their use in bone diseases in literature. The selected plants were studied for the positive effect on cell proliferation which is one of the important targets for the bone remodeling. Cell proliferative activity of aqueous and ethanolic extracts of selected plants was screened using MTT assay. Osteosarcoma cells (MG-63 cell line) and primary mesenchymal stem cells (MSCs) isolated from the rat femur were used for the MTT assay. The results of the above study showed significant ($P < 0.001$)cell proliferative activity of aqueous extract of seeds of *Nigella sativa*on MG-63 cells and MSCs and hence, supporting its ethnomedicinal use in the management of osteoporosis.

KEYWORDS: Osteoporosis, ethnomedicinal plants, MG-63 cell line, mesenchymal stem cells, MTT assay.

INTRODUCTION:

Osteoporosis is a major disease which has significant implications on public health. Osteoporosis is three times more common in women as compared to men[1]. In aged women, pathogenesis of osteoporosis is complicated and depends on many exogenous and endogenous factors. Age-related changes lead to lesions of hormonal status, regenerative system, especially in combination with negative endogenous factors[2]. Osteoporosis means porous bone, and it is characterized by fragile bone and deteriorated bone tissues[3]. Osteoporosis causes progressive bone loss, which renders the bones susceptible to fractures with minimal trauma and is popularly known as "the silent disease" because early symptoms are absent[4].

World Health Organization (WHO) defines osteoporosis as a bone mineral density of 2.5 standard deviations or more below the mean peak bone mass (average of young, healthy adults) as measured by dual-energy X-ray absorptiometry. Osteoporotic risk of fractures is associated with high mortality, morbidity and high medical expenses throughout the world[5], The financial burden of osteoporotic fractures includes direct costs (hospital acute care, in-hospital rehabilitation, outpatient services, long term nursing care) and indirect costs (morbidity, loss of working days). Some costs are difficult to quantify, e.g. deterioration of quality of life, and time spent by the family on the care of the patient[6].

The aim of the treatment is to prevent further bone loss in order to reduce initial and further risk of fractures. Many pharmacological agents, Vitamin D, calcium supplementation, biphosphonates, hormone replacement therapy, and selective estrogen receptor modulators are available for the treatment of osteoporosis[7]. The available treatments have several adverse effects; oral

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biphophonates cause gastrointestinal side effects such as abdominal pain, esophagitis, osteonecrosis of jaw and musculoskeletal pain. Estrogen therapy is associated with increased risk of cancer[8], and prolonged use of calcium leads to deposition of calcium in blood vessels and increased risk of cardiovascular diseases[9].

To overcome the wide range of side effects produced by these synthetic drugs, there is an increasing demand for 'green medicines' which are thought to be healthier and safer for the treatment of osteoporosis. There is a great demand of the herbsdue to their efficacy, safety and lesser side effects as compared to synthetic molecules. These herbs also have therapeutic role for age-related disorders like memory loss, immune disorders, etc. for which no modern medicine is available[10]. Various phytoconstituents such as flavonoids, alkaloids, carotenoids, terpenoids, sulphides, lignans, have been identified in various parts of these plants.

Since ancient time, many herbs are used in various bone related disorders like rheumatoid arthritis, osteodysplacia, osteoarthritis and osteomalacia. Based on the literature, roots of *Asparagus racemosus* (Ar), roots of *Hemidesmus indicus* (Hi), Bark of *Berberis aristata* (Ba), Fruits of *Emblica officinalis* (Eo), and *Nigella sativa* (Ns) were selected for their potential on bone cell proliferation. Two *in vitro* osteoblast model systems were used for the screening of selected plants viz., human osteoblast-like cells MG-63 and primary osteoblast cells isolated from rat femur. Cell viability was assessed by Trypan blue assay and cell proliferation assay was performed by MTT assay.

MATERIAL AND METHODS:

Collection and authentication of selected plants:

The fresh roots of *Asparagus racemes* and fresh fruits of *Emblica officinalis* were collected from the Botanical garden of Gandhinagar. The stem bark of *Berberis aristata*, roots of *Hemidesmus indicus* and seeds of *Nigella sativa* were purchased from Lallu Vrajlal Gandhi Herbal Store, Ahmedabad, Gujarat, India. The herbarium and voucher specimen of collected and procured crude drugs were prepared and submitted to the Pharmacognosy department of K.B. Institute of Pharmaceutical Education and Research, Gandhinagar. Freshly collected crude drugs were washed properly with water and dried. Dried drug materials were coarsely powdered and stored in a properly labeled airtight container until further use. All the crude drugs were authenticated by their morphological, microscopical and physicochemical parameters and compared with references.

Crude drug was powdered and evaluated for ash values (Total ash and acid insoluble ash), extractive values (alcohol and water extractive) and loss on drying for their authentication[11].

Preparation of Aqueous and ethanolic extracts:

Twenty gram of dried powdered drug was taken, and extracted with ethanol (100ml x 2) by heating under reflux on a water bath at 55˚C for 6 hours, and allowed to stand overnight and filtered. And for preparation of aqueous extract, 20 gram of dried powdered drug was extracted with water (100ml x 2) by heating under reflux on water bath for 6 hours, and allowed to stand overnight and filtered. Aqueous and ethanolic filtrates of each drug were concentrated to dryness on water bath temperature not exceeding 60˚C. Ethanolic and aqueous extract of each drug was labeled properly and stored in refrigerator till further use.

Procurement and maintenance of Cell line:

First the complete growth medium for the MG-63 cells and for primary mesenchymal stem cells (MSCs) was prepared using sterile liquid Dulbecco's minimum essential medium DMEM, 10 % Fetal Bovine Serum and 1% Antibiotic solution (10000 U Penicillin and 10 mg Streptomycin/ml). The prepared complete growth media was labeled as basal media and stored at 8ºC in a refrigerator for further use.

The human bone osteosarcoma cell line, MG-63 was used for the screening of extracts for their cell proliferation activity. MG-63 cell line was procured from NCCS, Pune prepared from 14 years human male bone with osteosarcoma. Cell line was maintained as per ATCC protocol throughout the work.

Sub culturing of Cells:

According to ATCC protocol cell line was allow to grow in DMEM supplemented with 1X antibiotic antimycotic solution and 10% FBS under standard growth conditions (temperature 37°C, 5% CO_2 and 95% humidity) in a CO_2 incubator. After confluent monolayer, cells were trypsinized with 0.25% trypsin–0.2% EDTA in Dulbecco's phosphate buffered saline (DPBS) and subcultured to obtain enough number of cells for proliferation assay. For the proper growth of the cell media was changed every alternate day.

Cell viability by Trypan blue assay[12-14]

The aliquot of 10µl of the trypsinized cell suspension was taken in a micro centrifuge tube and 1:1 mixture of the cell suspension was prepared by adding 10µl of the 0.4% trypan blue dye solution. It was gently mixed and kept at room temperature for about 5 min prior to use, the haemocytometer and the cover slip were sterilized with 70% (v/v) iso-propyl alcohol and were allowed to air-dry. Properly mixed cell suspension and trypan blue dye mixture was loaded on the chamber between the cover slip and the V-shaped groove in the chamber and

allowed to set. The living cells are as clear form and dead cells are seen blue in colour under inverted microscope. Cell viability was calculated by counting living cells and non living cells using following formula,

%Cell Viability=(no.of living cells/ total cell count)*100

Isolation of mesenchymal stem cells (MSCs) from rat femur[15-18]:

After the quarantine period, the animals were used for the experiment. The study was approved by the institutional animal ethics committee of K. B. Institute of Pharmaceutical Education and Research, Gandhinagar (KBIPER/ approval No: KBIPER/IAEC/ANIM/ 2016/577). The rats were euthanized by cervical dislocation under ether anesthesia, the femurs were removed aseptically and the soft tissue and muscles attached were cleaned-off from the femur. Femurs were cut from both ends and bone marrow was flushed with 2ml DMEM with help of a syringe with needle (27 gauge). Bone marrow cell suspension was filtered through sterile filter to remove cell debris and collected cell suspension was centrifuged at 1000rpm for 5 min. The MSCs were seeded in T-25cm^2 tissue culture flask. Primary cells (MSCs) were in were cultured DMEM supplemented with the 1X antibiotic-antimycotic solution and 10% FBS. MSCs were allowed to grow under standard growth conditions. The media was changed at every alternate day until the full confluence was achieved. Cells were trypsinized with 0.25% trypsin, 0.2% EDTA in Dulbecco's phosphate buffered saline and either subcultured at a split ratio of 1:2 in 25 cm^2 volume tissue culture flask or seeded in microtitre plates (96 well and 24 well plates) for different cell based assays.

Cell Proliferation activity by MTT assay[19-21]:

Cell proliferation activity of extracts was screened by osteoblast cell line MG-63 and MSCs. Cells were plated at the concentration of 1×10^4 cells/ml with DMEM in 96-well plate; Next day spent media was discarded carefully and the cells were treated with 3 different concentrations (1000µg/ml, 100µg/ml and 10µg/ml) of aqueous and ethanolic extracts of each drug in triplicate. After 48 h of incubation with drug extracts, the 20 µl of MTT (final concentration 0.5mg/ml) was added to each well and incubated for 4h at 37°C in dark place. Media was carefully pipetted out without disturbing the cell monolayer and 200 µl of DMSO was added to each well, the plate was shaken carefully and incubated for 1h in 37°C in dark place. Optical density (OD) of each well was measured at 570nm in microwell plate reader.

Statistical analysis:

Data was analyzed by applying one-way analysis of variance (ANOVA) followed by Tukey Test, using Graph Pad Prism software. The data was expressed as mean ± standard error of the mean (SEM). The results were considered statistically significant if the $P<0.05$.

RESULTS:

Authentication of selected plants:

Selected plants were authenticated by their physicochemical parameters and values were compared with the standard literature. Results of ash value, moisture content and extractive value are as shown in the Table 1.

Preparation of extracts:

Percentage yield of aqueous and ethanolic extracts of plant was as shown in Table 2.

Table 1: Physicochemical parameters of selected plants

Parameters → / Name of Plant ↓	Ash value (%w/w)		Loss on Drying (%w/w)	Extractive value (%w/w)	
	Total ash	Acid insoluble ash		Water soluble extractive	Alcohol soluble extractive
Roots of *Asparagus racemosus*	4.3 ± 0.19	0.45 ± 0.04	6.3 ± 0.31	33.7 ± 0.72	11.4 ± 0.42
Roots of *Hemidesmus indicus*	3.6 ± 0.31	0.28 ± 0.02	5.32 ± 0.47	9.6± 0.60	8± 0.42
Bark of *Berberis aristata*	12.1 ± 0.56	5.45 ± 0.32	5.98± 0.29	10± 0.54	5.5± 0.36
Fruits of *Emblica officinalis*	0.8 ± 0.35	0.04 ± 0.01	3.37 ± 0.63	10.2 ± 0.83	9.6 ± 0.64
Seeds of *Nigella sativa*	6.2 ± 0.65	0.4 ± 0.06	3.1 ± 0.42	7.5 ± 0.26	11.7 ± 0.67

Table 2 : Percentage yield of extracts

Name of Plant	Extract	% Yield of Extract (% w/w)
Asparagus racemosus (Root)	Aqueous (Ar A)	34.2
	Ethanolic (Ar E)	11.8
Hemidesmus indicus (Root)	Aqueous (Hi A)	9.4
	Ethanolic (Hi E)	8.3
Berberis aristata (Bark)	Aqueous (Ba A)	10
	Ethanolic (Ba E)	5.5
Emblica officinalis (Fruits)	Aqueous (Eo A)	10.5
	Ethanolic (Eo E)	9.1
Nigella sativa (Fruit)	Aqueous (Ns A)	8.1
	Ethanolic (Ns E)	12.0

Sub culturing of cells and Trypan blue assay:

The cells of the MG-63 cell line were sub-cultured at regular intervals when the cells in the tissue culture reached the confluency of 80-90%. Trypan blue is acid dye having two azo chomophore groups. Cell wall of the living cells is non permeable to trypan blue dye it only entre in the cell wall of nonliving cells. Total viable cell count and % cell viability by trypan blue was found to be $2.28*10^6$ cells/ ml and 98.21% respectively.

Cell proliferation assay:

Cell proliferative activity of the plant extracts was measured by MTT assay. Cell proliferation activity of each extract was evaluated both on osteoblast like cells of MG-63 cell line and normal cells of primary mesenchymal stem cells. Alendronate was taken as a positive control, and basal media was considered as a negative control. In our study we observed that, alendronate cause remarkably increase the proliferation of osteoblast like cells of MG 63 cell line as compare to the cells grown with basal media alone. Alendronate showed cell proliferation activity in dose dependent manner.

Aqueous and ethanolic extracts of roots of *Hemidesmus indicus* showed significant cell proliferation activity in dose dependent manner. Aqueous and ethanolic extract roots of *Hemidesmus indicus* showed significant cell proliferation potential ($P < 0.01$) at concentration 1000 µg/ml. Aqueous extract of seeds of *Nigella sativa* also showed highly significant proliferation potential ($P < 0.001$) at concentration 1000 µg/ml and signification proliferation ($P < 0.01$) at concentration 100 µg/ml and 10µg/ml. Aqueous and ethanolic extract of roots of *Asparagus racemosus*, bark of *Berberis aristata* and fruits of *Emblica officinalis* have no cell proliferation potential on cells of MG-63 cell line at any concentration (Shown in Figure 1).

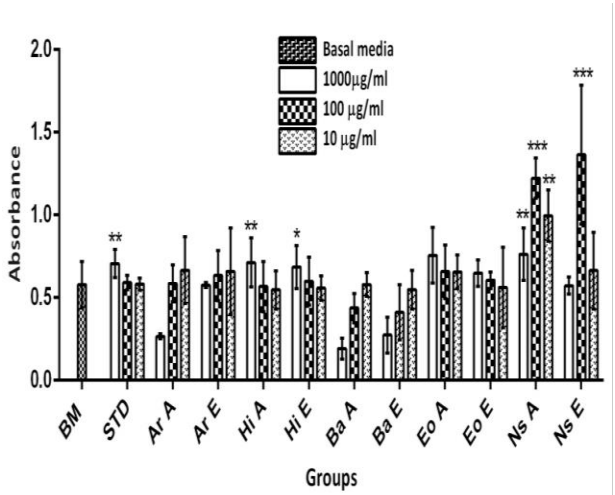

Figure 1: Cell proliferation activity of selected plant extracts on MG-63 osteoblast like cells

The values depicted in the graph are from higher to lower concentrations (i.e. 1000 µg/ml, 100µg/ml, and 10µg/ml) for each drug extract. **STD** = Alendronate standard **Ar A** = Aqueous extract of *Asparagus racemosus* **Ar E** = Ethanolic extract of *Asparagus racemosus* **Hi A** = Aqueous extract of *Hemidesmus indicus* **Hi E** = Ethanolic extract of *Hemidesmus indicus* **Ba A** = Aqueous extract of *Berberis aristate* **Ba E** = Ethanolic extract of *Berberis aristate* **Eo A** = Aqueous extract of *Emblica officinalis* **Eo E** = Ethanolic extract of *Emblica officinalis* **Ns A** = Aqueous extract of *Nigella sativa* **Ns E** = Ethanolic extract of *Nigella sativa* Values are expressed in mean ± SEM. n=3. Significantly different from *P < 0.05 **P < 0.01 ***P < 0.001 Vs Control (Basal media)

Cell proliferation potential of extracts of selected plants was also evaluated on normal cells isolated from the bone marrow of rat femur. Results of the study indicated that aqueous and ethanolic extract of roots of *Asparagus racemosus* have highly significant ($P < 0.001$) cell proliferation property at the concentration of 1000µg/ml and low concentration 100 µg/ml and 10µg/ml have significant ($P < 0.01$) cell proliferation potential. Aqueous extract of seeds of *Nigella sativa* and also showed excellent ($P < 0.001$) cell proliferation potential but it was not in a dose dependent manner. Cell poliferative potential of ethanolic extract of seeds of *Nigella sativa* is also significant at concentration 10µg/ml while other concentration have non significant cell proliferation. Ethanolic extract of fruits of *Emblica officinalis* have highly significant ($P < 0.001$) cell proliferation compared to its aqueous extract. Aqueous and ethanolic extract of roots of *Hemidesmus indicus* showed cell proliferation activity in dose dependent manner but not as significant as aqueous extract of roots of *Asparagus racemosus.* Aqueous and ethanolic extract of bark of *Berberis aristata* have no significant cell proliferation potential compared to other extracts (Shown in Figure 2).

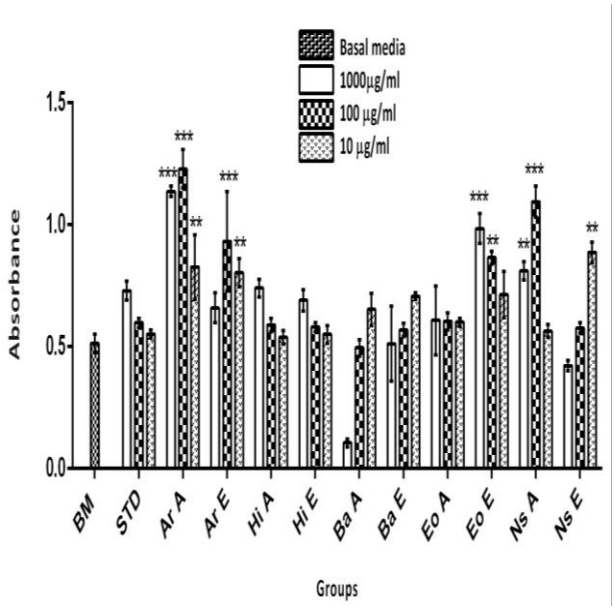

Figure 2: Cell proliferation activity of selected plant extracts on primary mesenchymal stem cells

The values depicted in the graph are from higher to lower concentrations (i.e. 1000 µg/ml, 100µg/ml, and 10µg/ml) for each drug extract. **STD** = Alendronate standard **Ar A** = Aqueous extract of *Asparagus racemosus* **Ar E** = Ethanolic extract of *Asparagus racemosus* **Hi A** = Aqueous extract of *Hemidesmus indicus* **Hi E** = Ethanolic extract of *Hemidesmus indicus* **Ba A** = Aqueous extract of *Berberis aristate* **Ba E** = Ethanolic extract of *Berberis aristate* **Eo A** = Aqueous extract of *Emblica officinalis* **Eo E** = Ethanolic extract of *Emblica officinalis* **Ns A** = Aqueous extract of *Nigella sativa* **Ns E** = Ethanolic extract of *Nigella sativa* Values are expressed in mean ± SEM. n=3. Significantly different from *P < 0.05 **P < 0.01 ***P < 0.001 Vs Control (Basal media)

DISCUSSION:

Osteoporosis constitutes a major public health problem, contributing significantly to morbidity, mortality and healthcare spending[22]. Though osteoporosis is a senile disease, it will be started in young age and showed in adulthood[23]. With increasing life expectancy the prevalence of osteoporosis is on rise and it poses a major public health issue[24].Natural menopause in women, making spontaneous cessation of estrogen has potent effect on the development and integrity of skeleton[25]. Lifestyle changes, modifications in consumption of food, regular exercise have beneficial effects on bone health[26]. The bone undergoes continuous turnover throughout life. Bone mass decreased due to activation of osteoclast which enhance bone resorption [27], and osteoblast are bone forming cells. Bone metabolism depends upon these two types of cells. The state of our bones is always close to equilibrium between bone formation and bone resorption. In osteoporosis there is imbalance between bone formation and bone resorption[28]. Currently available treatments for osteoporosis mostly include antiresorptive agents. Antiresorptive agents inhibit osteoclastic bone resorption and slow down loss of bone mass[29].

As ovarian hormone deficiency is a major risk factor for osteoporosis in the postmenopausal women, hormone replacement therapy (HRT), is the most effective treatment, but not preferred due to the risk of breast cancer and cardiovascular diseases. The other available therapeutic agents are also associated with adverse effects. So there is a search for the natural drugs in the treatment of osteoporosis. The advantages of the natural drugs are their easy availability, and negligible side effects.

Herbal drugs have been traditionally used in *Ayurveda* to accelerate the healing of bone fractures and to strengthen the bones. Five plants (roots of *Hemidesmus indicus*, roots of *Asparagus racemosus*, bark of *Berberis aristata*, fruits of *Emblica officinalis*) were selected on the basis of their use in bone disorder in literature.

For the treatment of osteoporosis, either there is increase in the activity of osteoblasts or there is reduction in the functioning of osteoclasts[30]. As osteoblast proliferation is one of the important parameter of bone formation so cell proliferative potential of the extracts of selected plants was screened by MTT assay.

MTT assay is a colorimetric assay that measures the reduction of yellow 3-(4, 5-dimethythiazol2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, colored (dark purple) formazan product. Cell lysate was prepared by using an organic solvent and the released, solubilized formazan reagent is measured spectrophotometrically. Since reduction of MTT can only occur in metabolically active cells the level of activity is a measure of the number of the viable cells.

Study suggests that the proliferative responses of normal cell or primary cell and human osteosarcoma cell lines are quite different[31,32]. So, in present work cell proliferative potential of each extract was evaluated by two *in-vitro* models using cells of MG-63 cell line and primary mesenchymal stem cells isolated from rat femur. Cells of MG-63 cell line are cancerous cells as it is obtained from 14 years old male with osteosarcoma and MSCs are normal cells. As bone marrow mesenchymal stem cells are the good source of bone forming cell-osteoblast, proliferation of MSCs is also important target for the treatment of osteoporosis [33].

In present investigation, it was observed that aqueous extract of seeds of *Nigella sativa* showed good proliferation potential in the osteoblasts of MG-63 cell line as well as primary MSCs of bone marrow, whereas the ethanolic extracts of fruits of *Emblica officinalis* showed cell proliferative activity only in MSCs. Root of *Hemidesmus indicus* also have cell proliferative potential in dose dependent manner in both *in-vitro* models but have less significant in MSCs compared to the cells of MG-63 cell line. Thus, from the present study it can be concluded that aqueous extract of seeds of *Nigella sativa* causes significant proliferation stimulation amongst all five-selected drugs. Immunoprotective activity of aqueous extracts of seeds of *Nigella sativa* also support the results of the study[34]. So it can be use for the treatment and management of osteoporosis. Further, the seeds of *Nigella sativa* can be used for the preparation of herbal formulation for the treatment of osteoporosis.

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RESEARCH ARTICLE

Formulation and Evaluation of Ocular in-Situ gelling system containing Ciprofloxacin and Naproxen Sodium

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ABSTRACT:

Ocular drug delivery was considered a challenging approach for many decades, due to the limitation in the bioavailability in addition to the common drawbacks of the conventional eye preparations represented with blurred vision, pre-corneal elimination and high variability. For this reason, six different ion- induced ocular in-situ gel were formulated containing combination of ciprofloxacin and naproxen using sodium alginate and gellan gum in three different concentrations (0.1, 0.3 and 0.5%) as ion cross-linking polymers in conjugation with HPMC as viscosity enhancer. These were characterized for clarity, pH and drug content, in addition to the evaluation of their in-situ gelling capacity and rheological properties. Moreover, in- vitro dissolution analysis and antimicrobial studies were performed to ensure the proper releases and the effectiveness of the formulas. The results revealed that all prepared formulas exhibited a clear, transparent appearance with acceptable pH and drug content. Furthermore, the prepared formula (F3) containing 0.5% sodium alginate was selected as the optimal formula, having best gelling capacity and viscosity which gives an ideal release profile for extended period up to 8 hours. Finally, the antimicrobial study proves the effectiveness of the prepared formula with greater zone of inhibition compared to the conventional ciprofloxacin eye drop. As a result, we can conclude the successful preparation of sustained release ocular in-situ gel of ciprofloxacin and naproxen that can be considered as an effective replacement to the available eye drops of both drugs which used to prevent bacterial infection and minimizing the inflammation followed the surgical operation of cataract.

KEYWORDS: Ciprofloxacin HCL, Ion cross-linking, Naproxen sodium, Ophthalmic in-situ gel, Sodium alginate.

INTRODUCTION:

The ophthalmic drug delivery system is extremely interesting and highly challenging task facing the formulator [1], which might be attributed to the various anatomical and physiological constraints of the eye represented by the relative impermeability of the corneal epithelial membrane, tear dynamics and nasolacrimal drainage resulting in a poor residence time of the drug at the site of action, and hence poor bioavailability of the drug [2,3]. As a result, most of the administered drug from the conventional ocular delivery systems, like solutions, suspensions and ointments, are no longer sufficient to ful fil the present day requirements of providing a constant rate delivery for prolonged time [4].

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Different delivery systems have been developed during the past decades to increase the corneal permeability and to prolong the contact time on the ocular surface. Successful results have been obtained with inserts and collagen shields. However, these have disadvantages of poor compliance, especially by old age people and many patients sometimes lose the device without noticing it [5]. For this reasons, new pharmaceutical formulations were investigated, including the ocular in-situ gel, which is a formulation that can be instilled easily and accurately as liquid drops into the eyes then undergo a sol-gel transition in the in the cul-de-sac of the eye, according to changes in specific physicochemical parameters. (Such asp H, Temperature, ionic strength) which is determined by the polymer used during the formulation [6,7].

In the present work a combination of antibiotic (ciprofloxacin HCl) and anti-inflammatory (naproxen sodium) were formulated as ocular in-situ gel to treat eye infection, where the naproxen was used to reduce the

inflammation in combination with ciprofloxacin to treat or prevents the infection associated with the inflammation. That Ciprofloxacin hydrochloride (CIP), is a fluoroquinolone derivative that acts by inhibiting DNA gyrase and topoisomerase IV, which are essential enzymes in the reproduction of bacterial DNA [8], hence it is used to treat external infections of the eye such as acute and sub-acute bacterial conjunctivitis, conjunctivitis, keratitis, kerato-conjunctivitis and corneal ulcers [9].

While Naproxen sodium (NAP.), a propionic acid derivative, is a non-steroid drug with established anti-inflammatory activity. As an ophthalmic solution, it is able to decrease aqueous levels of proteins, polymorphonuclear leukocytes and PGE2 following experimentally induced inflammation in rabbits. In addition, topical sodium naproxen has successfully maintained mydriasis during cataract operation and also effective in preventing and controlling ocular inflammation after the surgery [10].

The aim of this study was to formulate ocular in-situ gel, that undergo gelling via ion-induced mechanism with the aid of sodium alginate and gellan gum in combination with HPMC as a viscosity inducer to sustain the release of the two drugs (CIP the antibiotic that prevent infection, and nap the anti-inflammatory drug prevent pain and reduce inflammation) accordingly the ocular contact time would increase hence the drugs residency time increase as well, which in turn would reduce frequency of administration. Nevertheless to reduce the number of pharmaceutical products, eye drops, into one product hence improve the patient compliance.

MATERIALS AND METHODS:

Materials:

Ciprofloxacin HCl (CIP), naproxen sodium (NAP) (Samara drug industry, Iraq), hydroxyl propyl methyl cellulose (HPMC K4M), sodium alginate, gellan gum, benzalkonium chloride (Himedia, India), sodium chloride (NaCl), sodium bicarbonate ($NaHCO_3$), calcium chloride ($CaCl_2$) (Provizer pharma, India). All other solutions, chemicals and reagents were of analytical grade.

Preparation of the Ocular In-Situ Gel:

For the preparation of 50ml of CIP-NAP ocular in-siu gel, all the ingredients showed in table 1 were accurately weighed, followed by separately dissolving the viscosity inducer (HPMC K4M), in addition to sodium alginate and gellan gum in three different concentrations (0.1, 0.3, 0.5%) to a mixture of distilled water (D.W) and NaCl using a magnetic stirrer and allowed to hydrate overnight. On the other hand, the aqueous solutions two active ingredients (CIP and NAP) along with benzalkonium chloride added gradually to the mixture of the previous polymers and the pH was adjusted to 6.5 using HCl [11].

Table 1: Formulation of CIP-NAP ocular in-situ gel

Ingredients	F1	F2	F3	F4	F5	F6
CIP	0.3%	0.3%	0.3%	0.3%	0.3%	0.3%
NAP	0.2%	0.2%	0.2%	0.2%	0.2%	0.2%
HPMC K4M	0.5%	0.5%	0.5%	0.5%	0.5%	0.5%
Sodium alginate	0.1%	0.3%	0.5%	-	-	-
Gellan gum	-	-	-	0.1%	0.3%	0.5%
Benzalkonium chloride	0.01%	0.01%	0.01%	0.01%	0.01%	0.01%
NaCl	0.9%	0.9%	0.9%	0.9%	0.9%	0.9%
DW	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.

*Abbreviations: Ciprofloxacin HCl (CIP), naproxen (NAP), sodium chloride (NaCl), distilled water (DW).
*All the ingredients are expressed in w/v percent.

Characterization of the Prepared Ocular In-Situ Gel:

Fourier Transform Infrared Spectroscopy (FT-IR) Studies:

FT-IR spectrum for both, pure drugs and optimum formulation was analyzed in the range between 4000-500 cm^{-1}, by applying pressed-disk technique [12].

Gelling Capacity:

The prepared formulations of ophthalmic in-situ gel were evaluated for the gelling capacity by measuring the time for gelation and also the time required for the gel to dissolve, which was achieved after the addition of each formulation to a freshly prepared simulated tear fluid (STF) in a proportion of 25:7 at 37 ºC to simulate the ocular media [13].

Clarity:

Clarity test was performed by visual inspection of the prepared CIP-NAP ocular in-situ gel after swirling them against a black and white background under good light. The prepared formulations were detected for clarity, turbidity or the presence of any dispersed particles [14].

The pH Measurement:

The pH for the prepared in-situ gel formulas was detected using a calibrated pH meter [15].

Drug Content:

The drug content of CIP-NAP ocular in-situ gel was determined by dissolving 1ml of the prepared formulation in pH 7.4 phosphate buffer then

spectrophotometrically analyzed to determine the amount of each drug [16].

Rheological Study:
The viscosity of the ocular in-situ gel formulations is an important indicator for ease of administration and sustain the release of both drugs after administration. They are evaluated for each CIP-NAP in–situ gel before and after gelling at 25 and 37ºC for each formulation respectively using a Brookfield Viscometer in different rotational speed (30, 50, 75 and 100rpm) [17].

In-Vitro Dissolution Study:
The in vitro dissolution of the prepared in-situ gel formulations was performed by diffusion method using the classical laboratory procedure using the open ended glass tube. A celllophane membrane pre-soaked in the dissolution media was fixed in the open end of the glass tube, which resembles the donor compartment that fixed inside 100ml beaker containing 50ml of phosphate buffer pH 7.4 resembling the receptor compartment. 1ml of the preparation was allowed to diffuse via the cellophane membrane to the receptor compartment which was kept on magnetic stirrer at 37ºC.

5ml sample was withdrawn in each time for 8 hrs and analysed using UV dissolution apparatus at 322 and 230 nm to detect the concentration of both CIP and NAP respectively [18].

Antimicrobial study:
The antimicrobial effect of CIP from the optimum formula was compared to that of the commercial ciprofloxacin eye drop using agar diffusion test. In order to do that, five different phytopathogenic microorganisms were used in this test, out of these microorganisms, two are Gram-negative bacterium (*Escherichia coli* (*E. coli*) and *Acinetobacter*) and three are Gram- positive bacterium (*Streptococcus, Staphylococcus aureus* and *Staphylococcus epidermidis*) were collected from privet microbiology laboratory, Iraq. The pure cultures of these organism were in lyophilized or freeze-dried form, therefore, they were reconstituted by the addition of sterile water. This resulted in required suspension of microbial cultures. Sterility was also maintained during inoculation and for this purpose sterile loops were used to transfer the cells to liquid broth medium. The next step involved the incubation of the liquid cultures to ensure the optimum replication and growth of bacterial cells. Finally, they were stored in refrigerator for subsequent usage. Time given for incubation was 24 hours and thick spread of microbes was obtained for assays. The medium used for testing and maintaining the bacterial strains was Nutrient agar (NA).

This was followed by pouring a suitable dilution of sterile CIP solution (from both optimum formula and the commercial CIP eye drop) into a pressed well in the previously prepared agar media seeded with the test microorganisms. These solutions were allowed to diffuse from the cell for 2hrs, followed by incubation period of 24hr at 37ºC. The inhibitory effect of CIP from the optimum formula was evaluated by measuring the zone of inhibition (ZOI) and compared to that of the marketed CIP eye drop [19,20].

Statistical Analysis:
All results were analyzed statistically by (ANOVA) single factor to compare and evaluate the significance of the results, where ($P<0.05$) are considered to be significant.

RESULTS AND DISCUSSIONS:

Fourier transform infrared spectroscopy (FT-IR) analysis:
Studying the molecular effect of the drugs and the expedients on each other required FT-IR analysis, the test was performed for both drugs along with the optimum formulation (F3) are shown in figure 1. It clearly presents the characteristic peaks of the CIP at 3365 cm^{-1} which results from OH stretching vibration, 1392 cm^{-1} for O-H bending vibration, and the peaks at 1315 and 1267 cm^{-1} which represents the stretching vibration for ester and alkyl halide consequently [16].

On the other hand, FT-IR spectrum of nap clearly demonstrates the characteristic peaks of the drug at 1604cm^{-1} resulted from C=C aromatic stretching vibration, 1261 and 1028 for aryl-O stretching vibration, and the peak of C=C bending vibration that presented at 688 cm^{-1}[21,22]. Furthermore, the results of the optimum formula (F3) showed no significant shifting in the peaks, which clearly signifying the compatibility between the two drugs.

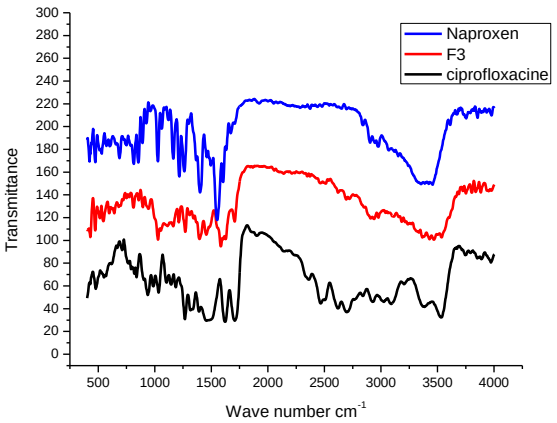

Fig. 1: The reconstructed Fourier transform infrared spectrum of the two drugs and the optimum formulation (F3)

Evaluation of the Gelling Capacity, Clarity, pH and Drug Content:

Ophthalmic in-situ gel formulations (F1-F6) were prepared using different concentrations of sodium alginate and gellan gum (0.1%, 0.3% and 0.5%) respectively. The results of gelling capacity, appearance, pH and drug content are shown in table 2. The results clearly demonstrate that all prepared formulations possess a clear appearance with an acceptable pH and drug content. Moreover, the gelling capacity were significantly increased upon increasing the concentration of both sodium alginate and gellan gum, with the formulation (F3) found to be the optimum one, having an immediate gelation and the gel persist for an extended period.

This can be explained due to the nature of the polymers (sodium alginate and gellan gums) that they possess carboxyl and hydroxyl groups that undergo cross linking upon any increase in the polymer concentration, resulting in increasing the within the polymer matrix, therefore strong bridges are generated that form a rigid matrix, hence the gelling strength [23].

Table 2: Results of gelling capacity, appearance pH and % drug content

Formula	Gelling capacity	Clarity	pH	%Drug content
F1	+	Transparent	6.53±1.93	98.05±1.45
F2	++	Transparent	6.56±1.03	99.43±2.09
F3	+++	Transparent	6.58±2.08	98.35±3.08
F4	-	Transparent	6.51±1.80	97.12±1.89
F5	+	Transparent	6.54±2.35	99.27±1.56
F6	++	Transparent	6.53±1.65	98.17±2.76

*****Abbreviations**:** No gelation (-), gelation occurred in a few minutes and remain for few hours (+), gelation immediate and remained for a few hours (++), gelation is immediate and last for extended period (+++). Results are expressed as mean ± SD, n=3.

Rheological Analysis:

For further optimization of the prepared CIP-NAP in-situ gel formulations, the viscosity of each formulation was measured before and after gelation in order to select the most suitable formulation that is easily administered with high viscosity after gelation. The results are shown in table 3 clearly indicated a significant increment in the viscosity of each formula upon increasing the concentration of sodium alginate and gellan gum, with formula (F3) containing 0.5% sodium alginate shows the best results [23].

Table 3: Rheological properties before and after gelation for the prepared ocular in-situ gel formulas (in centipoise)

Formula	Viscosity of solution (at 25 ºC)	Viscosity of in-situ gel (at 37 ºC)
F1	0.0155±1.72	83.3±1.37
F2	0.0347±1.08	210.5±2.98
F3	0.126±2.16	794.4±1.02
F4	0.0124±1.93	72.5±1.46
F5	0.0257±2.65	188.4±2.37
F6	0.0946±1.34	530.6±2.34

* Results are expressed as mean ± SD, n=3.

In-vitro Dissolution Analysis:

Results of in vitro release of both naproxen sodium and ciprofloxacin HCL from the formulated ocular in-situ gel formulas F1-F6 was illustrated in figures 2 and 3 respectively. The prepared formulations showed initial burst release of both drugs specially from formulations F1 and F4 containing 0.1% sodium alginate and gellan gum which decrease by increasing the polymers concentrations to 0.3 and 0.5%, a result which can be explained by the fact that as long as these insitu gelling systems were prepared in an aqueous media, hence the formed matrix after gelation was pre-hydrated, accordingly, hydration and water permeation could not control drug release [24,25]. Furthermore, a significant improvement in controlling the release of both drugs from the formulated CIP-NAP in-situ gel preparations upon increasing the concentrations of both sodium alginate and gellan gum, with F3 containing 0.5% sodium alginate giving the best result by retaining the drugs for prolong period up to 8 hours, which is correlated with the results of the gelling capacity study which proves that F3 giving an immediate gelation for extended period of time.

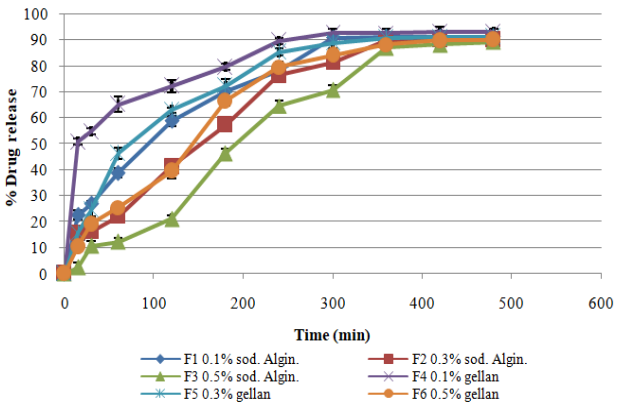

Fig. 2: In-vitro release analysis of naproxen sodium from ocular in-situ gel preparations (F1-F6) in pH 7.4 phosphate buffer (Results are expressed as mean ± SD, n=3).

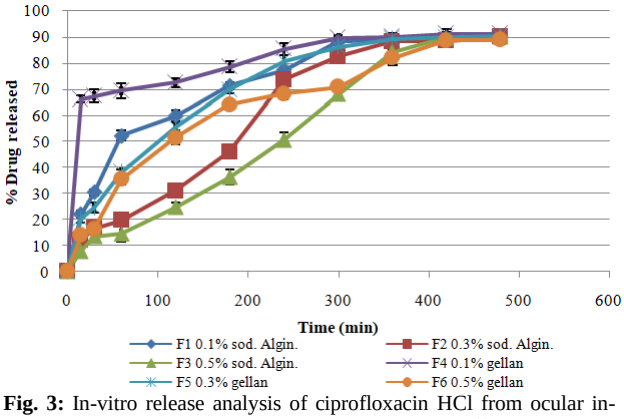

Fig. 3: In-vitro release analysis of ciprofloxacin HCl from ocular in-situ gel preparations (F1-F6) in pH 7.4 phosphate buffer (Results are expressed as mean ± SD, n=3).

Table 4: Antimicrobial effect of ciprofloxacin from both marketed and the optimum ocular in-situ gel formula (F3)

Type of bacteria	Type of Microorganisms	Minimum inhibitory concentration (mg/ml)	Zone of inhibition (mm)	
			Marketed antibiotic	Formulated Antibiotic
Gram +ve	*Streptococcus pneumonia*	2	10	12
	Staphylococcus aureus	1	14	15
	Staphylococcus epidermidis	0.5	15	17
Gram -ve	*Escherichia coli (E. coli)*	2	8	10
	Acinetobacter	2	8	8

Microbiological Study:

The antimicrobial effect of CIP in the prepared ocular in-situ gel and the antimicrobial effect of the marketed cip eye drops were shown in table 4. The results clearly shows that CIP retain its antimicrobial effect after formulation as in-situ gel, with a higher ZOI observed in most data that might result from the slow and prolong diffusion of the drug from the prepare in-situ gel [19].

CONCLUSION:

From our work, all the collected data proves the effective implementation of ocular in-situ gel containing CIP. and NAP., by using a combination of sodium alginate (0.5% w/v) as an ion cross-linking polymers with (0.5% w/v) HPMC K4M as viscosity enhancer, which can be efficiently administrated post surgically after cataract operation in order to prevent bacterial infection and reducing inflammation.

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RESEARCH ARTICLE

Development of Posaconazole delayed-release tablets by high shear melt Granulation Technique

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ABSTRACT:
Pharmaceutical industries, particularly small and medium scale enterprises (SMEs) always prefer a manufacturing process that involves conventional equipment and devoid of solvents due to commercial, safety and ecological reasons. However, some drug products like Posaconazole delayed-release tablets are currently manufactured by the processes that require complex/ costly equipment or a huge quantity of solvent and specifically skilled personnel. Aim of this study was to simplify delayed-release dosage form of Posaconazole, a BCS class IV drug having pH dependent solubility by the simple yet innovative manufacturing process. The study employed high shear melt granulation (HSMG) technique for manufacturing of drug product. Drug release controlling excipients namely Eudragit L100, triethylcitrate and Macrogol 6000 were selected, as these are suitable excipients for solventless processing at high temperature. Levels of drug release controlling ingredients were optimized using one factor at a time (OFAT) approach. Itwas revealed that drug release from the formulation was affected by the level of Eudragit L100, and Eudragit L100: Macrogol 6000 ratio. The optimized formulation was found satisfactory and reproducible with respect to all the studied pharmacotechnical parameters. The product was found stable at the accelerated storage conditions. In conclusion, Posaconazole delayed-release tablets can be prepared by conventional set of equipment, which are available at most of the pharmaceutical SME by using the explored HSMG technique.

KEYWORDS: Posaconazole, melt granulation, high shear, solventless, delayed-release, solid dispersion.

INTRODUCTION:
Notwithstanding advancement in processing techniques to prepare solid dispersion of drug in carrier polymer, small and medium scale pharmaceutical industries (SMPI) vacillating to manufacture such drug products due to higher technological investment to prepare such dosage forms. Hence, there is a craving need to explore a cost effective processing technique which does not involve any specialized equipment.

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Posaconazole delayed-release tablets is such a dosage form where in the drug product is molecularly dispersed in enteric polymer. Posaconazole is a newer anti-fungal drug, which used to treat life-threatening conditions caused by fungal infection.[1,2] It is a BCS class IV drug, which is having good solubility in acidic media, but having poor solubility in basic media. So, as described in patent application no. WO 2009/129300 A2, orally administered Posaconazole dissolves in stomach fluids and when this stomach fluid reaches the environment of the intestine (typically less acidic than about pH 6.4) a substantial amount of the dissolved posaconazole precipitates, hindering absorption in the intestine. It has been determined that in environments where the pH is about pH 6.4 or more basic, the solubility of posaconazole free base is less than about 1µg/mL.[3] Hence, in commercially available solid oral formulations, Posaconazole is formulated as solid suspension or solid solution in enteric coating polymers to enhance the solubility in alkaline pH and to protect the drug from acidic environment of stomach where it has

higher solubility.[3–7] The formulations mentioned in prior the art were processed by hot melt extrusion process. Some other prior arts also mentioned spray drying, freeze drying and wet granulation by top spray method. All of these techniques requires specific, expensive equipment and huge quantity of solvent(s). So, it becomes difficult for SMPI to manufacture such type of product.

Prior art:

Patent# WO2009129300 discloses compositions wherein Posaconazole is molecularly dispersed in hydroxy propyl methyl cellulose-derivative polymer by using hot melt extrusion. This patent states that Pharmacokinetic parameters of Posaconazole can be improved by preparing solid dispersion of the drug in enteric polymers such as HPMC-AS. The patent discloses drug: Polymer ration and a temperature range for hot melt extrusion process.[3]

Patent application# US20150231081 discloses the use of polymer other than HPMC derived polymer to prepare hot melt extrudes of Posaconazole solid dispersion. Here, the embodiments disclose use of methacrylic acid and ethyl acrylate polymer (Eudragit L 100-55) to prepare drug polymer extrudates.[5]

Patent# WO2017/032908A1 discloses the method of preparation of posaconazole formulation by top spray granulation method wherein posaconazole is dissolved in a common solvent with HPMCAS and Methacrylic acid – methyl methacrylate copolymer (1:1). The solution is sprayed over microcrystalline cellulose. Many prior arts also described in the patent wherein hot melt extrusion, freeze drying and spray drying processes are disclosed. However, these techniques require specific and expensive equipment which are not present in most of the pharmaceutical production plants.[6]

Patent application# EP3210599 A1 discloses the use of water soluble, neutral or anionic polysaccharide as precipitation inhibitor in solid solution of posaconazole in enteric polymer. Additionally, it also states that the process of melt extrusion should be carried out in temperature below 160°C because, beyond this temperature, posaconazole is degraded. Hence, as per the disclosure, the processing temperature can be lowered by using a non-enteric polymer other than a neutral or anionic polysaccharide. The patent also states that the presence of a sugar alcohol in the mixture that is subjected to hot-melt extrusion may increase the chemical stability of posaconazole.[8]

Patent# WO2015154718 discloses the composition of posaconazole and vinyl pyrrolidone-vinyl acetate copolymer or a polymer containing ethylene glycol units. Here also, it is noted that an enteric polymer must require to prepare solid dispersion. The solid dispersions are prepared by the process of hot melt extrusion.[9]

Kathuria A, found that Eudragit L100 and Eudragit S 100 polymers can be used for dry coating process. Here, they used Vector GXR-35 Rotary Granulator/Coater, which is again a complex equipment. However, it was revealed that polymer : plasticizer ratio 2:1 was required to produce the film using dry coating technique.[10]

Introduction to High shear melt granulation technique:

Melt granulation is a process by which pharmaceutical powders are efficiently agglomerated by use of a binder which melts during the process. This concept was reported in some research works in late 1970 and early 1980, but after that it is seems that it was not studied widely.[11,12] High shear melt granulation (HSMG) technique has been explored for its applicability like solubility enhancement and controlling the drug release. Some work regarding taste masking and compressibility improvement has also been reported.[13]

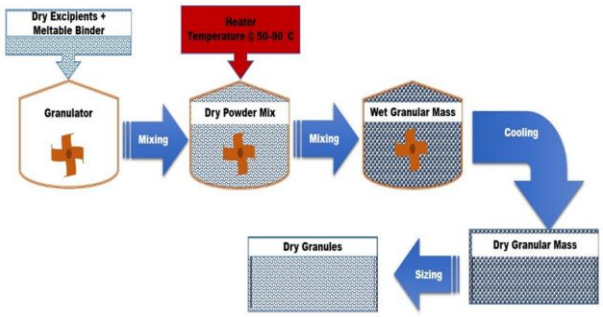


Figure 1 General processing flow of a high shear melt granulation process[12]

Following are some explored application of melt granulation techniques:

A sustained release matrix formulation of Verapamil HCl, a BCS class I drug was prepared using melt granulation technique. The matrix was prepared by waxes namely Glyceryl monostearate and stearic acid and combination thereof. The study revealed that the combination of the waxes was more drug release retarding than alone waxes. Further, it was also observed that drug release was decreased with increase in the level of waxes.[14] Similar way, a sustain release wax matrix tablet formulation of Metoprolol succinate was prepared. Here, hydrogenated vegetable oil, Compritol and Precirol were evaluated as release retarding ingredients. It was observed that release retardant effect was found in order of hydrogenated vegetable oil > Compritol > Precirol.[15] Solubility of Telmisartan was successfully improved by preparing its solid dispersion with alkalized using polyethylene glycol (PEG) as carrier. The solid

dispersion was prepared using hot melt method wherein drug, alkalizer, and PEG mixture was heated at 100°C with constant stirring. It was concluded that solid dispersion containing alkalizer may improve the dissolution of pH dependent drug like telmisartan.[16]

Solid dispersion of poorly soluble drugs in hydrophilic polymers is a well-explored technique to improve aqueous solubility of poorly soluble drug. Solid dispersion refers to a group of solid products consisting of at least two different components, generally a hydrophilic inert carrier or matrix and a hydrophobic drug. Primarily, solid dispersion improves solubility by exposing molecularly dispersed colloidal particles of drug substances to aqueous media which dissolves at a faster rate due to manifold increase in surface area. Carriers which are explored by researchers includes hydrophilic polymers like polyethylene glycol, polyvinyl pyrrolidone, polyvinyl alcohol, hydroxypropyl cellulose, hydroxypropyl methyl cellulose; pH dependant polymers like hydroxypropyl methyl cellulose phthalate and Eudragit; and some non polymeric materials like Urea, mannitol, gums.[17–21] Various types of solid dispersion and methods to prepare such solid dispersions are well explored by researchers. Melt granulation is one of the newer techniques to prepare solid dispersion wherein the solid dispersion can be prepared in a conventional high shear mixer. In this technique, binder which acts as carrier also, is heated with drug and other ingredients above its melting point to form solid dispersion. This process is more preferable due to its better process controls.[22] A solid dispersion of Aceclofenac was prepared using hot melt technique to enhance solubility of the drug. In this study, a mixture of drug and PVP K-30 was heated at 70°C to form molten mass. The optimized formulation demonstrated increased solubility of the drug substance.[23] A solid dispersion of nifedipine which was used as model drug was evaluated. In this study, solubility of nifedipine was successfully improved by preparing its solid dispersion in matrix of hydroxy propyl methyl cellulose K-4M and Eudragit RS-100. The solid dispersion was prepared by melt fusion technique wherein methanol was used as solvent.[24]

Looking at the craving demand of SMPI, an attempt has been made to demonstrate that Posaconazole delayed-release tablets can be prepared using conventional equipment and cost effectively by unique melt-granulation technique. Here, the excipients were selected based on literature study which includes various patents, peer reviewed articles, leaflet and approval package of marketed product, and excipient manufacturer's recommendations. Level of release controlling components viz. Drug: Enteric polymer and Enteric polymer: meltable binder cum hydrophilic polymer were optimized by one factor at a time (OFAT) approach. The targets were to achieve delayed drug release profile and the optimized product should be stable at accelerated condition (40°C/ 75%RH) for six months.

MATERIALS AND METHODS:

Materials*:*

Posaconazole amorphous was received as a gift sample from Amneal (Ahmedabad, India) and all other excipients namely Eudragit L100 (Evonik), tri-ethylcitrate (Vertellus), Polyethylene glycol 6000 (Clariant), Microcrystalline cellulose – Avicel PH 102 (FMC), Methocel E5 (Colorcon India), Cros-carmellose sodium (FMC), Aerosil (Evonik) and Magnesium stearate (Peter Greven) were received from Piramal Pharmaceutical developments services (Ahmedabad, India). All other reagent used were of analytical grade.

Preparation of solid dispersion:

Composition of the solid dispersion was optimized. Ratio of the drug release controlling components viz. Posaconazole: Eudragit L100 and Eudragit L100: PEG 6000, were optimized using one factor at a time (OFAT) approach. Level of Triethylcitrate (TEC) was fixed at 50% of Eudragit base on cited literature.[10] Formulae of Posaconazole: Eudragit L100 and Eudragit L100: PEG 6000 ratio optimization trials are tabulated in Table 1 and Table 2 respectively. The trial batches were manufactured using melt granulation technique as mentioned in Figure 2.

Table 1 Formulae of Posaconazole: Eudragit L100 ratio optimization trials

Batch#	**P2**		**P3**		**P4**	
Batch Detail	**Posaconazole: Eudragit L100 ratio**					
	1 : 1		**1 : 1.5**		**1 : 2**	
Ingredient	**g/ batch**	**% w/w**	**g/ batch**	**% w/w**	**g/ batch**	**% w/w**
Posaconazole (amorphous)	100	26.67	100	21.62	100	18.18
Eudragit L100	100	26.67	150	32.43	200	36.36
Triethylcitrate (TEC)	50	13.33	75	16.22	100	18.18
Polyethylene glycol 6000 (PEG 6000)	25	6.67	37.5	8.11	50	9.09
Microcrystalline cellulose (AVICEL PH 102)	100	26.67	100	21.62	100	18.18
Total	375	100.00	462.5	100.00	550	100.00

Table 2 Formulae of Eudragit L100: PEG 6000 ratio optimization trials

Batch#	**P5**		**P3**		**P6**		**P7**	
Batch Detail	**Eudragit L100 : PEG 6000 ratio**							
	1 : 1.5		**1 : 0.15**		**1 : 0.35**		**1 : 0.45**	
Ingredient	**g/ batch**	**% w/w**	**g/ batch**	**% w/w**	**g/ batch**	**% w/w**	**g/ batch**	**% w/w**
Posaconazole (amorphous)	100.0	22.35	100	21.62	100.0	20.94	100.0	20.30
Eudragit L100	150.0	33.52	150	32.43	150.0	31.41	150.0	30.46
Triethylcitrate (TEC)	75.0	16.76	75	16.22	75.0	15.71	75.0	15.23
Polyethylene glycol 6000 (PEG 6000)	22.5	5.03	37.5	8.11	52.5	10.99	67.5	13.71
Microcrystalline cellulose (AVICEL PH 102)	100.0	22.35	100.0	21.62	100.0	20.94	100.0	20.30
Total	447.5	100.00	462.5	100.00	477.5	100.00	492.5	100.00

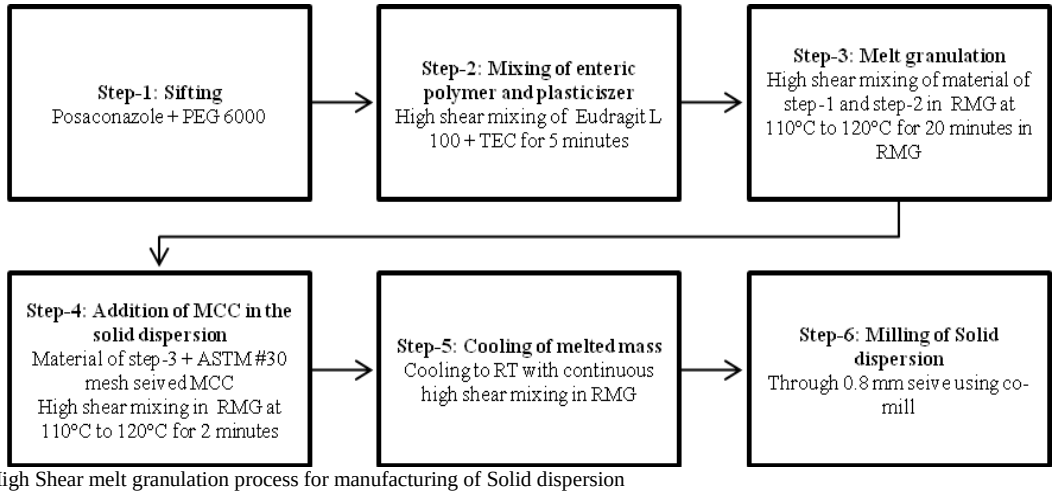


Figure 2 High Shear melt granulation process for manufacturing of Solid dispersion

Table 3 Formula of Posaconazole delayed-release tablets 100 mg

Batch# P8			
Sr. No.	**Ingredients**	**Qty/tablet (mg)**	**%w/w**
Solid dispersion			
1	Posaconazole (amorphous)	100.0	14.29
2	Eudragit L 100	150.0	21.43
3	Triethylcitrate	75.0	10.71
4	Polyethylene glycol	52.5	7.50
5	Microcrystalline cellulose (AVICEL PH 102)	100.0	14.29
Extra-granular			
6	Microcrystalline cellulose (AVICEL PH 102)	159.5	22.79
7	Hypromellose (Methocel E5)	35.0	5.00
8	Croscarmellose sodium	14.0	2.00
9	Colloidal silicon dioxide (Aerosil 200 Pharma)	7.0	1.00
Lubricant			
10	Magnesium stearate	7.0	1.00
Core Total		700.0	100.00
Film-coating (3% w/w weight gainof core tablets)			
11	Opadry® II Yellow	21.0	3% of core
12	Purified water	q.s.	--
Total		721.0	--

Preparation of tablet:

The optimized solid dispersion was compressed in to tablets using commonly used excipients. The tablets were then film-coated by spraying aqueous dispersion of a ready-mix coating material over the rolling bed of compressed tablets in auto-coater. Formula of the film-coated tablets is tabulated in Table 3.

Assay:[25]

Standard preparation:

Posaconazole reference standard was accurately weighed and dissolved in methanol to yield concentration of 25.0 µg/mL, this was diluted to 10.0 µg/mL using same solvent.

Sample preparation:
Ten tablets were crushed using mortar-pastel. The 50 mg equivalent crushed powder was weighed and dispersed in 50 mL Acetone with continuous stirring for 30 minutes and then centrifuged for 5 minutes. The supernatant was diluted with methanol to prepare 10 µg/mL solution.

Standard and samples were analyzed in a double beam UV-spectrophotometer at wavelength of 260nm.

Dissolution method:[26]
Dissolution method for Posaconazole method was used as recommended by office of generic drugs, US-FDA. Following is the dissolution method for Posaconazole DR Tablets:
Apparatus: USP II (Paddle), 75 rpm
Media:
Acid Stage: 0.01 N HCl, 750 mL
Buffer Stage: 50 mM phosphate buffer, pH 6.8 with 0.37 % Polysorbate 80 (after 120 minutes, to the acid stage, add 250 mL of 0.2M Phosphate Buffer, 1.46% Polysorbate 80), 1000 mL

Media:
Acid Stage: 0.01 N HCl, 750 mL
Buffer Stage: 50mM phosphate buffer, pH 6.8 with 0.37 % Polysorbate 80 (after 120 minutes, to the acid stage, add 250 mL of 0.2M Phosphate Buffer, 1.46% Polysorbate 80), 1000 mL

Aliquots measuring 10mL were withdrawn at each time-point, filtered through 0.45µ Millipore membrane filter and analyzed by UV-spectrophotometer after appropriate dilution to yield the sample solution having concentration in a range of 5.0µg/mL to 25.0µg/mL.

Stability study:
The optimized batch (Batch# P8) was packed in 40 CC, Round, HDPE bottle with child resistant cap. Thirty tablets were packed in each bottles and the packed tablets were kept on stability in accelerated condition (40°C±2°C/75% RH ± 5%) for 6 months.

Alcohol dose dumping study:
Co-administration of alcoholic beverages may affect drug release form a modified release solid oral dosage form leading to rapid dumping of complete or fraction dose. Dose-dumping can pose a significant risk to patients, either due to safety issues or diminished efficacy or both. Generally dose-dumping is observed due to a compromise of the release-rate-controlling mechanism. Hence, regulatory agencies are recommending that in-vitro dissolution studies should be conducted to determine the potential for dose dumping. So, a dissolution study was carried out on the optimized batch (Batch# P8) as per US-FDA recommendation.[27, 28]

RESULTS AND DISCUSSION:

Optimization of Posaconazole: Eudragit L 100 ratio
Dissolution profiles of Posaconazole: Eudragit L 100 ratio optimization trials are graphically represented in Figure 3. Here, it can be observed that in batch# P2, the drug was released more than 10% in acidic media, which means that enteric protection is not appropriate. Further, in this batch, drug was not dissolved completely in buffer stage. So, here, the level of Eudragit L100 is not appropriate. In batch# P3 and P4, the drug release was satisfactory in acid and buffer stage. However, in batch P4, the drug release profile was slower when compared to batch# P3. Hence, 1: 1.5 proportion of Posaconazole: Eudragit L 100 was selected.

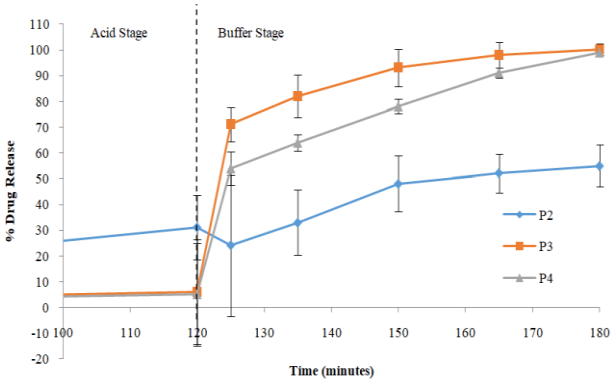


Figure 3 Comparative dissolution profiles of Posaconazole : Eudragit L 100 ratio optimization trials

Optimization of Eudragit L100: PEG 6000 ratio:
In batch# P5, the impeller of RMG was overload and jammed. This means that more PEG is required for better processibility when material is in the molten state. Batch# P3 and P6 shown satisfactory drug release profiles in acid and buffer stage dissolution as observed in Figure 4. However, the drug was completely (more than 85%) dissolved within 15 minutes in batch# P6. Further, in batch# P7, more than 10% drug was dissolved in the acid stage which may be due to more amount of PEG. Hence, 1 : 0.35 proportion of Eudragit L100 : PEG 6000 was selected.

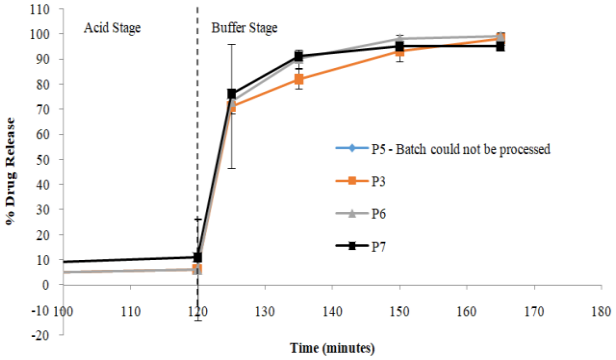


Figure 4 Comparative dissolution profiles of Eudragit L100 : PEG 6000 ratio optimization trials

Physical characterization data of Posaconazole DR tablets 100mg:

The optimized batch P6 was reproduced as batch# P8 and solid dispersion of this batch was evaluated for compression process as per formula given in Table 3. Physico-chemical characterization data of tablets are given in Table-4. The dissolution profile of solid dispersion and tablets are given in Figure 5. Physico-chemical characterization data and dissolution profile of Posaconazole delayed-release tablets 100 mg were found satisfactory. Dissolution profile of batch# P6 and P8 were found comparable, which means that the formula and process for preparation of Posaconazole-Eudragit L100 solid dispersion are reproducible. Drug release profiles of solid dispersion and tablets are comparable which revealed that compression of solid dispersion in tablets does not have any impact on the drug release profile.

Table 4 Physical characterization data of Posaconazole DR tablets 100 mg: Optimized batch (Batch# P8)

Weight (N=20) (mg) (Individual Minitablets) **Target: 7 mg ± 1 mg**	
Average	701.30
Minimum	694
Maximum	711
Thickness (N=20) (mm)	
Average	6.08
Minimum	6.00
Maximum	6.13
Hardness (N=20) (N) **Target: 25N ± 5 N (for average)**	
Average	204.0
Minimum	179
Maximum	223
Friability (%) **Target: NMT 1.0%**	0.17
Disintegration time (N=6) (min'Sec")	1'24"
Assay **(Target: 90.0% to 110.0%)**	97.9
Loss on drying	2.24

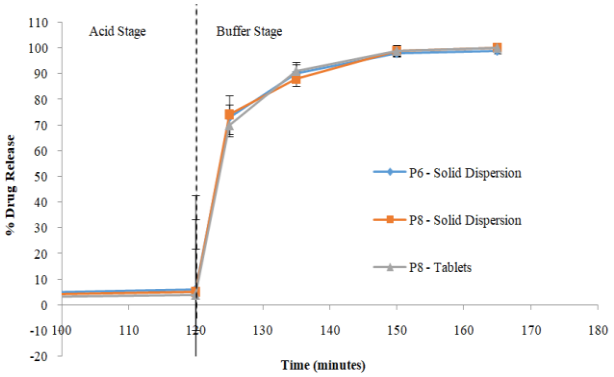


Figure 5 Comparative dissolution profiles: Solid dispersion of Batch# P6, solid dispersion of batch# P8 and tablets of batch# P8

Stability study:

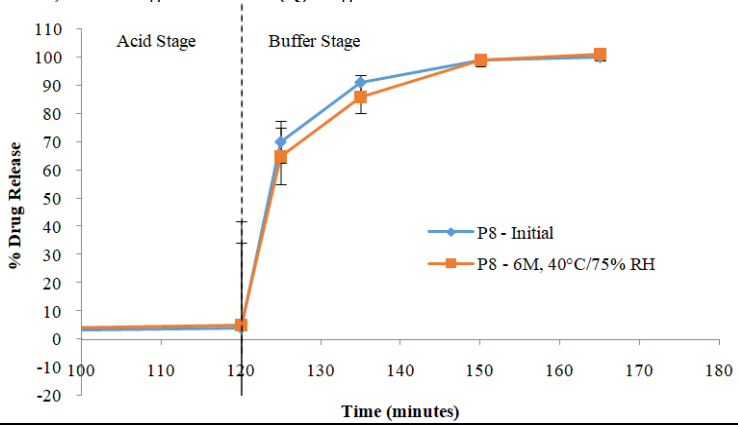
Stability study data of the optimized formulation (Batch# P8) is summarized in Table 5. There was no significant change observed in tablet hardness and assay after 6 month storage at accelerated condition compared to their initial values. Further, dissolution profiles at initial and after 6 months at accelerated condition were also comparable. So, it can be concluded that the drug product is stable.

Table 5 Stability study data Posaconazole delayed-release tablets 100 mg

Batch# P8
Pack style: 40CC, Round, HDPE bottle with CR cap, 30 counts
Stability condition: 40°C ± 2°C/75% RH ± 5%

Test	Specification	Initial	Accelerated condition, 6 months
Tablet Hardness (avg)	-NA-	204 N	198 N
Assay	90.0% - 110.0%	97.9%	98.1%

Dissolution Conditions (N=6):
Specification:
Acid stage: NMT 10% at 120 min, Buffer stage: NMT 75% (Q) drug release in 30 min



Alcohol Dose dumping study:

Comparative dissolution profiles of Posaconazole from the delayed-release tablets in dissolution media with 0% v/v and 40% v/v alcohol are given in below Table 6. The data revealed that the drug was not dissolved more than 10% when studies without alcohol and with 40% v/v alcohol. This reveals that dose is not dumping from the dosage form in alcohol induced dose dumping study.

Table 6 Comparative dissolution profiles: Alcohol dose dumping study

Batch#	**P8**		**P8**	
Alcohol concentration in dissolution media	**0% v/v**		**40%v/v**	
Time (minutes)	**% Drug Released**	**min - max**	**% Drug Released**	**min - max**
30	1	0 – 2	1	0 – 2
60	1	0 – 2	1	0 – 3
90	2	1 – 4	6	4 – 8
120	5	4 – 7	8	6 – 10

Dissolution Conditions (N=6):
Apparatus: USP II (Paddle), 75 rpm
Media: 0.01 N HCl, 750 mL with or without alcohol

CONCLUSION:

From the dissolution profiles and other pharmaco-technical parameters of the optimized trial, it can be concluded that the drug product, Posaconazole Delayed-release Tablets was successfully developed. The optimized formulation was reproducible, stable and was satisfactory with respect to alcohol induced dose dumping study. It can also be concluded that Posaconazole delayed-release tablets can be prepared by melt granulation technique using conventional equipment. The explored melt granulation process was found capable to replace the existing complex process which required costly and complex equipments.

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RESEARCH ARTICLE

Level of Cellular Damage of patients with renal Failure during Dialysis

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ABSTRACT:

Background: Type II Diabetes Mellitus (T2DM) is characterized by insulin resistance at the level of skeletal muscle and fat tissue and muscle and liver, which leads to increase the level of glucose in the blood. This type of diabetes is not dependent on insulin, which affects adults or diabetes resistant ketosis, when the pancreas continues to produce insulin, but there is an imbalance in insulin receptor cells that does not respond to insulin, causing improper hepatic glucose metabolism. Dialysis is considered one of the most important strategies to deal with the decline of the kidneys from the performance of their vital functions, which leads to renal failure. Dialysis is an artificial mechanism by which nitrogen waste and liquid and toxic solvents are removed from the circulation. When the kidneys fail to do this properly, in all types of dialysis; blood is reacted with an artificial solution similar to human plasma and through a semipermeable membrane occurs the diffusion of liquids and dissolved substances. lt involves access to the blood circulation through either a venous or central venous artery catheter or a vaccination where the patient's blood pumping occurs through a blood removal device where two-way diffusion of atoms between dialysis and blood is allowed through a semipermeable membrane. **Subjects:** From the beginning of December 2018 to the end of May 2019, 158 individuals residents of Najaf and Karbala Governorates were included to participate in the current study. **Method:** Sandwich-ELISA technique was applied to estimate levels of Nitric Oxide, Superoxide Dismutase, GR in the sera samples of the study groups using kits that prepared by Elabscience Company, China. **Results:** The results of the present study recorded significant difference ($p < 0.05$) of NO, SOD, and GR concentrations measured in the samples of the two study groups. When comparing male and female subgroups implicitly, a significant difference ($p = 0.000$) of NO levels was recorded when patients of both genders compared together. Similarly, the study recorded a significant variation ($p = 0.001$) for NO levels when comparison was carried out between the males in the study subgroups, on the other side no such result was observed ($p = 0.062$) when the two women subgroups were compared together. The current study found a high significant difference ($p = 0.000$) when SOD in the samples of diabetic females was compared with their healthy partners in the control group, the highest mean of SOD (1.360 ng/ml) was observed in the group of diabetic females who underwent hemodialysis during the completion of the current work, while the highest concentration of a separate sample (4.493ng/ml) was found in a 66-year-old patient who had diabetes at the age of 35 and undergoing hemodialysis only for the second time. The study indicated a significant increase ($p < 0.05$) in the concentration of GR in males compared to females in both study groups. The highest mean of GR (1.561ng/ml) was observed in the group of healthy males, while the highest concentration of a separate sample (2.532ng/ml) was found in a 46-year-old patient who had diabetes at the age of 35 and undergoing hemodialysis since 6 months. In the patients group, positively significant correlation ($r = 0.697$ at $p < 0.005$) was observed for the concentrations of NO as well as GR to the age. More than four-fifths (92 of the total patient cases, at $p < 0.005$) of people with diabetes and dialysis as an adjunct to kidney function showed a positive correlation between NO and GR concentrations, while the relationship was shown to be negative between SOD and GR.

KEYWORDS: Renal Failure, Dialysis, T2DM, NO, SOD, GR.

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INTRODUCTION:

The adjustment of NO (NO) metabolic rate is mainly significant in type 2 diabetes, for the reason that activation of NOS has accomplished under insulin control by the Akt-pathway. Consequently, NO

disturbances generation can be a result of insulin resistance that affects correspondingly the vascular response. Reported researches have shown that impaired NO metabolic rate has been found in T2DM, predominantly in the existence of nephropathy. A decreased urinary excretion of NO related products, like nitrites and nitrates, together termed as **NOx** were testified in type 2 diabetic patients with nephropathy. On the other hand, micro albuminuria has been linked with impaired endothelial function in type 2 diabetic subjects. Hyperglycemia may also be a factor in the lessened NO generation in T2DM, since high glucose inhibited endothelial NOS action in the glomeruli, through a protein kinase C–associated mechanism. Additionally, high glucose or the accompanying advanced glycosylation end products reduced NOS expression. Urinary NOx production has decreased in non diabetic renal disease. Correspondingly, increased plasma nitrate concentrations were testified in T2DMin addition to the metabolic syndrome. For the reason that the stimulation of NOS action by insulin is impaired in muscle of type 2 diabetic patients, examinations about response to the hormone of whole-body NO production in T2DM has key relevance [Cohen2005].

Augmented oxidative stress has the major role in the diabetes etiology besides its complications [Antonio 2000]. In diabetes, persistent hyperglycemia motivates the generation of ROS from numerous resources [Liang-Jun 2014], accordingly, diabetes typically causes augmented formation of ROS and debilitated antioxidant defenses. SOD catalyzes the transformation of O_2^- into H_2O_2.Under hyperglycemic circumstances, endothelial cells generate elevated levels of O_2^-. overproduction of O_2^- can inhibit glyceraldehyde-3-phosphate dehydrogenase that stands for a significant enzyme of the glycolytic pathway. This causes buildup of glucose and other intermediate metabolites of this alleyway and moves to other alternative alleyways of glucose metabolism accompanied by augmented creation of advanced glycation end products [Chia-We 2015].

Antioxidant enzymes stand for a foremost resource to protect in contradiction of reactive oxygen species produced by macrophages, neutrophils and eosinophils. These enzymes are mainly imperative in contradiction of chronic infections. GR (GR) (E.C. 1.8.1.7)[Kavita 2013] is one of a chain of enzymes which serves to maintain glutathione in the reduced form. In vitro, this enzyme can function with either NADH or NADPH as hydrogen donor [Zhu2018]. Glutathione (GSH) and GR stand for parts of the GSH redox cycle that keeps cells in contradiction of damage by oxidants. GR has a dominant role in glutathione metabolic rate and as such is a would-be target for different therapies.GR has FAD and a disulphide at its active site. The catalytic reaction necessitates drop of the site by NADPH, making a semiquinone of FAD, a sulphur radical and thiol. GR has involved some attention as a typical instance of flavin enzymes, and numerous efforts were prepared to regulate the order of attachment of substrates and releasing of its products. After reduction of the active site by NADPH, NADP is feasibly released after or before the catalytic steps including glutathione. The relation among reaction rates and substrate concentrations put forward primary release of NADP. Conversely, the competitive inhibition exposed by NADP headed for NADPH suggests release of NADP after condensed glutathione [Sergey 2012, Sáenz 2016, Yusen 2018]. Partial GR deficiency was first described in patients who had undergone drug-induced hemolysis, but whose red cells had normal G-6-PD activity. Subsequent studies showed, however, that subsequently partial GR deficiency was also observed in patients who had a variety of hematologic disturbances, both drug-induced and idiopathic, and a bewildering array of hematologic and nonhematologic diseases. Included in the disorders which were associated with deficiency of this red cell enzyme were diseases, such as hemophilia, leukemia, hemoglobin C disease, and Gaucher's disease-disorders which by no stretch of the imagination could be due to the enzyme deficiency [Sikanyika, 2019]. It was suggested, therefore, that GR deficiency might be secondary to some other disturbance common to these patients [Langbein 2017].

SAMPLES AND METHODS:

Samples: During the period from the beginning of December 2018 to the end of May 2019, 158 individuals residents of Najaf and Karbala Governorates were included in the current study. The participators were classified into two groups depending on their healthy to: patients with renal failure caused by diabetic complications undergoing hemodialysis and controls. The first group included 108 patients between the age of 20 and 80 (54.160±13.347), while the second group included 50 healthy persons between the age of 22 and 65 years (26.140±6.940). Total data about the study groups were summarized in Table 1.

METHOD:

Sandwich-ELISA technique was applied to estimate levels of Nitric Oxide, Superoxide Dismutase, GR in the sera samples of the study groups using kits that prepared by Elabscience Company, China.

Table 1: Levels (Mean±S.D.) of Age (Year) in The Study Individuals

Study Groups (n)	Gender (n)	Age (Year) Mean ± S.D.	Min.-Max. Range	p-value
Patients *108*	Male 71	54.380 ± 12.827	24-80 56	*0.786* *For 1 vs 2* *0.000* *For 1 vs 3* *0.808* *For 3 vs 4* *0.000* *For 2 vs 4*
	Female 37	53.730 ± 14.468	22-72 50	
Controls *50*	Male 38	26.370 ± 7.295	16-43 27	
	Female 12	25.420 ± 5.900	20-38 18	

1: Male Patients with Renal Failure, 2: Female Patients with Renal Failure, 3: Healthy Male Control, and 4: Healthy Female Control. The Mean Difference is Significant at 0.05 Level

RESULTS AND DISCUSION:

The results of the present study recorded a statistically significant difference (p = 0.000) of NO concentration measured in the samples of the two study groups, as shown in Tables 2.

Table 2: Levels (Mean ± S.D.) of NO Concentrations (µmol/L) in the Sera Samples of Renal Failure Patients and Controls Groups

Study Groups (n)	NO Concentrations (µmol/L) Mean ± S.D.	Min.-Max. Range	p-value
Patients *108*	2.952 ± 0.932	0.110-4.463 4.353	*0.000*
Controls *50*	1.618 ± 1.098	0.186-4.506 4.320	

The Mean Difference is Significant at 0.05 Level:

Elevated glucose levels promote the production of reactive oxygen species as a result of auto-oxidation of glucose, metabolism, and the development of advanced glycosyl products [Brownlee 2001]. Uncontrolled blood sugar leads to a decrease in NO metabolism, which can be the cause of total and micro vascular complications [Dara 2017]. The dialysis procedure itself stimulates the cytokine induced by any synthase and also because of platelets that generate high concentrations of NO due to high urea. NO is an important metabolic product of the oxidation process. No excessive production can be cytotoxic, and is caused by the interaction of NO with reactive oxygen species and nitrogen, leading to the formation of peroxynitrite anion, tyrosine protein nitrogen, and the production of hydroxyl radical [NO in renal health NO is a cytotoxic molecule responsible for complications of dialysis and results in nitrogen stress in these patients, because it is a highly reactive free radical [Anila 2017]. The regulation of NO metabolism is particularly important in case of type 2 diabetes, because NO synthase (NOS) activation is controlled by insulin through the Akt pathway [Ranganath 2008]. High NO production may indicate insufficient blood purification, due to the common effect on the pathways of disposal through the renal duct. Therefore, changes in renal function, highlighted by changes in creatinine concentration, will be accompanied by changes in serotoxic NO [Reddy 2015].When comparing male and female subgroups implicitly, the study showed the absence of statistical differences (p > 0.05) when comparing male with female in the control group, while a significant difference (p = 0.000) of NO levels was recorded when patients of both genders compared together. Similarly, the study recorded a significant variation (p = 0.001) for NO levels when comparison was carried out between the males in the Study subgroups, on the other side no such result was observed (p = 0.062) when the two women subgroups were compared together, as demonstrated in Table 3. The renal blood vessels of men become more dependent on NO with age compared to gynecological diseases, suggesting that any renal disease that interferes with NO production may over time cause kidney damage to progress more quickly in men for women [Chien 2019].

Table 3: Levels (Mean ± S.D.) of NO Concentrations (µmol/L) in the Study Subgroups

Study Groups (n)	Gender (n)	NO Concentration (µmol/L) Mean ± S.D.	Min.-Max. Range	p-value
Patients *108*	Male 71	3.062 ± 1.102	0.129-4.463 4.334	*0.000* *For 1 vs 2* *0.001* *For 1 vs 3* *0.062* *For 3 vs 4* *0.086* *For 2 vs 4*
	Female 37	1.743 ± 0.573	0.110-2.732 2.622	
Controls *50*	Male 38	1.953 ± 1.168	0.186-4.506 4.320	
	Female 12	1.506 ± 1.052	0.530-4.441 3.911	

1: Male Patients with Renal Failure, 2: Female Patients with Renal Failure, 3: Healthy Male Control, and 4: Healthy Female Control. The Mean Difference is Significant at 0.05 Level

Significant difference (p < 0.05) was noticed at the two study's groups were compared together, when SOD concentration was examined, as shown in Table 4.

Table 4: Levels (Mean ± S.D.) of SOD Concentrations (ng/L) in the Sera Samples of Renal Failure Patients and Controls Groups

Study Groups (n)	SOD Concentrations (ng/L) Mean ± S.D.	Min.-Max. Range	p-value
Patients *108*	1.278± 0.988	0.115-4.493 4.378	*0.012*
Controls *50*	0.704±0.416	0.014 - 1.991 1.977	

The Mean Difference is Significant at 0.05 Level:
Statistically, no significant differences ($p > 0.05$) were found when SOD comparing in both genders, implicitly; in the study groups. Similarly, the present study did not record significant differences in the statistical comparison between males with diabetes and their healthy peers in the control group. In contrast to what was observed previous comparisons, the current study found a high significant difference ($p = 0.000$) when SOD in the samples of diabetic females was compared with their healthy partners in the control group, as illustrated in Table 5. The highest mean of SOD (1.360ng/ml) was observed in the group of diabetic females who underwent hemodialysis during the completion of the current work, while the highest concentration of a separate sample (4.493ng/ml) was found in a 66-year-old patient who had diabetes at the age of 35 and undergoing hemodialysis only for the second time. On the other hand, the lower mean concentration of this enzyme (0.741ng/ml) was recorded in the healthy male subgroup, as well as the lowest concentration of this enzyme (0.014ng/ml) was noticed in the sample of a healthy man at 35 years of age.

Table 5: Levels (Mean±S.D.) of SOD Concentrations (ng/L)in the Study Subgroups

Study Groups (n)	Gender (n)	SOD Concentrations (ng/L) Mean ± S.D.	Min.-Max. Range	p-value
Patients	Male	1.083 ± 0.879	0.115-4.493	*0.066*
108	71		4.378	*For 1 vs 2*
	Female	1.360 ± 1.003	0.141-4.419	*0.053*
	37		4.278	*For 1 vs 3*
Controls	Male	0.741 ± 0.416	0.014-1.991	*0.093*
50	38		1.977	*For 3 vs 4*
	Female	0.586 ± 0.211	0.264-1.602	*0.000*
	12		1.338	*For 2 vs 4*

1: Male Patients with Renal Failure, 2: Female Patients with Renal Failure, 3:Healthy Male Control, and 4: Healthy Female Control. The Mean Difference is Significant at 0.05 Level

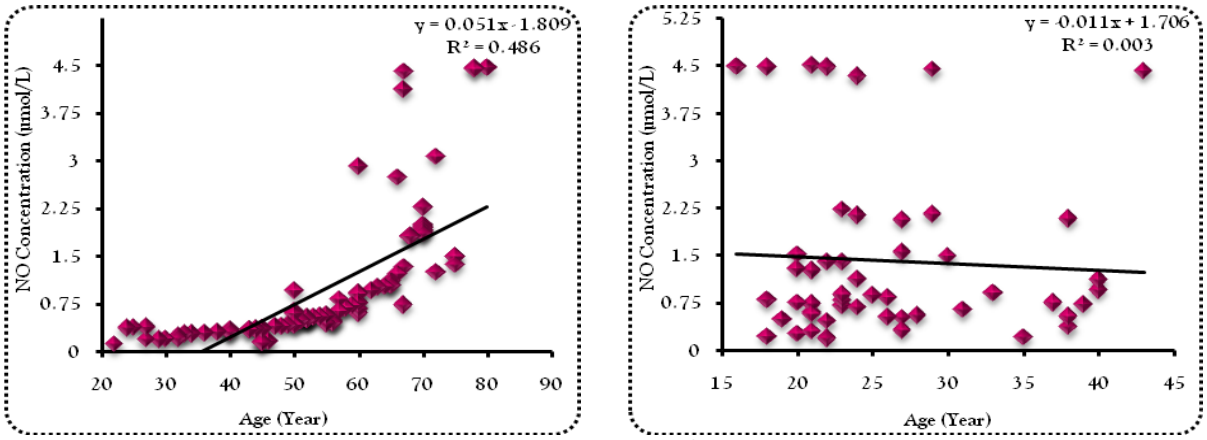
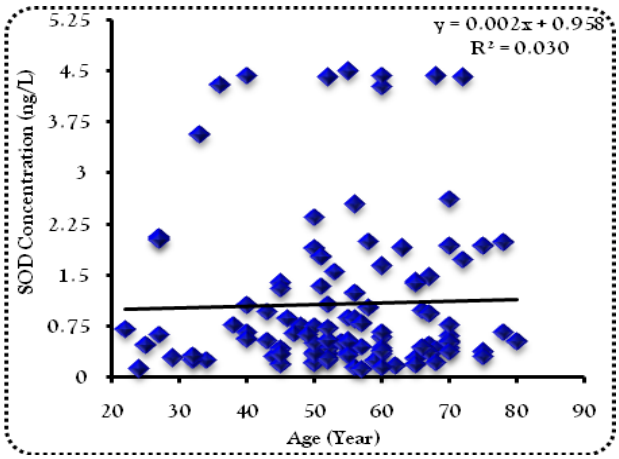


Figure 1: Relationship Between Age and NO Concentration in Sera Samples of (A): Patients and (B): Healthy Individuals

In the patients group, positively significant correlation ($r = 0.697$ at $p < 0.005$) was observed for the concentrations of NO to the age as shown in Figure 1 A, while no such correlations were noted at this relation examined in the control group (Figure 1 B).

Despite the significant increase in levels of this enzyme in patients with type 2 diabetes mellitus with renal failure and in the different age groups, this increase was not consistent with the age of infected samples. Similarly, in the healthy individuals group where the study did not record any association between the level of enzyme and the age of healthy individual. Based on the results shown in Figure 2 A and B, the study was unable to find any statistically acceptable correlation for the age of study subjects with naturally produced SOD enzyme levels or as an immune response to renal failure.



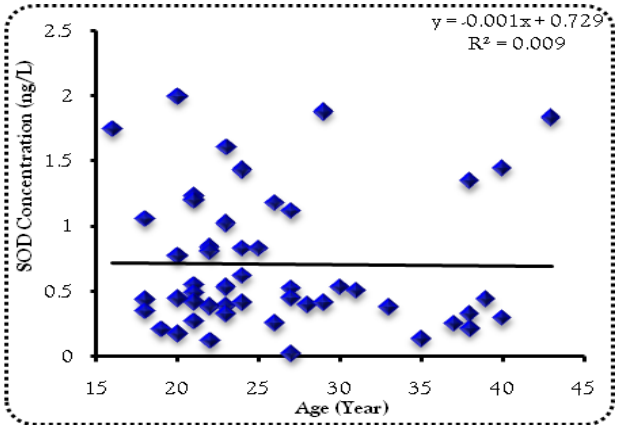


Figure 2: Relationship Between Age and SOD Concentration in Sera Samples of (A): Patients and (B): Healthy Individuals

According to linear regression test that's applied on the two study groups, respected positive correlation ($r = 0.72$ at $p < 0.001$) was observed when GR concentration was correlated to the age of cases in the group of diabetic patients with renal failure, as shown in Figure 3 A. On the other side, non significant negative correlation was recorded when the GR in the sera of healthy control samples was related to their age, Figure 3B illustrated the detailed results.

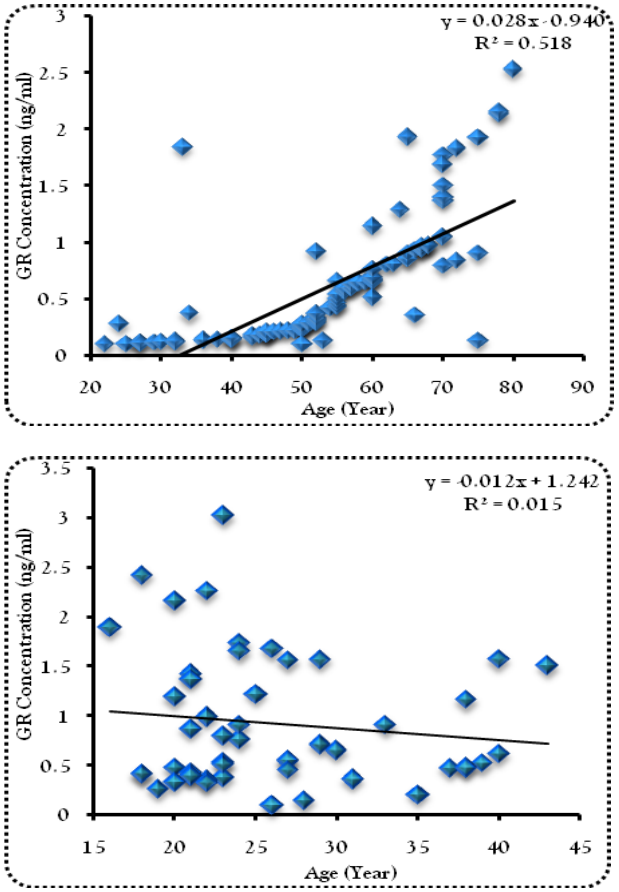


Figure 3: Relationship Between Age and GR Concentration in Sera Samples of (A): Patients and (B): Healthy Individuals

More than four-fifths (92 of the total patient cases, at $p < 0.005$) of people with diabetes and dialysis as an adjunct to kidney function showed a positive correlation between NO and GR concentrations, as illustrated in Figure 4 A. With the same manner, a significant positive correlation was shown in approximate 66% of the healthy individuals, when the same parameters were correlated (at $p < 0.05$) together in the controls group, as illustrated in the Figure 4 B.

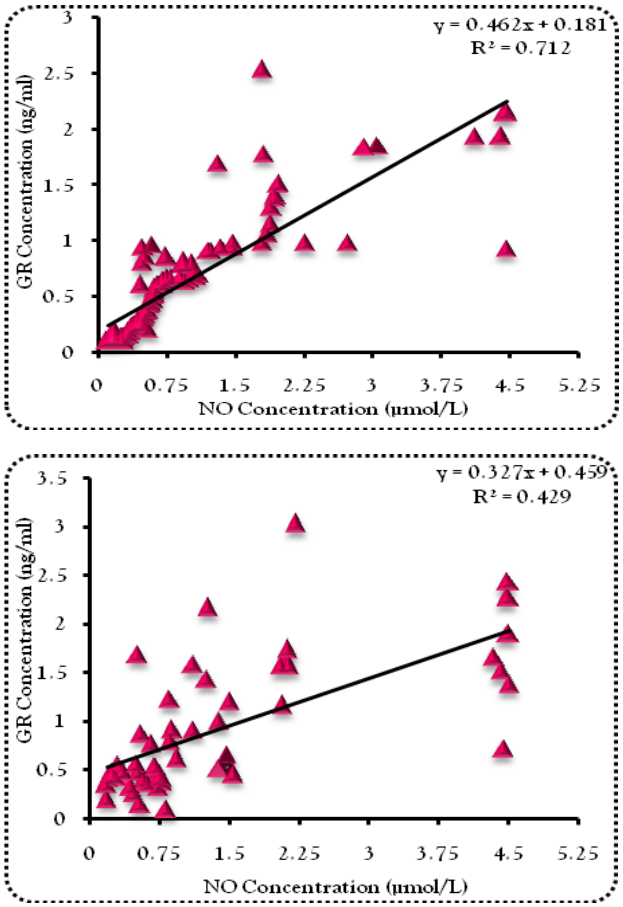


Figure 4: Correlation of NO with GR Levels in The Sera Samples of (A): Patients and (B): Healthy Groups

Results of the present study showed the absence of statistical significance when the correlation between SOD and NO in the diabetic group was carried out as shown in Figure 5 A. On the other hand, the study illustrated a positive correlation ($r = 0.58$ at $p < 0.05$) between the two parameters measured in the controls group (Figure 5 B).

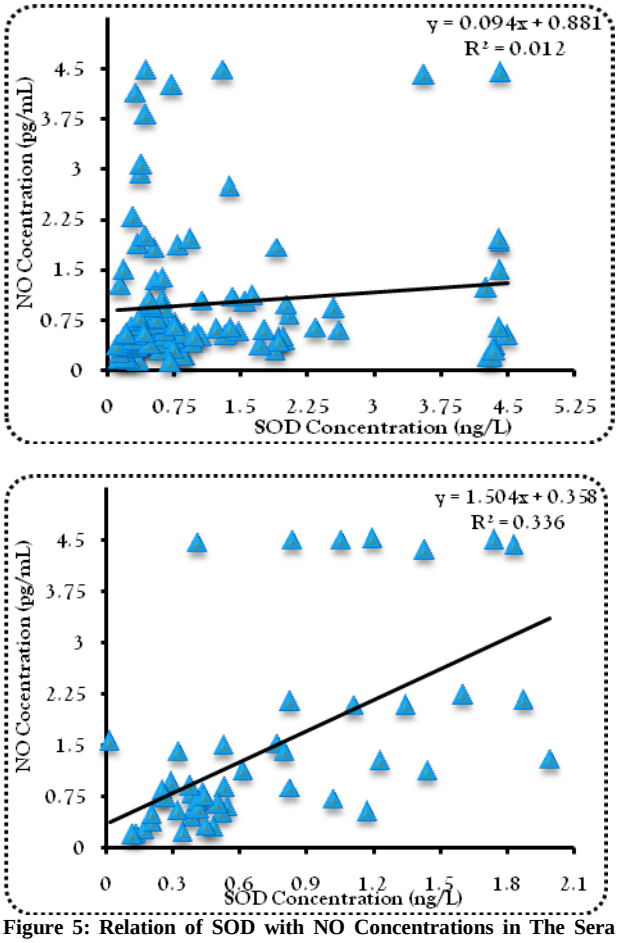


Figure 5: Relation of SOD with NO Concentrations in The Sera Samples of (A): Patients and (B): Healthy Groups

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RESEARCH ARTICLE

Optimization of Repaglinide Controlled Release Floating Tablet

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ABSTRACT:
The aim of present investigation was to develop efficient controlled release floating tablet (CRFT) of Repaglinide. Floating dosage form for gastric retention has potential to use as controlled-release drug delivery systems which providing opportunity for both local and systemic drug action. The tablets were prepared by using wet granulation techniques using PVP K 30, NKG and Carbopol 934 P. A 3^2 full factorial design (CCD) was applied to optimize two independent variables at three different levels by varied response variables. Two independent variables i.e. amount of NKG (i.e., polymer X1) and amount of Carbopol 934 P (i.e., polymer X2) were varied at three different levels that was coded for low, medium and high (-1, 0, 1 respectively). The response variables T6 (cumulative % amount of drug released in 6 hr) (Y1), T12 (cumulative % amount of drug released in 12 hr) (Y2), Q50 (time in minutes required to 50% of drug released) (Y3), FLT (Y4), TFT (Y5), and Swelling Index after 12 hr (Y6) were selected for present study. ANOVA study was also employed to optimize for best fitted quadratic model. Compressed matrices exhibited Super case-II transport drug release kinetics approaching zero-order, as the value of release rate exponent (n) varied between 0.9430 and 1.0133. Formulation A4 was the optimized best formulation from the response surface plot and contour plot of all the formulation.

KEYWORDS: Carbopol 934 P, ANOVA, CCD, FLT, NKG, TFT.

INTRODUCTION:
For the present study, the aim was to develop controlled release floating dosage form to increase the gastric residence time for the drug which leads to increase the bioavailability of drug. Many literatures were suggested that the most convenient method of controlled delivery of drug is undoubtedly oral, but oral controlled release of the drug for an extended period of time that exhibits more absorption in stomach and upper small intestine, has not been successful with conventional approaches. So, it has been decided to develop the controlled release floating dosage form as a novel approach for the drug delivery and the tablet is well known as most convenient dosage form among all oral drug delivery.

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Finally it was decided to develop a controlled release floating tablets (CRFT). [1-4] Repaglinide is a novel antihypertensive agent, widely absorbed from the stomach and upper part of the small intestine. It has shorter elimination half life (0.8hr to 1hr), so necessity to frequent administration and bioavailability can be improved by making the drug completely absorbed in the stomach and upper part of the small intestine. CRFT of Repaglinide was developed using Natural Karaya gum (NKG) and Carbopol 934 P. [5,6]

NKG is a natural gum and it is obtained from gummy extrudes from stem bark of Natural Karaya belongs to the family of sterculiaceae. It is freely soluble in water via hydration and practically insoluble in absolute ethanol. It is used as suspending agent, viscosity enhancer and rate controlling polymer in controlled release dosage. [7-10] Carbopol 934 P is a synthetic high molecular weight cross linked water soluble polymer of acrylic acid, which is known as "Carbomer". It is freely soluble in water and alcohol. It is used as cross linking agent for controlled release matrix as a rate controlling

polymer, stabilizing agent in emulsion, thickening and viscosity modifying agent.

Optimization study was done to determine the appropriate concentration of NKG and Carbopol 934 P in combination as a controlled release polymer and aim was to predict individual effect of both polymers (NKG and Carbopol 934 P) at different concentration level. A 3^2 full factorial design was selected to optimize two independent variable at three different levels by varied response variables. Experimental trials were performed at all nine possible combinations. Two independent variables i.e. amount of NKG (polymer X1) and amount of Carbopol 934 P (polymer X2) were varied at three different levels that was coded for low, medium and high (-1, 0, 1 respectively). The response variables were measured by a multiple factorial regression analysis using the best fitted quadratic model for each trial and it was carried out in MS EXCEL 2007. Various computations required for current study using response surface plot and contour plot were carried out by employing software Design Expert version 8.0.7.1 A statistical model incorporating interactive and polynomial terms was utilized to evaluate the responses. [11,12]

$$Y = b0 + b1X1 + b2X2 + b12X1X2 + b11X12 + b22X22$$

Where, Y is the dependent variables, b0 is the arithmetic mean response of the nine runs, and b1 is the estimated coefficient for the factor X1. The main effects (X1 and X2) represent the average result of changing one factor at a time from its low to high value. The interaction terms (X1X2) show how the response changes when two factors are simultaneously changed. The polynomial terms (X12 and X22) are included to investigate non-linearity. [13-17]

MATERIALS:

Repaglinide was obtained as a gift sample from Zydus Cadila Healthcare limited, Ahmedabad. Natural Karaya Gum (NKG) was obtained as a gift sample by Medicinal natural products research laboratory, University Institute of Chemical Technology, Mumbai. PVP K 30 obtained as a gift sample from Alembic limited, Vadodara. Carbopol 934 P was obtained as gift sample from Corel Pharma Chem, Ahmedabad. NaHCO3, Lactose, Talc, Mg. Stearate and IPA used in the present study were provided by.

METHODS:

Preparation of Repaglinide Controlled Release Floating Tablet:

Repaglinide controlled release floating tablets were prepared by wet granulation techniques using different concentrations of various polymers. To prepare tablet, weighed all ingredients except talc and magnesium stearate and shifted through sieve no 40 then blend uniformly in glass mortar with pestle. After sufficient mixing, the blend was wetted by adding sufficient quantity of isopropyl alcohol as a granulating agent. Prepared wet mass was granulated by passing through sieve no 18. Prepared granules were dried at 50 0C – 60 0C for 20 min in hot air oven. After drying, dried granules were lubricated by adding sufficient quantity of magnesium stearate and talc for 5 min. The tablets were compressed using 6 mm punch on 8 station rotary punching machine. Experimental Design [18,19,20]

A central composite design (CCD) was employed for the optimization of Repaglinide controlled release floating tablets. A 3^2 full factorial design was selected to optimize two independent variables at three different levels by varied response variables. Experimental trials were performed at all nine possible combinations. Two independent variables i.e. amount of NKG (polymer X1) and amount of Carbopol 934 P (polymer X2) were varied at three different levels that was coded for low, medium and high (-1, 0, 1 respectively). The response variables T6 (cumulative % amount of drug released in 6 hr) (Y1), T12 (cumulative % amount of drug released in 12 hr) (Y2), Q50 (time in minutes required to 50 % of drug released) (Y3), FLT (Y4), TFT (Y5), and Swelling Index after 12 hr (Y6) were selected for present study. The experimental design with corresponding formulations is outlined in Table 1. Floating Properties [21].

To measure the floating properties, five tablets from each formulation were selected randomly and placed in beaker containing 250 ml of 0.1 N HCL (pH 1.2). The temperature was maintained at 37 ± 0.5 0C. The time by which the tablet started to float on the surface of medium for FLT and entire duration of time by which the tablet constantly remained on the surface of the medium for TFT was noted. The Floating lag time (FLT) and Total Floating Time (TFT) of tablet of each formulation is shown in Table 2.

Swelling Study [22-24]:

The extent of swelling can be measured in terms of percentage weight gain by the tablet. Five tablets from each formulation were selected randomly for the swelling study. Each tablet individually weighed (W0) and separately placed in beaker containing 100 ml of 0.1N HCL (pH 1.2). The tablet was removed from each beaker after 1 hour of time interval and excess surface solvent from the tablet was wiped out carefully with filter paper. Each swollen tablet was reweighed (Wt) and the swelling index (SI) is calculated using the following formula,

$$\text{Swelling index (SI)} = [(Wt - Wo) / Wo] \times 100$$

Where,
Wt = Final weight of tablet at time t (mg),
Wo = Initial weight of tablet (mg)

The value of swelling index for the tablet of each formulation is given in Table 2.

In Vitro Dissolution Study [25]:
The In-vitro dissolution study for the tablet of each formulation was conducted as per United States Pharmacopoeia type II apparatus. The rotating paddle method was used to study the drug release from the tablets. Dissolution medium 900ml of 0.1 N HCl (pH 1.2) was placed in dissolution vessel. The release was performed at 37 0C±0.5^{0}C and at a rotational speed of paddle about 50rpm. Tablets were placed in each dissolution vessel. The 5ml samples were withdrawn at the time interval of one hour for 16 hrs. The collected samples were filtered through Whatman filter paper No. 40 and analyzed for drug content by UV Spectrophotometer. The absorbance for each sample was measured at 207nm and the concentration of drug present was calculated using calibration plot of Repaglinide. Then, the cumulative percentage amount of drug released after each time interval was calculated using the formula,

Cumulative Amount of Drug Release = C × DF × DM

Where,
C = Concentration of drug (µg/ml),
DF = Dilution Factor is 1,
DM = Dissolution Medium (900ml) Statistical analysis [12]

Statistical optimization of Repaglinide tablet was done by design expert software, Version 8.0.7.1. the study type was response surface, 9 runs were applied to the design type central composite and design model was selected as quadratic. The quadratic model is best fitted for the results to determine the effect of independent variable on response variables. There was considerable difference observed in minimum and maximum values of each response variable with respect to the independent variables. By applying two way ANOVA with 95% confidence level, their predicted values were found for each response variables. The value of $P < 0.05$ was considered to be significant. To demonstrate graphically the influence of each factor on responses, the response surface plots and Contour plots were generated.

RESULTS AND DISCUSSION:

From the preliminary study, it was found that NKG and Carbopol 934 P were efficient polymer to achieve controlled drug releasing property by forming swellable matrix with the drug. Therefore, optimization study was applied to find best possible concentration of both polymer for the present investigation. The formulations were designed by 3^2 full factorial design which is shown in Table 1. Amount of NKG and amount of Carbopol 934 P were selected as independent variables and it was coded as X1 and X2 respectively. Both variable optimized by varied at three different level.

The matrix tablets of designed formulation were prepared by wet granulation method. Developed tablets were evaluated for various response variables. The response variables T6 (cumulative % amount of drug released in 6 hr) (Y1), T12 (cumulative % amount of drug released in 12 hr) (Y2), Q50 (time in minutes required to 50% of drug released) (Y3), FLT (Y4), TFT (Y5), and Swelling Index after 12 hr (Y6) were selected for present investigation. The results of all response variables are shown in Table 2.

The values of T6 was varied from 30.89% to 43.39%, T12 was varied from 57.93% to 84.19%, Q50 was varied from 408 min to 612 min, FLT was varied from 76 seconds to 95 seconds, TFT varied from 16 hrs to 22 hrs, SWI was varied from 96% to 130%. The quadratic model is best fitted to determine the effect of independent variable on response variables. There was considerable difference observed in minimum and maximum values of each response variable with respect to the independent variables. By applying two way ANOVA with 95% confidence level, their predicted values were found for each response variables and it was shown in Table 3.

Drug release profile from all the developed formulation was applied for model dependent kinetics by providing the kinetic treatment and it was exhibited Super case-II transport drug release kinetics approaching zero-order, as the value of release rate exponent (n) varied between 0.9430 and 1.0133. The kinetic treatment of drug release profile for all the formulation A1 to A9 was shown in Table 4.

Table 1: Selected Factor Combinations as per 3^2 full factorial design

Code	Coded level		Actual values (mg)	
	X_1	X_2	X_1	X_2
A_1	-1	-1	10	6
A_2	-1	0	10	8
A_3	-1	1	10	10
A_5	0	-1	14	6
A_5	0	0	14	8
A_6	0	1	14	10
A_7	1	-1	18	6
A_8	1	0	18	8
A9	1	1	28	10

Table 2: The results of each response variables as per 3^2 full factorial design

Code	Code	Code	T_6	T12	Q50	FLT	TFT	SWI
	X_1	X_2	(%)	(%)	min	Sec	hrs	(%)
A1	**-1**	-1	43.39	84.19	408	80	16	96.89
A2	-1	0	37.48	73.89	462	79	17	104.2
A3	-1	1	37.68	73.27	474	76	19	113.7
A4	0	-1	40.09	80.12	450	83	18	102.5
A5	0	0	38.41	76.27	456	82	20	110.9
A6	0	1	34.41	69.18	522	79	21	123.7
A7	1	-1	40.88	74.76	453	95	19	106.9
A8	1	0	34.48	66.39	534	90	20	126.7
A9	1	1	30.89	57.93	612	87	22	130.3

Table 3: Significant level and predicted values of each response variables

Response	Name	Units	Obs	Analysis	P-value	Predicted Value
Y_1	T6	%	9	Polynomial	0.0497	39.93
Y_2	T12	%	9	Polynomial	0.0241	78.48
Y_3	Q50	min	9	Polynomial	0.0346	451.33
Y_4	FLT	Sec	9	Polynomial	0.0036	81.56
Y_5	TFT	hr	9	Polynomial	0.0064	18.56
Y_6	SWI	%	9	Polynomial	0.0257	103.43

Table 4: Kinetic treatments to dissolution profile for each formulation A1 to A9

Code	Zero Order		Hixon Crowell		Korsemeyer Peppas			Higuchi Plot	
	(R^2)	K_0	(R^2)	K_H	(R^2)	n	K_k	(R^2)	Kp
A1	0.9996	6.9659	0.9564	0.6225	0.9996	0.967	0.9534	0.9654	0.0342
A2	0.9979	6.2374	0.9592	0.5961	0.9966	1.0133	0.7602	0.9626	0.0381
A3	0.9984	6.1665	0.9576	0.5898	0.9974	0.9987	0.7834	0.964	0.0386
A4	0.9999	6.6667	0.9591	0.6106	0.9993	0.9795	0.8786	0.9628	0.0357
A5	0.9991	6.3956	0.9606	0.602	0.9975	0.9983	0.8014	0.9619	0.0371
A6	0.9992	5.7928	0.9638	0.5748	0.9941	1.006	0.7019	0.9585	0.0409
A7	0.9973	6.2349	0.9509	0.5891	0.9985	0.976	0.8559	0.9696	0.0383
A8	0.9995	5.4715	0.9535	0.5484	0.9978	0.9452	0.7954	0.9675	0.0437
A9	0.9991	4.8097	0.9534	0.5147	0.994	0.943	0.7014	0.9678	0.0497

Response Y1

T6 = + 56.065 + 1.03 * X1 - 3.953 * X2 - 0.143 * X1* X2 -
0.014 * X12+ 0.26 * X22

The regression co efficient was found from the ANOVA study and it was found that the negative effect of X2 coefficient while positive of X1 coefficient at low level on the response variable but at high level opposite results were found. It was ment that the concentration of Carbopol 934 P was not created much impact on drug release rate when it compared with the concentration of NKG at low level in the formulations. Negative coefficient was found in combination of both variables and it was suggested that when the concentration of polymer to drug was increased, the drug release from the dosage was decreased. When the concentration of NKG was increases, the drug release rate was significantly reduced. It was found from the response surface plot and contour plot shown in Figure 1. Response Y2

T12 = +73.068 + 6.17 * X1 - 4.87 * X2 - 0.185 * X1 * X2 -
0.22 * X12+ 0.27 * X22

The regression equation was suggested that the effect of variable X1 and X2 on response Y2. From the Figure 2, it was found that at the low level the effect of variable X1 on the drug release was more considerable than the variable X2. But at high level both are equally significant on the response variable. The negative coefficient was found for the combination of X1 and X2 suggesting that the cumulative percentages of drug release was significantly reduced by increasing the concentration of independent variables in combination.

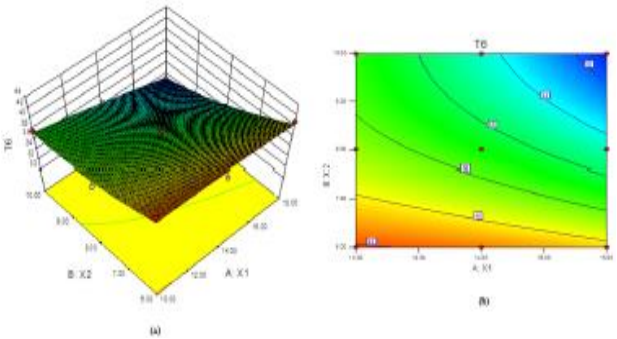

Figure 1: (a) Response surface plot and (b) Contour plot for response Y1

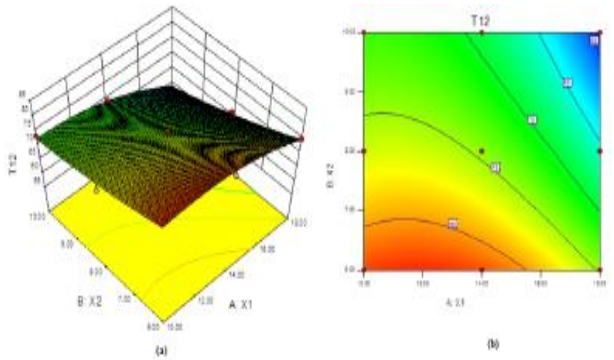

Figure 2: (a) Response surface plot and (b) Contour plot for response Y2

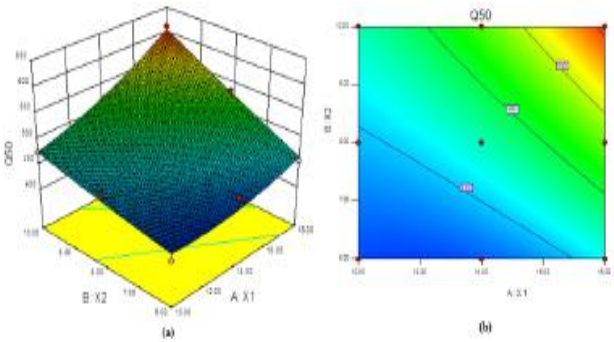

Figure 3: (a) Response surface plot and (b) Contour plot for response Y3

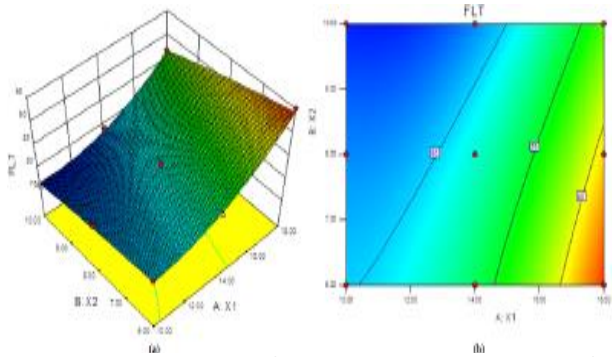

Figure 4: (a) Response surface plot and (b) Contour plot for response Y4

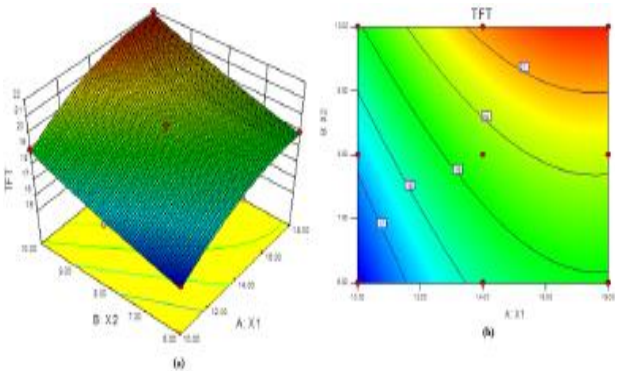

Figure 5: (a) Response surface plot and (b) Contour plot for response Y5

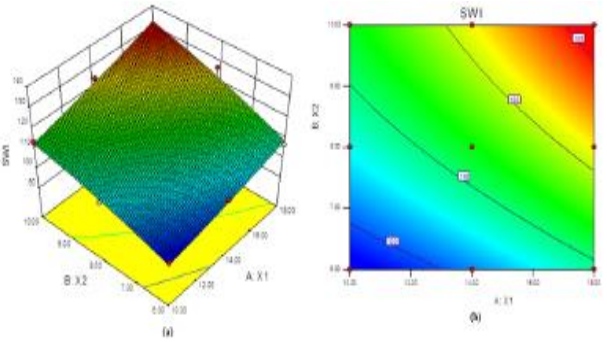

Figure 6: (a) Response surface plot and (b) Contour plot for response Y6

Response Y3

Q50 = +670.71 - 38.0 * X1 - 25.94 * X2 + 2.91 * X1 * X2 + 0.91 * X12 + 0.6 * X22

The regression equation was suggested that the effect of variable X1 and X2 on response Y3 was found negative. It might be indicated that the effect of selected variable on response (Y3) was not significant individually at low level. But the positive coefficients in the equation were indicated that the significant effect observed when the selected variables were used at high level as well as in combination. From the Figure 3, it was found that at the low level the individual effect of variables X1 and X2 on the response Y3 were not considerable significant. But at high level both are equally significant on the response variable. And in combination also significant response was found. Hence, this results might be reveled that the time required for 50 % drug release was enhanced with the concentration of polymer (NKG and Carbopol 934 P) to drug in the dosage increasing.

Response Y4

FLT = + 90.10 - 3.0 * X1 + 1.75 * X2- 0.13 * X1 * X2 + 0.198 * X12- 0.08 * X22

The regression equation was suggested that the variable X2 was more significant than the variable X1 because negative co efficient was found for variable X1 by ANOVA. It was suggested that FLT was enhanced when the level of NKG in the formulations was enhanced. And, opposite effect was found by X2 variable because the value of its coefficient was positive. It was indicated that the value of FLT was reduced when the level of X2 variable enhance. From the Figure 4, it was reveled that the level of X2 variable more significant because the FLT value lower towards the direction of higher level of X2 variable than X1 variable.

Response Y5

TFT = + 0.76 + 1.83* X1+ 0.083* X2 + 2.67 * X1* X2 - 0.052* X12+ 0.042 * X22

The coefficient for both variables was found to be positive at low level, high level and in combination. It was suggested that there was linear relationship observed

on response variable by the selected X1 and X2 variable. From the Figure 5, it was found that gradually rises the value of TFT as the concentration of both polymer NKG and Carbopol 934 P increases. But, it was also indicated that the level of X2 variable was more predominant than the value of X1 variable. Because the response direction move towards the higher level of X2 variable than X1 variable.

Response Y6

SWI = +50.45 - 0.91 * X1 + 8.64 * X2 + 0.21 * X1 * X2 + 0.047 * X12- 0.40 * X22

The coefficient of X2 variable was found to be positive at low level but X1 variable coefficient was negative. It was suggested that X1 variable move towards the predicted value positively with the concentration of Carbopol 934 P while negatively observed with the concentration of NKG at low level but at high level vise versa results were obtained. Both variable might be affecting SWI significantly but the effect of X2 variable was more predominant than X1 variable. From the Figure 6, it was found that the response value was increased by increasing the level of both variable (X1 and X2).

CONCLUSION:

Controlled release floating tablets of Repaglinide with NKG and Carbopol 934 P were prepared and optimized using central composite experimental design (3^2 Full Factorial Design) and multiple response optimizations. The quantitative effect of these factors on the release rate could be predicted by using polynomial equations. The model was found to be satisfactory for describing the relationships between formulation variables and individual response variables. The experimental values of each response variables obtain from the optimized formulation were very close to the predicted values. The developed tablets were found desirable drug release kinetics and found to be zero order. Formulation A4 was found to be best optimized formulation because of its desirable drug release kinetics and other response variables.

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RESEARCH ARTICLE

Analysis methods for medications improving Cerebral circulation

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ABSTRACT:

The objective of the study was to evaluate the efficacy of medical treatment in patients diagnosed with acute cerebrovascular accident (ACVA) included in the REGION inventory. The research was carried out in 2019 in 4 municipal primary health-care facilities in Moscow (Russia) and included the processing of past medical histories in two groups of totally 750 patients. Group I involved 400 patients treated against ACVA before 2017, and Group II comprised 350 patients treated from 2017 to 2019 inclusive. Patients of both groups were compared across the frequency of medications prescription before admission to the clinic with ACVA diagnosis, during outpatient treatment up to six months, and after discharge. Comparative analysis was performed based on telephone surveys and applying the Morisky Green medications adherence assessment scale. Before admission to the clinic, patients from the second group compared to those from the first one was 2.9 times more frequently prescribed with statins ($p \leq 0.01$), within six months of treatment outpatients received those 4.3 times more often ($p \leq 0.001$) and anticoagulants – 6.2 times ($p \leq 0.0001$). After discharge, patients were assigned to therapy with statins 2.5 times more frequent in the second group of the patient population ($p \leq 0.01$). Significant variables in therapeutic efficacy between patients in Group I and II have been established. Starting from 2017, the quality of ACVA medical care has significantly improved compared to that in 2009 – 2017.

KEYWORDS: acute cerebrovascular accident, stroke, statins, anticoagulants, Morisky Green scale.

INTRODUCTION:

Diseases associated with cerebral circulation disorders are among the most dangerous in the adult population[1,2]. Thus, stroke incidence in the world averages 3 cases per 100.000 population[3]. In case when cerebral circulation disorders become chronic, its treatment becomes one of the most challenging tasks that therapists and neurologists might face[4].

Specifics of these diseases management is the complex approach to its treatment, in particular, the graded prescription of medications relative to known risks of pathology development such as arterial hypertension, cerebral hyperperfusion, or neuronal degradation[5].

The problem of various cerebral circulation disorders is sufficiently covered by numerous scientific studies. Most papers focus on the pathogenesis of the disease as well as on prevention strategies, early diagnostics, and therapy tactics[6-8]. To date, one of the discussion topics remains neuronal protection from damages caused by cerebral circulation insufficiency[9]. Non-invasive methods for estimating the intensity of metabolic processes in brain tissues and blood flow rates are

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effective enough to assess the degree of cerebral circulation disorder[10]. Also, the selection of neuroprotectors adequate to the complex pathogenesis of the disease can greatly improve the life quality of patients[11].

Cerebral circulation disease is characterized by persistent changes in general and focal brain functions, which increase the risk of disability or death. Thus, brain lesions associated with vascular dysfunction are considered as a major factor in adult mortality[12]. The risk factors of vascular malformations are quite widespread and include behavioral aspects like nervous exaggerations or stressful situations, and physiological like inadequate physical activity, smoking, excessive alcohol consumption, or fat and carbohydrates abundance in nutrition. That is, a lot of people are exposed to risk factors for cerebral circulation diseases. The unfavorable environmental situation and the growth of the urban population can be attributed to external factors. It is known that stroke incidence in rural areas is twice as lower as that in cities[13]. As mentioned above, acute cerebrovascular circulation disorders are a multi-component disease that includes brain stroke and transient ischemic attack (TIA). It is known to be the second disease in the world after myocardial infarction leading to increased mortality and the first-place disease causing disability[14-16]. The incidence of stroke in Russia is one of the highest in the world counting 91 cases per 100.000 population with 25% mortality rate, which is significantly higher than in other countries[17-19]. In modern medicine, drug therapy is capable of reducing the probability of stroke recurrence and mortality rates[20].

According to World Health Organization, stroke is one of the most common epidemiological diseases widely spread in the world[21] assuming high mortality and disability rates. Thus, one in five patients is unable to move on his own, about one-third need external assistance, and only 20% can resume full employment[22]. For ACVA recurrence, a slight decline in the incidence rate has been reported in recent years being equal to 0.9 for every 1000 people[23]. However, 25% of patients with recurrent circulation disorders prematurely die[24]. This suggests a high relevance of primary and secondary prevention of the entire spectrum of cerebral circulation disorders. The most common type of stroke is ischemic with 5 times more frequent morbidity rate than hemorrhagic one[25]. Generally, ischemic stroke is associated with such diseases as atherosclerosis and arterial hypertension, but as the most common reason is reported atherothrombosis, which is characterized by high progression and overall wide prevalence. Atherosclerosis is directly related to changes in blood vessels. In particular, disorders of the cardiovascular system are associated with the integrity violation of the atherosclerosis plaque. The main cause for such disorders is various degradation processes in the endothelium[26]. Endothelium directly interacts with blood (humoral), participates in the formation of vascular tone, and affects the anti-thrombogenic properties of the vascular wall. Besides, the endothelium is engaged in platelet aggregation and regulates their adhesion indices. Modern conceptions tend to relate the occurrence and progression of atherosclerotic plaque to continuous damage in the endothelium, which leads to platelet adhesion, the migration of smooth muscle cells from the vascular wall, and the formation of fibrous plaque[27]. The damage of endothelium cells occurs not only on functional and physiological level but within structure as well[27]. This is usually associated with bacterial invasions initiated by such microorganisms as Chlamydia pneumonia and Helicobacter pylori, as well as by certain viruses like cytomegalovirus, herpes virus, etc.

The prevention of cerebral circulation disorders is known to be primary and secondary[28]. Primary refers to a set of prophylactic measures performed before a stroke or heart attack happens, while secondary is provided already upon the stroke. Prevention involves particular interventions of accustomed lifestyle like avoiding bad habits along with monitoring blood pressure, blood glucose concentration, and blood lipid components. It also involves taking anticoagulant and antiaggregant drugs comprising overall antithrombotic therapy. In primary prophylactic, the use of anticoagulants is not always justified. In particular, Warfarin drug is used at the risk of stroke in patients diagnosed with non-valvular atrial fibrillation (NVAF). In the secondary prevention aspirin and clopidogrel are applied for some patients. For example, in the treatment with aspirin and other drugs of this group the probability of recurrent acute cerebrovascular circulation accident reduces by 15%. At that, the aspirin dose may vary within a very wide range between 50 and 1500mg per day. Clopidogrel is effective at a dosage of 75mg per day, and its efficacy indices are comparable to those of aspirin intake. It should be considered though that by aspirin treatment, the probability of hemorrhagic complications, in particular, gastrointestinal and intracranial hemorrhage, is 0.10% within a year of aspirin treatment against 0.07% without it. Noteworthy is that longer aspirin intake increases significantly the likelihood of hemorrhage. The same patterns have been recorded for the clopidogrel. At combined aspirin and clopidogrel treatment, the incidence of hemorrhagic complications is reported to be 2–3 times higher[29]. Still, the number of prevented ACVA cases prevails over hemorrhagic risks, which indicates a clear benefit of the antiaggregants application.

Some researchers consider that the use of neuroprotectors and other drugs improving cerebrovascular circulation can significantly influence the life quality of patients[7,22]. Existing studies, however, are not sufficient to draw unequivocal conclusions. Comparative analysis and the results specification are required to develop a unified therapy strategy according to international standards. This has determined the relevance of this paper. As material for the research was used past medical histories of numerous patients staying at hospital care. Given that Russia is one of the countries with very high incidence rates of strokes and other cerebrovascular circulation disorders, the samples of patients for thorough analysis enlarges. This will undoubtedly contribute to drawing reasonable conclusions and improving the existing situation. The analysis in a sample of patients with cerebral circulation disorders will allow evaluating adequately the therapeutic tactics used in Russian primary health-care facilities and revealing its compliance or noncompliance with the world standards.

The purpose of this study was to evaluate the efficacy of drug treatment in patients with cerebrovascular circulation disorders included in the largest registry on this disease – REGION. The objectives of the study were to compare the effectiveness of ACVA therapy at different stages, namely, before admission to the clinic, during the first six months of outpatient treatment, and after discharge from the clinic. Besides, a separate task was to check patients' adherence to the prescribed therapy after discharge.

MATERIAL AND METHODS:

Materials:

The study was conducted in 2019 in Moscow (Russian Federation) retrospectively and randomly. A total of 4 databases from 4 urban primary health-care facilities were surveyed. The registry included the medical histories of 750 patients diagnosed with acute cerebrovascular circulation accident (ACVA). All patients enrolled into the study were assigned to 2 groups. Group I included 450 patients, who suffered and was treated against ACVA between 2009 and 2017, and Group II formed 300 patients diagnosed with ACVA after 2017 up to 2019 inclusively. The research was performed with respect to generally accepted moral and ethical standards. Agreement on anonymity and non-disclosure of personal information were concluded with each of the survivors according to accepted legal provisions. In occasioned death of a patient, similar procedures were conducted with the consent of the immediate family. The study did not include data on 153 patients, who did not give their consent to the processing of information, and, thus, are not count in mentioned above 750 examined participants.

The criterion for inclusion was the diagnosis of stroke of hemorrhagic or ischemic nature. ACVA diagnoses associated with pathological processes in brain vessels, presence of brain tumors, hemorrhagic stroke, or subdural hematomas of traumatic origin were accepted as exclusion criteria.

Acute cerebrovascular circulation accident was first observed in 82% (615 people) of patients from both groups including 385 from the Group I and 230 from Group II. The rest 135 people (28%) suffered from ACVA resurgence, namely, 82 persons in the first group and 53 in the second one. The primary aim of the study was to detect the difference in prescribed medications depending on the diagnosis limitation rather than the repeatability rate among patients. This specified the division into two groups. The ratio of ischemic or hemorrhagic strokes and transistor ischemic attacks was 83.0% (623 patients), 13.6% (102 patients), and 3.4% (25 patients), respectively.

The gender and age distribution of patients is presented in Table 1.

Table 1: Distribution of patients in groups by age and sex

Group	Male patients, number of patients and %*	Average age, years	Female patients, number of patients and %*	Average age, years
I	256 (56.9 %)	69.2 ±1.9	194 (43.1 %)	70.2 ± 2.2
II	173 (57.7%)	67.4 ±1.4	127 (42.3 %)	68.8 ± 2.0

*100% comprise all patients in this group.

No significant age or gender differences in both groups were found. The number of male patients in both groups is 1.3 times higher at $p \leq 0.05$.

Methods of research:

The obtained information from case histories was transferred to Microsoft Excel 2013 software (Microsoft Inc., USA) for further processing. The analysis was based on individual patient's characteristics like age and gender, as well as physiological factors like diagnosed ACVA pathology, pharmacotherapy applied, methods of clinical examinations (laboratory and instrumental), and availability and compliance with the standards of medical documentation. The results were compared for patients before admission to the clinic, 6 months after admission, and after discharge based on remote observation with the help of surveys.

Prescribed and performed treatment for patients of both groups was investigated. Information about patients who have already undergone treatment in the clinic and stay at home was obtained through direct interviewing by phone patients themselves or their relatives in case of apparent cognitive impairment in the patient. At that, to

determine the patient's compliance with the prescribed medication therapy, a medication adherence Morisky Green survey was applied.

Statistical analysis:

Collected information was processed in Statistica v.10.0 software (StatSoft Inc., USA) using standard statistical parameters, i.e., % of patients in each group taking prescribed medications. The allocation in the sample did not correspond to standard and, thus, specified the necessity of applying non-parametric methods of analysis. Therefore, the chi-square criterion was used to determine the validity of symptom frequency between the two groups, and by employing the McNemar test was calculated the significance of variables in the frequency of prescribed therapy. The variables are considered significant at $p \leq 0.05$.

RESULTS:

An Before admission to the clinic, both groups of patients were most frequently put on angiotensin-converting enzyme (ACE) inhibitors, as well as angiotensin receptor blockers (ARBs), beta-adrenoblockers (BAs), and statins (Table 2).

Table 2: Indicators of pharmacotherapy frequency in patients diagnosed with ACVA before admission to the clinic

Group of medications and diagnosis	Group I, %	Group II, %
ACE** inhibitors, ARBs; chronic HF	28.7	58.0*
ACE inhibitors, ARBs; PC	26.1	38.9
BAs; chronic HF	14.8	34.0*
BAs; PC	20.9	38.4
Statins; IHD	9.1	26.2*
Statins; PC	10.9	25.9
Statins; ACVA	9.1[1]	12.8[1]
Anticoagulants; AF	8.8	15.0
Antiaggregant; IHD, no AF.	23.7	43.1*
Antiaggregant; PC, no AF.	31.5	26.6
ACE* inhibitors; ACVA	40.8 [1]	35.5 [1]

* Variables are significant compared to Group I; [1] – % of patient with recurrent ACVA;
**ACE – angiotensin-converting enzyme; ARB – angiotensin receptor blocker; HF – heart failure; PC – postinfarction cardiosclerosis; BA – beta-adrenoblocker; IHD – ischemic heart disease; ACVA – acute cerebrovascular accident; AF – atrial fibrillation;

There was a significant difference in prescriptions between Group I and II for ACE inhibitors in favor of the latter (2 times more frequently at $p \leq 0.01$), as well as for BAs (2.3 times at $p \leq 0.01$). These medications were prescribed for the diagnosis of chronic HF. For heart ischemia cases among patients in Group II, statins were prescribed more often (2.9 times at $p \leq 0.01$) compared to Group I. At the same diagnosis without AF, antiaggregants were essentially more frequently prescribed (1.8 times, at $p \leq 0.05$) in Group II. No significant variables in treatment tactics was recorded between the two groups of ACVA patients. At outpatient treatment during six months, ACVA patients from both groups were more frequently admitted to ACE inhibitors, BAs, and antiaggregants (Table 3).

Table 3: Indicators of pharmacotherapy frequency in patients diagnosed with ACVA six months after admission to the clinic

Group of medications and diagnosis	Group I, %	Group II, %
ACE** inhibitors, ARBs; chronic HF	30.6	58.8*
ACE inhibitors, ARBs; PC	21.4	35.7
BAs; chronic HF	18.6	40.0*
BAs; PC	26.5	37.5
Statins; IHD	9.9	42.5*
Statins; PC	18.3	35.1
Statins; ACVA	10.6 [1]	44.7 [1]
Anticoagulants; AF	3.1	19.3*
Antiaggregant; IHD, no AF.	26.8	55.5*
Antiaggregant; PC, no AF.	40.7	56.7
ACE* inhibitors; ACVA	28.7	45.7*

* Variables are significant compared to Group I; [1] – % of patient with recurrent ACVA;
**ACE – angiotensin-converting enzyme; ARB – angiotensin receptor blocker; HF – heart failure; PC – postinfarction cardiosclerosis; BA – beta-adrenoblocker; IHD – ischemic heart disease; ACVA – acute cerebrovascular accident; AF – atrial fibrillation;

At that, significant variables were observed at assigning ACE inhibitors in favor of Group II (1.9 times more often at $p \leq 0.05$) and BAs (2.1 times at $p \leq 0.05$) with the accompanying HF diagnosis. The greatest difference between the groups in favor of Group II was noted when prescribing statins for IHD therapy (4.3 times more often at $p \leq 0.001$) and anticoagulants for AF therapy (6.2 times more often at $p \leq 0.0001$). Antiaggregants for treating IHD were prescribed 2.0 times more frequently in Group II ($p \leq 0.05$). For other drugs, no significant variables between the groups was recorded.

Based on the data in Tables 1 and 2, it can be concluded that patients in Group I with acute cerebrovascular circulation accident received more effective medicinal therapy both before admission to the clinic and during outpatient treatment for at least six months. That is, the quality of medical care for ACVA diagnosis slightly improved since 2017 compared to the previous period of 2009-2017.

Table 4: Indicators of pharmacotherapy frequency in patients diagnosed with ACVA after discharge from the clinic (based on results of telephone survey)

Group of medications vs. diagnosis / Medications prescribed	Group I, %	Group II, %
ACE** inhibitors, ARBs; chronic HF	54.7	63.9
BAs; chronic HF	25.7	34.5
Calcium antagonists	14.5	18.6
Diuretics	22.6	22.8
Statins	19.4	48.9*
Anticoagulants (oral intake)	3.5	6.7
Antiaggregants	45.6	49.8
Cardiac glycosides	6.9	6.8
Nootropics	13.1	15.2
Antioxidants	-	14.2
Polypeptides	0.8	3.4

*Variables are significant compared to Group 1
**ACE – angiotensin-converting enzyme; ARB – angiotensin receptor blocker; HF – heart failure; BA – beta-adrenoblocker;

Finally, patients staying at home on continuous pharmacotherapy as recommended most frequently were assigned to treatment with ACE inhibitors or BAs in case of accompanying AF diagnosis (Table 4). Diuretics, statins, and antiaggregants were also quite often prescribed. Among patients of Group I, no antioxidant intake was recorded.

Significant variables between the groups in favor of Group II was noted only for patients receiving statins, namely, 2.5 times at $p \leq 0.01$. For the rest, there are no significant variables in patients taking prescribed medications in both groups, i.e., all patients adhered to proposed prescriptions.

The obtained data on adherence to the prescribed medication therapy are presented in Table 5.

Table 5: Results on medications adherence in patients

Scores*	Group I	Group II
0	32.9	14.4**
1	15.1	9.4
2	20.9	13.7
3	11.4	17.2
4	19.7	45.3**

*Scores are given according to the Morisky Green scale;
** The variables between Groups I and II are significant.

The results of Morisky Green testing revealed that among the patients with 4 scores following the prescribed therapy, those from the second group dominated by 2.3 times compared to the patients from the first group ($p \leq 0.0001$). Among the patients from Group II though, a 2.2 times lower number of patients was scored with 0 points ($p \leq 0.0001$), which corresponds to very low compliance with the prescriptions (0-1).

As seen, the number of patients following the prescribed medicinal treatment for ACVA diagnosis has increased since 2017.

DISCUSSION:

The data obtained and handled through this research from the REGION inventory showed a significant improvement in the quality of medical and outpatient care provided. However, the evaluation was performed in a limited number of primary health-care facilities and may not reflect the overall trend in Russian medical sphere, especially, in regional and district centers of the country. First of all, the reported improvement concerns the increased frequency of assigning patients with medications related to forecast-modifying pharmacological therapy, in particular, ACE inhibitors, statins, and anticoagulants. This, to a greater extent, applies rather to patients diagnosed with HF and IHD than those with ACVA. The disadvantages of the applied therapeutic tactics concern the frequency of statins prescription for ACVA patients. The same tendencies are typical for anticoagulants that are widely used in AF treatment, which is known to be one of the main risk factors for emerging cerebral circulation disorders. To meet international standards, anticoagulants prescription frequency at AF is recommended to be increased by at least 3.5–4.5 times compared to already known data. This will certainly contribute to improving the quality of primary and secondary ACVA prevention. Besides the results of this research, similar trends in insufficient secondary prophylaxis have been observed in some other studies[30,31].

Despite the reported improvement in the quality of medical therapy provided for patients with cerebral circulation disorders, its level remains insufficient, at least in Russian health-care services. The frequency of assigning antiaggregants, statins, and anticoagulants has increased the most. The second factor defining the low efficacy of secondary ACVA prophylactic therapy is the unsatisfactory level of conscious and serious medication adherence of some patients after discharge from the clinic.

In some studies, an imbalance between laboratory and instrumental methods for ACVA diagnostics was also noted[32]. Generally, if laboratory tests are carried out in accordance with requirements, the instrumental diagnostics is performed untimely and with inconsistency to the available international standards. This, in turn, leads to late diagnosis of stroke and severe negative consequences for the patient. Patients' nonadherence to the prescriptions after outpatient treatment results in the risk of stroke recurrence and premature death. Late stroke diagnostics disables in-time identification of the stroke type, leads to incorrect or absent thrombotic treatment tactics, and, eventually, too late decision to transfer the patient to the neurosurgery department.

The mentioned above arguments are highly relevant due to the constant increase of stroke incidence with a tendency to move towards younger ages[33]. This is particularly true for individuals over 25 years, among whom the probability of stroke is 2–3 times higher with each subsequent decade[34,35]. The most common reasons for such rejuvenation are frequent stressful situations[36] and sedentary lifestyles, thus, frequent walks are a prerequisite for primary prevention of acute cerebrovascular accidents[37]. ACVA, in particular ischemic stroke, is the main cause of high mortality among the elderly and senile population. Besides, the results of long-term monitoring have confirmed that an increased risk of exposure to ACVA exists among persons engaged in mental work[38]. An unbalanced diet,

in particular, the prevalence of fat in the diet, and diabetes mellitus are highly common reasons for the development of this disease[39].

CONCLUSIONS:

Significant variables between the quality of therapeutic tactics for patients from Group I treated against acute cerebrovascular circulation accident before 2017 and for patients Group II being under treatment after 2017 were established. In particular, before assignment to the health-care facilities, patients from the second group suffering from IHD were 2.9 times more frequently prescribed with statins rather than those from the first group ($p \leq 0.01$). This tendency is more expressed within six months of outpatient treatment. Thus, in Group II, statins were prescribed 4.3 times more often ($p \leq 0.001$) and the use of anticoagulants was noted to be 6.2 times higher ($p \leq 0.0001$) at an accompanying AF diagnosis.

After discharge, significant variables between the two groups were recorded only for statins intake, namely 2.5 times at $p \leq 0.01$. Medications adherence assessment results according to the Morisky Green scale showed that Group II prevailed among the patients who scored a maximum of 4 points (2.3 times at $p \leq 0.0001$), although they were 2.2 times less frequently scored with 0 points ($p \leq 0.0001$). Thus, until 2017, patients with ACVA diagnosis failed to follow the prescribed therapy, as a rule.

CONFLICT OF INTEREST:

The authors declare no conflict of interest.

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RESEARCH ARTICLE

Pharmacognostic Study of the galls of wild representatives of *Quercus robur L.*, created by insects

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ABSTRACT:

Background: Galls - an extraordinary phenomenon for a plant, when in response to the penetration of the inhumane biological organism, a new structure is formed which differs from the plant itself in its morphological, chemical and functional state. In folk medicine, oak hills were considered as a raw material suitable for various domestic and therapeutic. **Purposes:** duplication of animal skin, making ink, used as tea, stopping bleeding and wound healing. Different pharmacopies of the World at different times included time articles on various types of Galls. Oak galls of wild plants are not used and almost not being studied. **Methods:** Plant material (galls) was collected from 4 forest areas in the Kiev region in September-October 2017. Pharmacognostical study, physicochemical and phytoconstituent study was performed following standard Pharmacopoeial procedures. **Results:** The main thing is the presence of a capsule in a loose parenchyma gall. When wetting a break with iron chloride or iron ammonium alum, in the form of 1% solution appears black and blue staining. The revealed three zones: the epidermal, parenchymal, consisting of several layers and sclerenchymal, which surrounds the inner capsule. The following groups of biologically active substances have been identified: ascorbic acid, glucose, proteins, tannins, catechins, saponins and alkaloids. **Conclusion:** Diagnostic features are the presence of crystals of calcium oxalate in the outer, dense layer of the parenchyma, the middle region and in the internal parenchymal cells of the capsule. It is established that the amount of tannins is 70-75%. The amount of ascorbic acid is comparable to that in *Rosae fructus* and is 0.2%. The present study shows the prospects of using wild galls in Europe as alternatives to the import of *Gallae turcicae*, *Gallae chinensis* and *Gallae pistaceae* and the development of new drugs on their basis.

KEYWORDS: Oak galls, Pharmacognosy, phytochemistry, ethnomedicines, medicinal plants.

INTRODUCTION:

Galls - an extraordinary phenomenon for a plant, when in response to the penetration of the inhumane biological organism, a new structure is formed which differs from the plant itself in its morphological, chemical and functional state. That is what opens up an extensive field of activity for both biologists and pharmacists. It is already known that in galls there is a large number of tannins up to 80%, this is among the natural objects has no analogues.

Tanning material are polyphenolic compounds, which, with demand, together with saponins, take the first place in the pharmaceutical market[1]. The primary source of tanning material, as active pharmaceutical ingredients, is medicinal plants. Certain polyphenolic compounds are not always well known, but there are differences between polyphenols in different species of plants[2].

Oak nuts for ages used in the form of broths for the treatment of burns and frostbites, with severe diarrhea, external and internal bleeding. Strong broth rinsed the mouth with stomatitis and paradontosis, used to heal cracks on the lips, rubbed into the roots of the hair to strengthen them, treated them with lichens, eczema, trophic ulcers. The galls was used for lotions for inflammation of the eyes, with hemorrhoids, varicose, mixed with decoction of a larch sponge drank with pulmonary tuberculosis. Most of the pharmacologic effects confirmed by scientific publications concern the

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Quercus infectoria gall. Preparations from this raw material are widely used in India, the countries of Asia Minor, in Iran. Galls are known in Malaysia as a "Manjakani" and are used in combination with other herbs as a drinking medication by women after childbirth to restore the elasticity of the uterine wall[3]. In Indian traditional medicine, they are widely known as majupal and are used as a powder in the treatment of toothache and gingivitis[4,5]. For gallium Q. infectoria, astringent, antidiabetic·[6] antitremorine, local anesthetic[7], antiviral,[8] antibacterial,[9] antifungal,[10] larvicidal[11] and anti-inflammatory[12] are scientifically confirmed. The crude gall powder of Q. infectoria was active against S. aureus and B. subtilis, whereas methanol and aqueous extracts were active against S. epidermidis. Acetone extracts also inhibit the growth of S. aureus, S. epidermidis and B. subtilis. The most sensitive bacteria to the action of ethanol and water extract from the galls of the Q. infectoria is P. aeruginosa[13]. Iranian researchers have shown that methanol extracts from Q. infectoria are highly effective as antibacterial agents for removing biofilms in dental practice. These results prove that the biologically active substances Q. infectoria galls can be useful for the prevention and treatment of caries, since they have an inhibitory effect and prevent the growth of the S. mutans carriogenic bacterium[14]. Methanol and aqueous extracts of Q. infectoria were tested for anticandion activity against five species of Candida albicans, Candida krusei, Candida glabrata, Candida parapsilosis and Candida tropicalis. Fungicidal action was noted[15]. Chloroform extract of bile powder Quercus infectoria has good antifungal activity against Aspergillus and Pencillium species[16]. The antihistamine activity of extracts of oak galls was first noted by Hungarian scientists in 1950[17]. These results were later confirmed in the study of alcohol extracts on guinea pigs against the aerosol of 5-hydroxytryptamine[18]. However, the extracts studied were unrefined, which caused side toxic effects. The release of the active principle was achieved through the use of extracts of organic solvents and column chromatography (Sephadex LH-20 and silica gel). A preliminary study of the structure of this chemically pure substance using mass spectrometry, thin-layer chromatography, base hydrolysis and electrophoresis showed that it is most likely a piperonylic acid ester. The substance, injected intraperitoneally at doses of 4mg/kg guinea pigs 5 hours prior to exposure to 0.15% histamine aerosol, significantly reduced the bronchoconstrictor effect of histamine[19]. Umachigi et al.[20] found significant antioxidant activity in Q. infectoria extracts, since the extract inhibited the activity of removing superoxide and 1,1-diphenyl-2-pyrldetrazyl (DPPH), and tyrosinase activity[21]. It is assumed that flavonoid and phenolic compounds extracted from Q. infectoria galls have potential antioxidant activity. Galls Q. infectoria possess powerful suppression of free radicals and powerful antioxidant activity in trials in both chemical and biological models. Polyphenols can act on the metabolism of bones, modulating the proliferation of osteoblasts, differentiation and mineralization, as well as the function of osteoclasts[22]. This combined effect of polyphenols on osteoplast and osteoclasts is useful for supporting the bone remodeling process, which is a key endpoint in the treatment of osteoporosis.

MATERIALS AND METHODS:

Plant collection:

We have studied the galls collected in 4 forest areas in the Kiev region in September-October 2017. Three forest tracts, located in the Irpen forestry between Nikolaevka and Khmelnaya villages of the Kiev-Svyatoshinsky district, the fourth - in the Claudian larches near the village of. Dibrova.

Forest no. 1. On the forest area of 5 hectares are oak-pine crops aged 15-16 years old. The growth density is about 1 person per 20-40 m2. 234 specimens of Quercus robur were examined. The height of the trees is 3.5-5 m. Galls are found on 19 fruit-bearing individuals. Galls are mostly located on the middle side shoots more often, are found in aggregation up to 6 pcs per leaf. Number of galls on one tree from 3 to 17 pcs. (maximum 48). A total of 205 pieces (338 grams) were collected, the average weight of 1 piece-1,64 + 0,72 grams, a diameter of about -1,8 + 0,9cm.

Forest no. 2. On the area of the forest of 3 hectares are rarely growing pine-oak crops aged 12-15 years. Density of 1 person per 50 m2. Separately, Corylus avellana L. and Sambucus nigra L. are detected. 78 specimens of Quercus robur L., 3-4 m high, were detected, 12 of them were found to be feral individuals. Number of galls per person from 3 to 28 pieces. In total, 112 pcs (204 grams) were collected, the average weight of 1 pc-1.89 + 0.57 grams, with a diameter of about 2.1 + 0.75cm.

Forest no. 3. Forest area of 2 hectares with birch-pine-oak plantations 18-20 years old. The density of growth is 1 person per 30-40 m2. We examined 49 individuals in height of more than 5 m. Galls were detected in 9 individuals, amounting to 737 units per person. Noted aggregation galls at closely spaced branches and leaves 0.5-2.5 cm in diameter. Total collected 155 pieces (286 g), the average weight of 1 pc-1.84 + 0.63 grams, a diameter of 0.66 2,1+ cm Forest №4. Forest area of 1.2 hectares with birch-pine-oak plantations. The specimens of Quercus robur L. are marked with aggregations of galls up to 6 pieces per sheet. A total of 336 pcs (571 g), average weight 1 pc-1,7 + 0,38 grams, diameter 1,7 + 0,73 cm, are collected.

Pharmacognostical Study:
Microscopic analysis was carried out for fresh raw materials. Cutting of raw material was made by a blade. To clarify the drug, it was coagulated in the clarifying fluid: a solution of chloral hydrate and a 5% solution of sodium hydroxide. For histochemical reactions, a solution of Lugol (for starch grains), a Molush solution (for detection of inulin), a solution of iron-ammonium alum (for the detection of tannins) was used. The microscopic structure was studied using the ULAB trinocular light microscope with an increase of 40, 100 and 400 times. Photos were taken by Canon EOS 550 SLR. Macroscopic observations were made with naked eyes and centimeter scale was used for measurement.

Physicochemical Parameters and Qualitative Analysis:
Qualitative analysis for the presence of various secondary metabolites dissolved in water and alcohol extract was done as per reference. [практикум].

RESULTS AND DISCUSSION:

Macroscopic evaluation:
The galls were collected under natural conditions and visibly as indicated in Section 3. In nature, galls often form accumulations on leaves up to six formations (Fig. 1). In the process of drying the gall, the appearance of adult insects of galls pathogens was observed in Fig. 2, which left their shelter.

Figure 1. Aggregation of galls on *Quercus robur* leaves

Figure 2. Adult insect of the asexual generation of *Cynips quercustolii*

A macroscopic analysis showed that the investigated whole galls were spherical in diameter from 0.5 to 2.5 cm in weight from 0.3 to 2 grams. The dimensions were set after 15 measurements were statistically processed with the establishment of a confidence interval. The surface is wrinkled, bare, with a golden hue from yellow to brown. The color and shape were set with the unaided eye under daylight, the nature of the surface under the magnifying glass with a tenfold increase. In the galls had a short basal stem. In some formations in the upper part there are conical projections. Most have holes created by adult insects. The hole leads into a cylindrical channel, reaching the central capsule. The inner part is loose, porous, in the center there is a capsule, sometimes with the remains of an insect, the color on the break from light to dark brown. The smell when scraping, scraping, trituration in a mortar and wetting with water is absent. The taste is sweet and sour, strongly astringent. When wetted with iron chloride or iron ammonium alum in the form of 1% solutions, black-blue staining was observed.

Microscopic evaluation:
Microscopic analysis revealed the following zones: epidermal, parenchymal, consisting of several layers, and sclerenchyma, which surrounds the inner capsule.

Basic cells of the epidermis are polyhedral, more often quadrangular (Fig. 3A). Cell walls are unevenly thickened, clearly visualized in the form of beads (Fig. 3D). Stomatal is rare, the guard cells are much smaller than the subsidiary ones, which are anomocytically located and do not differ in size and shape from the basic cells of the epidermis (Fig. 3C). In the parietal part of the cell, parts of the protoplast with a yellow pigment are visible. Simple and glandular trichomes on the surface of the epidermis is not found. In some areas, under the epidermis, there are powerful sclerenchymal protrusions (Fig. 3B).

The epidermis adjoins the outer zone of the parenchyma, which consists of irregularly shaped cells arranged irregularly (Fig. 4A). The walls of the cells are thin without thickening. The middle part of the parenchyma is represented by cells of oval shape, tightly adjacent to each other (Fig. 4C). In cells there are rare inclusions of calcium oxalate in the form of prismatic crystals. In this layer there are sites of sclerenchyma and individual sclerenchyma cells (Brachkleleids). The main part of the parenchyma is represented by long parenchymal cells with numerous air cavities (Fig. 4B).

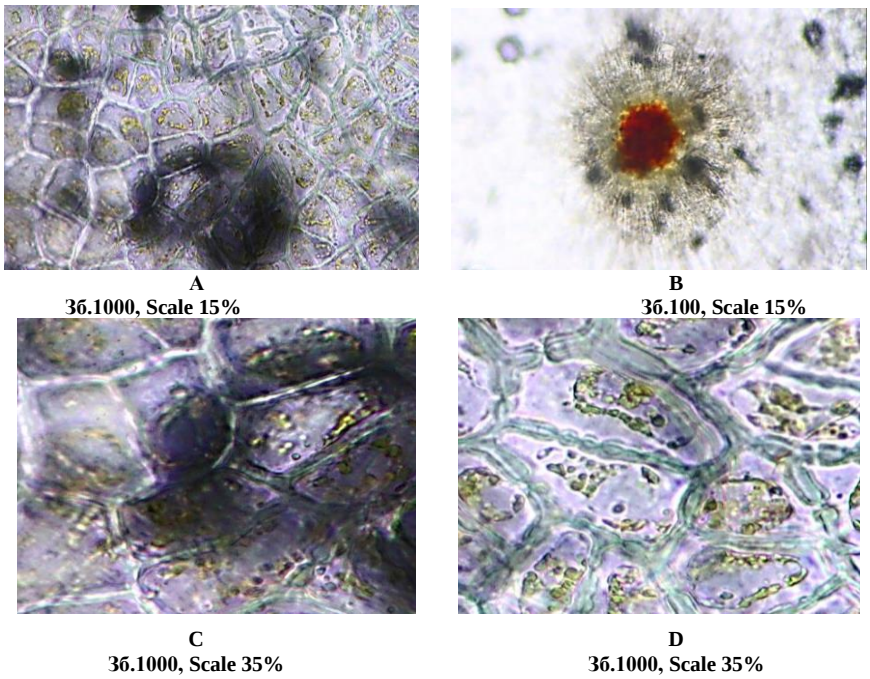


Figure 3. The structure of the epidermis *Quercus roburis gallae*: A - the basic cells of the epidermis; B - sites of sclerenchyma; C - stomata; D - thickening of the cell wall.

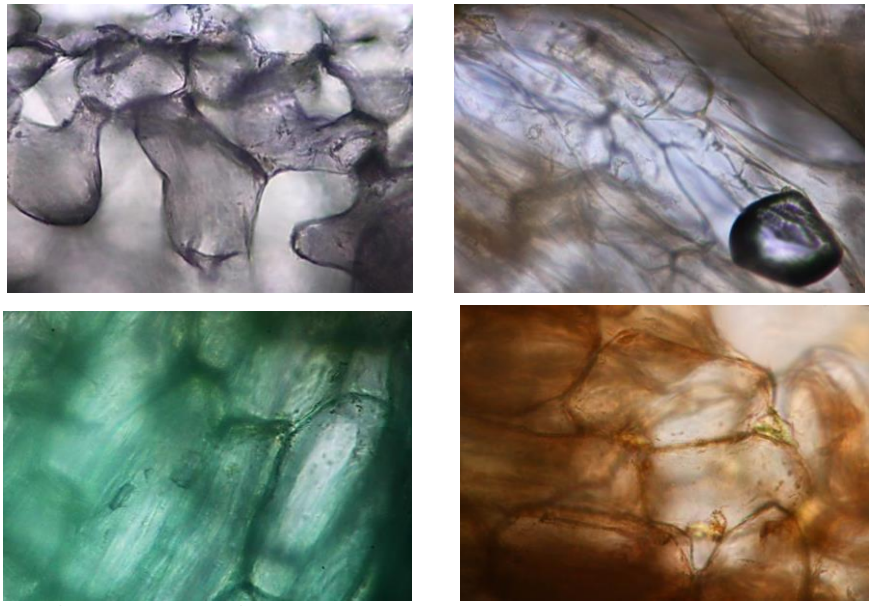


Figure 4. Differentiation of the parenchyma of *Quercus roburis gallae* (3б.1000, Scale 15%): A - subepidermal layer; B - dense layer of parenchyma; B - aerenchyma; D - internal parenchyma.

Closer to the central capsule is a small inner zone. It is formed by thick-walled parenchymal cells (Fig. 4D), in some there are numerous starch grains. There are brachy- and astrosclereide.

Around the central cavity is a ring of sclerenchyma, with a variety of shapes and sizes of sclereids (Fig. 5). Present, rectangular, ovate, filiform, stellate cells. Small sclereids have strongly thickened walls with depressions, the lumen is large, usually filled with dense brown contents. Large sclereids are strongly elongated. All parenchymal cells of the inner zone of the capsule contain starch.

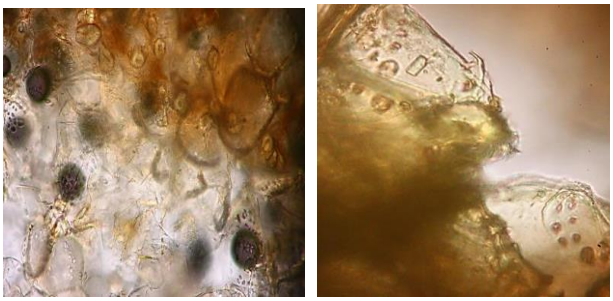
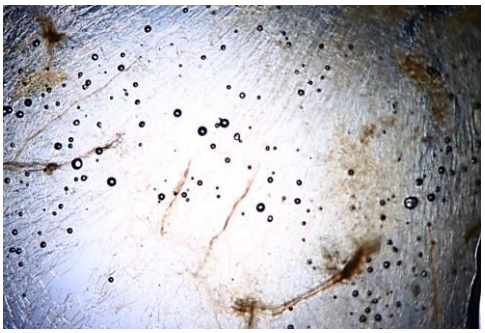


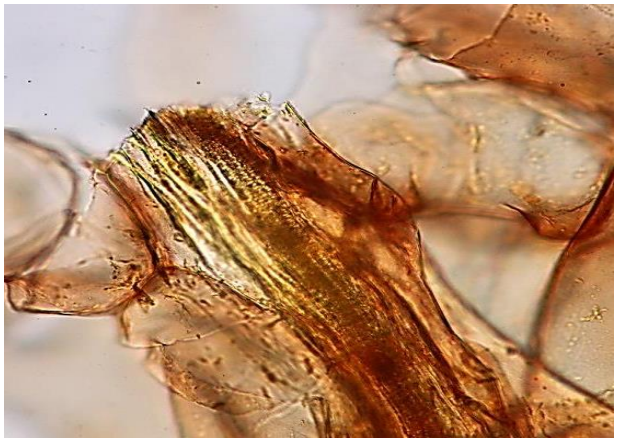
Figure 5. Structure of the shell of the inner capsule *Quercus roburis gallae* (Ɜб.1000, Scale 15%), cells with starch and druses.

Microscopic analysis revealed the presence of calcium oxalate crystals in the outer, dense layer of the parenchyma, the middle region, and prismatic crystals in the inner parenchymal cells of the capsule (Fig. 4C and Fig. 5).

Figure 6 shows an image of a scrap of conductive elements with a section of a vessel having a mesh thickening. In general, the vascular bundles are distributed without a system, permeating all zones of the gall. The anatomical structure of oak galls demonstrates that the pathologically altered tissues of the host plant are fully adapted to life support and protection of the larva of *Cynips quercustolii*. The presence of extensive sections of sclerenchyma in the subepidermal region and a continuous layer of stony cells of the inner capsule perform a protective function, with sclereids pathologically diverse in form.



Ɜб.100, Scale15%



Ɜб.1000, Scale35%

Figure 6. Scraps of the conductive elements of *Quercus roburis gallae* and the site of the vessel.

There are also forms mainly represented in leaves (astrosclereids, filiform sclereids) and mostly in fruits of plants of Brachysclereids. The main parenchyma with large thin-walled cells and air-sacrificial cavities provide the larva with air and moisture, in addition the overgrown parenchyma layer protects the larva from parasitoid damage. Starchy grains, which are abundantly present in the innermost zone of the parenchyma, provide the larva with the necessary nutrition.

Quality phytochemical analysis:

The metamorphosis of leaf tissues under the influence of the salivary glands of the larva Cynips quercustolii leads to irreversible changes not only of the anatomical structure of the affected tissue, but certainly and primarily to changes in their biochemical composition. The composition of the saliva that triggers these processes remains unknown until now.

High content of tannins in Gallae Turcicae and Gallae Chinensis, which are traditionally present in the pharmaceutical market, determined the primary identification of tannins in galls collected by us with subsequent phytochemical screening of biologically active compounds. The results of qualitative reactions are presented in the table 1.

Table 1. Identification of biologically active substances *Quercus roburis gallae.*

Qualitative reactions to tanning material		
Reaction	Observations	Conclusions
With a solution of iron ammonium alum	Black-blue staining	There are tannins, mainly hydrolysable
With gelatin solution (1% solution).	There is a haze that disappears when adding excess gelatin	There are tannins
With alkaloids (2% solution of papaverine hydrochloride)	A white precipitate forms	
Qualitative reactions to saponins		
With lead acetate (10% lead acetate solution)	Brown sediment	There are saponins
The reaction of Salkovsky (with chloroform)	The chloroform layer turns yellow	
Lafon reaction (with 10% copper sulfate solution)	Green staining	
Sanya reaction (with 0.5% alcohol solution of vanillin)	Orange staining	
With cholesterol (1% alcohol solution)	White precipitate	
With barite water	Brown sediment	
Foaming	An abundant and persistent foam forms	

Qualitative reactions to alkaloids		
With phosphomolybdic acid	Blue sediment	There are alkaloids
With silicotungstic acid	Clear sediment	
With a solution of p-dimethylaminobenzaldehyde in concentrated sulfuric acid.	Reddish-brown color	
With Lugol's solution	Brown sediment	
With 1% aqueous solution of picric acid	Yellow sediment	
Murexide test	The reaction is negative	Purine alkaloids are absent
Qualitative reactions to flavonoids		
Cyanidic reaction	The reaction is negative	Flavonones, flavones, flavonols are absent
Reaction with iron (III) chloride	Blue staining	There are tannins
Reaction with vanillin in concentrated hydrochloric acid	Red staining	There are halocatechins
Wilson reaction	The reaction is negative	5-hydroxy-, 5-methoxyflavones and flavonols are absent

Phytochemical analysis established the presence of tannins, the most specific is the reaction with gelatin, when adding 1% solution of gelatin to the extract first appears turbidity, and with excess of the reagent - disappears. Tannins can be attributed to hydrolyzable, since a blue color is obtained when iron ammonium alum is added (in the presence of condensed tannins, green predominates). Along with tannins, saponins have been discovered. All qualitative reactions carried out to identify saponins produced a positive result. Saponins were previously identified in other types of gall. Based on the analysis - these substances are steroidal in nature. Since, according to the literature, saponins in other organs of *Quercus robur* are not described, we believe that they can be attributed to substances associated with damage to the tissues of the leaf by the insect larva. Qualitative reactions revealed the presence of alkaloids, which also agrees with the analysis of other species of gall, with no purine alkaloids in our studies. With respect to flavonoids, the presence of only reduced phenylchromane derivatives, namely gallocatechin (positive reaction with vanillin solution) was established. Reactions to oxidized phenylchromone derivatives gave a negative result both in the cyanidic reaction and in the Wilson reaction. It is known that the leaves of oak contain oxidized flavonols quercetin, kaempferol and isorhamnetin. It is known that flavonoids are the chemical basis of co-evolution of interaction with phytophagous insects and often act as food attractants and repellents, and in each specific case the interaction of plant-phytophagous is determined by the composition of flavonoids. Thus, the flavonoid composition of pathologically modified tissues changes. Reconfirmation of the presence of catechin in Quercus roburis galls collected in the Kiev region, namely, gallocatechin, will open up the possibility of using this raw material to produce P-vitamin, hypocholesterolemic, immunomodulating, antioxidant, cytoprotective drugs.

CONCLUSION:

To standardize *Quercus roburis gallae*, we have studied and presented the macro-diagnostic features of medicinal plant material. The main thing is the presence of a capsule in a loose parenchyma gall. When wetting a break with iron chloride or iron ammonium alum, in the form of 1% solution appears black and blue staining.

An analysis of the anatomical structure of *Quercus roburis gallae* revealed three zones: the epidermal, parenchymal, consisting of several layers and sclerenchymal, which surrounds the inner capsule. Diagnostic features are the presence of crystals of calcium oxalate in the outer, dense layer of the parenchyma, the middle region and in the internal parenchymal cells of the capsule. Starchy grains abundantly present in cells of the inner zone of parenchyma. In the stony layer of the capsule there are sclereids of various shapes. In Quercus robius gallae, the following groups of biologically active substances have been identified: ascorbic acid, glucose, proteins, tannins, catechins, saponins and alkaloids.

CONFLICT OF INTEREST STATEMENT:

We declare that we have no conflict of interest.

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RESEARCH ARTICLE

Soluble 1:1 Stoichiometry curcumin binary complex for potential apoptosis in human colorectal adenocarcinoma cells (SW480 and Caco-2 cells)

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ABSTRACT:

This study investigates the solid dispersion (SD) of curcumin (CMN) enhance the solubility, which can be employed for the treatment of colorectal cancer (CRC) greater to pure CMN. Solid dispersion (SD) prepared by a hot melt method using CMN with several carriers of poloxamers (P-407 and P-188), gelucire 50/13 (GLR) and mannitol (MNT). Prior, phase solubility studies were performed with drug and carriers. The SD characterized by *in vitro* drug release and novel dyeing test. Additionally, the cytotoxicity and apoptosis studies resolved to utilize the colorectal adenocarcinoma cell lines. The result showed that CMN-P-407 complex produced significant properties towards solubility (318±14.46 fold) and dissolution (91±0.43% at 30 min). The IC_{50} value for complex found to be 74 and 52µM/mL, while that for pure CMN ranged from 146 and 116µM/mL on the SW480 and Caco-2 cells respectively. Apoptosis study revealed that the cells are undergoing cell death by apoptosis and the small number of necrosis. The profound efficiency of soluble 1:1 stoichiometry curcumin binary complex (CMN-P-407 SD) indicated its potential application for CRC treatment by showing a higher capability of inhibiting cell growth compared to that of pure CMN.

KEYWORDS: Curcumin, Phase solubility studies, Colorectal cancer, MTT assay, Hoechst staining.

INTRODUCTION:

Primarily, colorectal cancer (CRC) is the major type of cancer in both women and men. In men, CRC is the third most common type of cancer and in women the second most typical type of cancer, and the third highest cause for death worldwide especially, in developed countries (60% of all CRC cases). The risk factors of CRC include colon polyps, long-standing ulcerative colitis, and genetic family history, and it is readily diagnosed in people aged 65–74[1].

Several studies have found that curcumin (CMN) shows a wide variety of pharmacological activities and has been considered as one of the most chemo-preventive agents that can induce apoptosis in numerous cellular systems. It is also reported to have the potential impact against an assortment of cancer cell growth[2]. As a result of many toxicological studies, the drug is proved to be non-toxic even at high concentrations.

The enhancement of the solubility of insoluble drugs is an important one among the most challenging task in present-day research. The diverse physicochemical methodologies that have been practice to enhance the solubility addition of surfactants by nanosizing and micronization, reduce the particle size, improve of wettability of powders, utilization of prodrugs and its salts, the liposome approach[3], and solid dispersion (SD).

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This research is aimed for (i) the utilization of different carriers in the preparation of SD, (ii) the prepared complex evaluated for aqueous solubility and *in vitro* dissolution, and (iv) upgrading CMN in hostile to cancer action by altering its mild cytotoxic profile against the SW480 and Caco-2 cell lines of CRC.

2. EXPERIMENTAL:

2.1. Materials:

Following chemicals were purchased: curcumin (CMN; purity > 99%; SRL Pvt. Ltd, Maharashtra, India), poloxamers (P-407 and P-188; BASF Corporation, Mumbai, India) and mannitol (MNT; S.D. Fine Chem. Pvt. Ltd, India). Gelucire 50/13 was gift samples from Gattefosse Pvt. Ltd, Mumbai, India. The colorectal adenocarcinoma cell lines of SW480 and Caco-2 were obtained from National Center for Cell Science (NCCS), Pune, India. Other reagents and chemicals utilized were based on the grade of analytics.

2.2. Phase Solubility Studies:

The study of phase solubility is done by the approach explained by Higuchi and Connors[4]. Briefly, CMN is done by adding an excess amount of CMN in 25 mL aqueous solutions of different concentrations (1 to 15 %) to the carriers (P-407, P-188, GLR, and MNT). The Eppendorf tubes containing the arrangement is set in a water bath at a constant temperature of 25 and 37± 0.5 °C for 24 h until the point that balance is accomplished, being shaken often at 30-minute interims. Hence, the content filtered by the Millipore membrane filter (0.45µm), is diluted appropriately, and absorbance is measured at 425nm with UV-spectrophotometer (Agilent Cary 60 UV-Vis Spectrophotometer, USA.). The complexation constant ($K_{1:1}$) is ascertained (Eqn. 1) by using slope and intercept evaluation of the phase solubility curve, where the intercept is the intrinsic solubility of the drug.

$$K_{1:1} = \frac{\text{Slope}}{\text{Intercept (1-Slope)}} \quad \text{.....(1)}$$

Also, the change in enthalpy (ΔH) on complexation determined from Van't Hoff equation, (Eqn. 2)

$$\ln(K_2/K_1) = \Delta H\,(T_2-T_1)/(RT_2T_1) \quad \text{.....(2)}$$

Where K_2, K_1 and T_2 and T_1 have referred to the stability constants and corresponding temperatures in Kelvin of 37 and 25 °C respectively. The change in Gibbs pure energy (ΔG) and entropy (ΔS) upon complexation /solubilization were computed from the equations (Eqn. 3 and 4) respectively.

$$\Delta G = -RT \ln K \quad \text{.....(3)}$$

where R is the gas constant (R = 8.314J/mol K)

$$\Delta S = \frac{\Delta H - \Delta G}{\Delta T} \quad \text{.....(4)}$$

2.3. Preparation of SD and PM:

The solid dispersions (SD) of varying compositions (1:3 to 1:7) were prepared by a melting method by adding CMN to the molten carrier at 70°C with continuous stirring of 700 RPM for 15 min until a homogeneous dispersion obtained. The resultant melt was allowed to solidify, cooled at room temperature (28°C), pulverized, sieved (# 120; 150-125µm) and stored at 25°C in a desiccator[5]. The physical control blends of the same compositions prepared by utilizing a mortar and pestle as indicated by the guidelines of geometrical mixing, following by screening.

2.4. Aqueous Solubility study:

Milli Q water by means of excess amount of test samples (CMN, PM and SD) in a volumetric flask were placed in a water bath at a constant temperature of 25 and 37±0.5°C for 24 h and shaken in between at 30 min intervals[6]. In this way, the substance was filtered through a Millipore membrane filter (0.45µm), diluted appropriately, and the UV absorbance was measured.

2.5. *In vitro* drug release:

The dissolution study was done at 37±0.5°C in 900mL of double distilled water at 50rpm by USP dissolution apparatus II (DS 8000, Lab India, India). The sample placed into the jar and time set as zero. At every 5min time interval up to 30 min, 5mL of samples were withdrawn and filtered by Whatman filter paper (pore size 11µm) then, the dissolved amount of CMN was determined spectrophotometrically. A correction factor represented for the cumulative dilution initiated by replacement of the sample with a unique medium to keep up the sink condition[7].

2.6. Dyeing experiment:

A simple novel test facilitates how efficiently compound (usually colored) get dissolved in an aqueous medium [8]. Briefly, 10mg of CMN and its SDs (equivalent wt.) was added to 15mL of double distilled water then sonicated for 5 min then filtered, formerly pictures of the solutions had taken. The white linens clothes of similar size (8 × 4.5 cm^2) were soaked into the above solution, it has already diluted to 50mL for 1.5 h.

2.7. *In vitro* cytotoxicity study:

The cytotoxicity of samples against SW480 and Caco-2 cell were estimated utilizing an MTT assay. Briefly, 96-well culture plates used for seeding the cells at a cell density of 5 x 10^3 cells/mL (200µL/well). DMSO solution used as a solvent control, after 24 h of incubation in different concentration of complexes, the MTT solution (5mg/mL, 20µL/well) was added to the media, followed by incubation at 37°C for 4 h. The

obtained purple formazan product dissolved by 100µL of DMSO solution to each well[9]. The absorbance estimations of each well were estimated at 570nm utilizing a plate reader (Bio-Rad, iMark, USA). The IC_{50} value was estimated, as the complex concentration is necessary to reduce the absorbance to a half the amount of that control.

2.8. Apoptosis study by AO/EB staining:

Acridine orange (AO) and ethidium bromide (EB) staining method used to investigate apoptotic morphology with some modifications. Briefly, the cells were treated with the IC_{50} concentration of compounds for 24 h were collected and washed with chilly PBS. The cell shots were resuspended and diluted with PBS to a concentration of 5 x 10^5 cells/mL and mixed with 25µL of staining solution (3.8µM of AO and 2.5µM of EB in PBS) on a clean slide. Quickly analyzed under a fluorescent microscope (Carl Zeiss, Axioscope 2plus, Germany) with UV filter (450-490nm). Three 100 cells for each sample were counted for live, apoptotic or necrotic by staining the nucleus structure, membrane integrity and percentage calculated[10]. Morphological variations were also observed and photographed (400x magnification).

2.9. Apoptosis study by Hoechst staining:

The Sw480 and Caco-2 cells were cultured in separate 6-well plates and treated with IC_{50} concentrations of samples. Control and treated cells were collected after 24 h of incubation and stained (Hoechst 33258 stain; mg/mL; aqueous) at room temperature for 5 min[11]. The fluorescent microscope fitted with a 377- 355 nm filter, randomly 300 cells observed with 400x magnification.

3. RESULTS:

3.1 Phase Solubility Studies

A standard linear curve obtained as a result of the concentration ranging from 5.97 × 10^{-5} to 5.7 × 10^{-4} mM and 8.14 × 10^{-5} to 7.8 × 10^{-4} mM for CMN at 25 and 37^{0}C respectively (Table 1). The result is revealing A_L type phase-solubility profile[12].

The apparent $K_{1:1}$ computed from the slope and intrinsic intercept values of solubility curves acquired by plotting concentration (% w/v) of dissolved CMN against concentration (% w/v) of the carrier (Fig. 1a and b). The stability constant (Ka) attained from the complex ranked in the order of 25 and 37°C as P-407 (631.9 and 524.9M^{-1}) > P-188 (436.48 and 388.28 M^{-1}) > GLR (100.14 and 112.05 M^{-1}) > MNT (10.88 and 11.90 M^{-1}). Entropy (ΔS), Gibbs pure energy (ΔG) and enthalpy (ΔH) were additionally ascertained from PS chart.

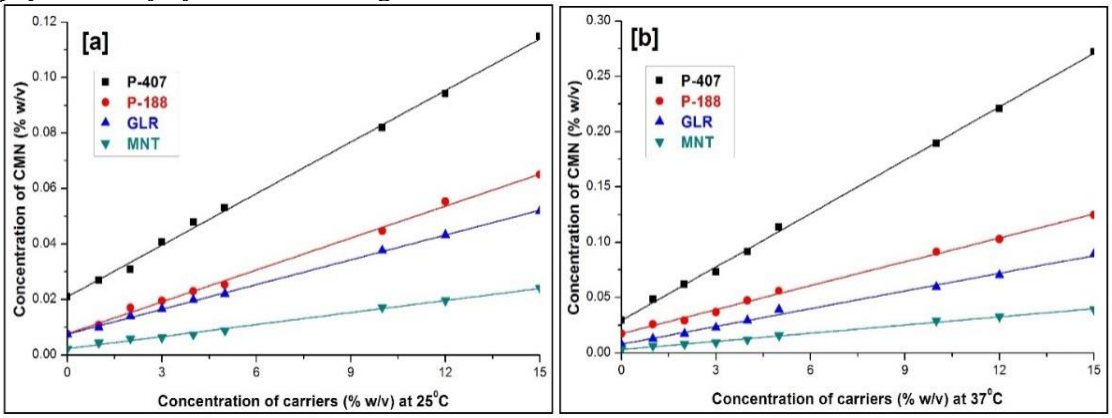

Figure 1: Phase solubility diagram of CMN in water (a) at 25 and (b) 37 °C in the presence of P-407, P-F188, GLR, and MNT.

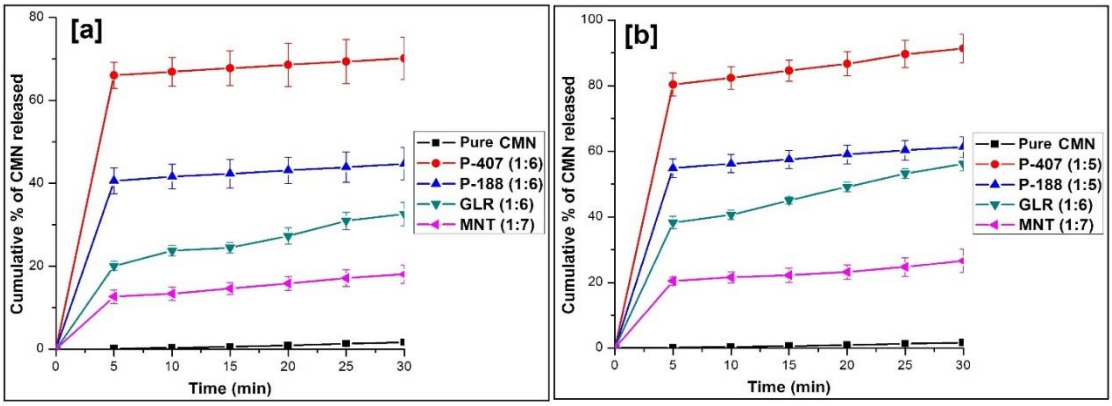

Figure 2: *In-vitro* release of CMN from (a) PM and (b) SD (mean ± SD, n = 3)

3.2 Aqueous solubility:

The effect of carrier concentration on the solubility of pure CMN in Milli Q water at 37°C found to be 0.004 mg/mL for 24 h. The solubility of CMN-SD (1:3 to 1:7) was enhanced by ~270-322 and ~169-195 fold with P-407 and P-188 respectively (Table **2**), due to strong surface-active property surface of drug molecules well adsorbed by the carrier[13].

3.3 *In vitro* dissolution Studies:

The mean dissolution curves of CMN and SD presented in Fig. 2. It is evident that rate of dissolution of pure CMN was very slow (1.62%) at the end of 30 min because of its native (high hydrophobicity - floats on the medium) characteristics, could prevent to contact with the bulk of the solution. The rate of dissolution of CMN-SD twofold framework with respective carriers demonstrated high burst release (20-80%) in the initial 5-6min showing perfect complex developed with the carrier.

Figure 3: Photograph of solution of (a) (i) CMN (ii) CMN- P-407 SD (iii) CMN- P-F188 SD and (iv) CMN- GLR SD (b) Photograph of cotton clothes dyed in the solution of (i) CMN (ii) CMN- P-407 SD (iii) CMN- P-F188 SD and (iv) CMN- GLR SD

After the burst release, the constant rate release profile observed with all SDs (Fig. 2b). To explore further solubility and dissolution, P-188 which has a lower molecular weight than P-407, (P-188 of average MW 8500 compared with the average MW of 12600 for P-407) used. The CMN-GLR SD (1:6) showed a considerable release rate of about 56.15% at the end of 30 min. At MNT-SD complex obtained were not satisfactory in comparison to other SDs[14].

3.4 Dyeing effect:

The CMN and CMN- SDs of P-407, P-188, and GLR showed in Fig. 3, when 10mg CMN added to 15mL of water, CMN float on the water due to the lipophilicity nature of the drug. However, the solution was a bright and characteristic color of poloxamers complex with CMN. Hence, GLR showed yellow color (Fig. **3a**). Fig. 4**b** shows that the cloth dyed in the solution was colorless (CMN), characteristic deep yellow (P-407), yellow (P-188) and pale yellow color (GLR) respectively[15].

3.5 MTT assay:

The cytotoxic results of the MTT-reduction assay of pure CMN and CMN-P-407(SD) on SW480 (Fig. **4a**) and Caco-2 (Fig. 4b) cells lines are shown the IC_{50} value for SD was estimated around 74µM/mL and 52µM/mL, while that of pure CMN ranged from 132µM/mL and 116µM/mL respectively.

3.6 Analysis of cell death:

The cytotoxic effect caused by CMN-P-407(SD) occurred in Sw480, and Caco-2 treated are shown as control or viable cells appeared as bright green color and having uniform chromatin with an intact cell membrane that they did not undergo any apoptotic changes. The stained cells characterized by SD caused more effective cell death than pure CMN.

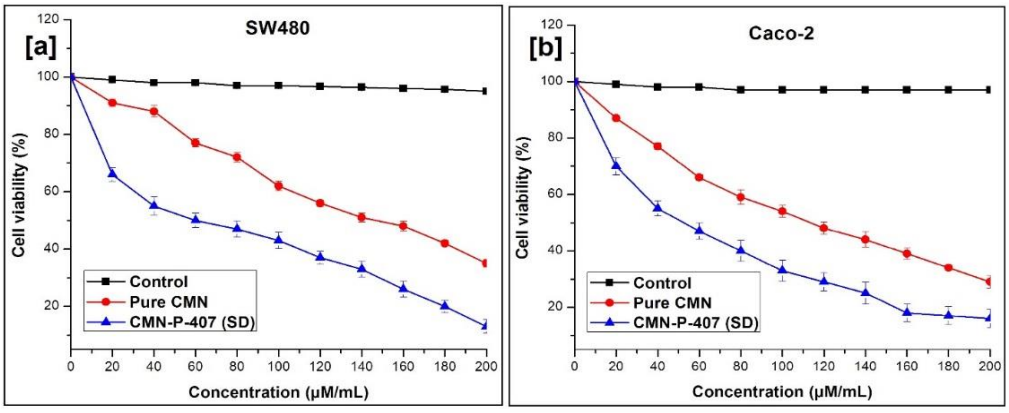

Figure 4: *In vitro* cytotoxic effect of CMN and CMN-SD on (a) SW480 and (b) Caco-2 cells lines. (Mean ± SD, n = 3)

Increased apoptotic cells and necrotic type of cell death also appeared in both samples (Fig. **5e**).

After treatment with IC_{50} concentrations of the complex for 24 h, the Sw480 and Caco-2 cells were observed for cytological changes[16]. The manual count of normal and abnormal, i.e., apoptotic cells in percentage from Hoechst 33258 staining in Sw480 and Caco-2 cells image illustrated in Fig. **5II; c** and **d**.

4. DISCUSSION:

The PS study acknowledged as a valuable data on the effect of the various carrier responsible for the solubility of CMN. The solubility of the CMN expanded straightly as a component of carrier fixation, i.e., increasing temperature and concentration of carrier, the solubility of CMN increased probably due to the changes in the interaction forces, such as hydrophobic forces and Vander Waals and between CMN and carriers. The slope of the PS diagram obtained (>1) in all carriers indicated the 1**:**1 complex stoichiometry[17]. From all the carriers, poloxamers and GLR showed ideal complexation constant in the ranges of 100 to 1000 M^{-1}, in contrary, MNT showed the weak interaction with CMN [18]. The figured estimations of ΔG were revealed negative in all carriers, thus confirmed spontaneity of binding and decreased with increase in the carrier's molecular weight. The calculated values of ΔH were found negative (exothermic) in P-407 and P-188 complexes except for the solubility system with GLR and MNT (Table 1).

Similarly, the ΔS value in CMN-GLR and CMN-MNT system found to be marginally high (6 and 4 J/mol K) showing that the reaction type is endothermic[19]. The very strong binding constant of poloxamer due to a polyoxyethylene-polypropylene block copolymer nonionic surfactant with an HLB value of 18-23. Polypropylene oxide (PPO) for the most part frame a focal hydrophobic center wherein methyl group associated employing Vander Waals force with CMN undergoing solubilization[20]. However, solubility due to polyoxyethylene oxide (PEO) blocked by hydrogen bonding interaction of either oxygen with water molecules. Endothermic (+ΔH) process of binding with high (+ΔS) value (6.3 J/mol/deg.) of GLR enhanced wettability while surrounded with a hydrophilic matrix, by reduction of interfacial tension among drug and water. The solubility system of the CMN increased with an increase in concentrations indicating the solvent properties of GLR for the drug[21].

An exception was noticed from MNT where high (+ΔS) value (4.4 J/mol/deg.) obtained due to ligand molecules are ionized, and water molecules are less ordered. The binding process is endothermic (+ΔH); ΔH favors ΔG and ΔS, and spontaneity ensured by negative ΔG refers to a least complex formation between MNT and CMN. These obtained results established that the soluble complex formed between hydrophilic carriers with lipophilic drugs.

The aqueous solubility of GLR binary system showed passionate enhancement of solubility around 121-144 fold higher than pure CMN could be due to more hydrogen bonding of water molecules to electron-rich GLR chain containing oxygen atoms (Table 2). In contrary, MNT complex showed odder enhancement of solubility 16-23 fold due to less interaction with drug molecules[22].

The *in vitro* release demonstrate after the burst release, the constant rate release profile observed with all SDs. These may occur due to the metastable supersaturation of CMN in the wet carrier matrix during dissolution.

Table 1: Thermodynamic parameters of CMN with various carriers at 25 and 37 °C (mean ± SD, n = 3)

Carrier	T (°C)	Intercept (Mm)	Ka (M^{-1})	ΔG (kJ/mol)	ΔH (kJ/mol)	ΔS (kJ/molK)
P-407	25	$5.70*10^{-4}$	631.97 ± 25.808	-15.98 ± 0.097	-11.9 ± 0.518	0.01368 ± 0.002
	37	$7.8*10^{-4}$	524.91 ± 20.814	-16.15 ± 0.023		
P-F188	25	$2.06*10^{-4}$	436.48 ± 17.122	-15.06 ± 0.224	-7.50 ±0.490	0.02538 ± 0.007
	37	$4.72*10^{-4}$	388.28 ± 16.871	-15.37 ± 0.245		
GLR	25	$2.04*10^{-4}$	100.14 ± 6.179	-11.41 ± 0.215	7.20 ± 0.875	0.06245 ± 0.006
	37	$2.12*10^{-4}$	112.05 ± 7.124	-12.16 ± 0.256		
MNT	25	$5.97*10^{-5}$	10.88 ± 1.439	-5.78 ±0.076	7.35 ± 0.868	0.04399 ± 0.002
	37	$8.14*10^{-5}$	11.90 ± 1.594	-6.33 ± 0.089		

Table 2: Aqueous solubility data of CMN-SD

Aqueous solubility (mg/mL)					
D:C ratio*	1:3	1:4	1:5	1:6	1:7
Pure CMN at 37 °C		0.004 ± 0.0003			
P-407	1.0812 ± 0.022	1.110 ± 0.0162	1.21 ± 0.0239	1.25 ± 0.0225	1.29 ± 0.0264
P-F188	0.6791 ± 0.028	0.7017 ± 0.016	0.7423 ± 0.024	0.7601 ± 0.022	0.7798 ± 0.026
GLR	0.4872 ± 0.028	0.4919 ± 0.016	0.5288 ± 0.024	0.5525 ± 0.025	0.5791 ± 0.024
MNT	0.0671 ± 0.028	0.0698 ± 0.025	0.0795 ± 0.022	0.0812 ± 0.032	0.0912± 0.034

* Drug: carrier ratio

Aside from non-ionic attributes, viscosity suggested a perfect part in the dissolution of the drug by delaying the contact of the drug with the dissolution medium. At CMN-SD with MNT obtained were not satisfactory in comparison to other carriers because the solubility due to a polar effect of carbohydrates and the formation of a hydrogen bond may improve solubilization[23]. The result showed the limited dissolution rate of MNT might be incomplete amorphization of drug particles compared with pure drug powder.

Among all carriers, P-407 showing better physicochemical properties, subsequently these complex developed for cytotoxic contribution. The results from MTT assay specifies that the complex revealed better cytotoxic activity than pure CMN[24]. These findings could be due to the effect of the variations in the cellular uptake profile leading to the better action of SD as suggested by Manju and coworker[25]. The results of this assay indicate that the complex of SD can efficiently deliver the drug to SW480 and Caco-2 cells using active targeting via endocytic process shared with improved cytotoxicity and aqueous solubility of CMN-SD. The cell viability with both SD and pure CMN decreases with increasing the concentration of cytotoxicity, whereas SD was significantly better than that of pure CMN.

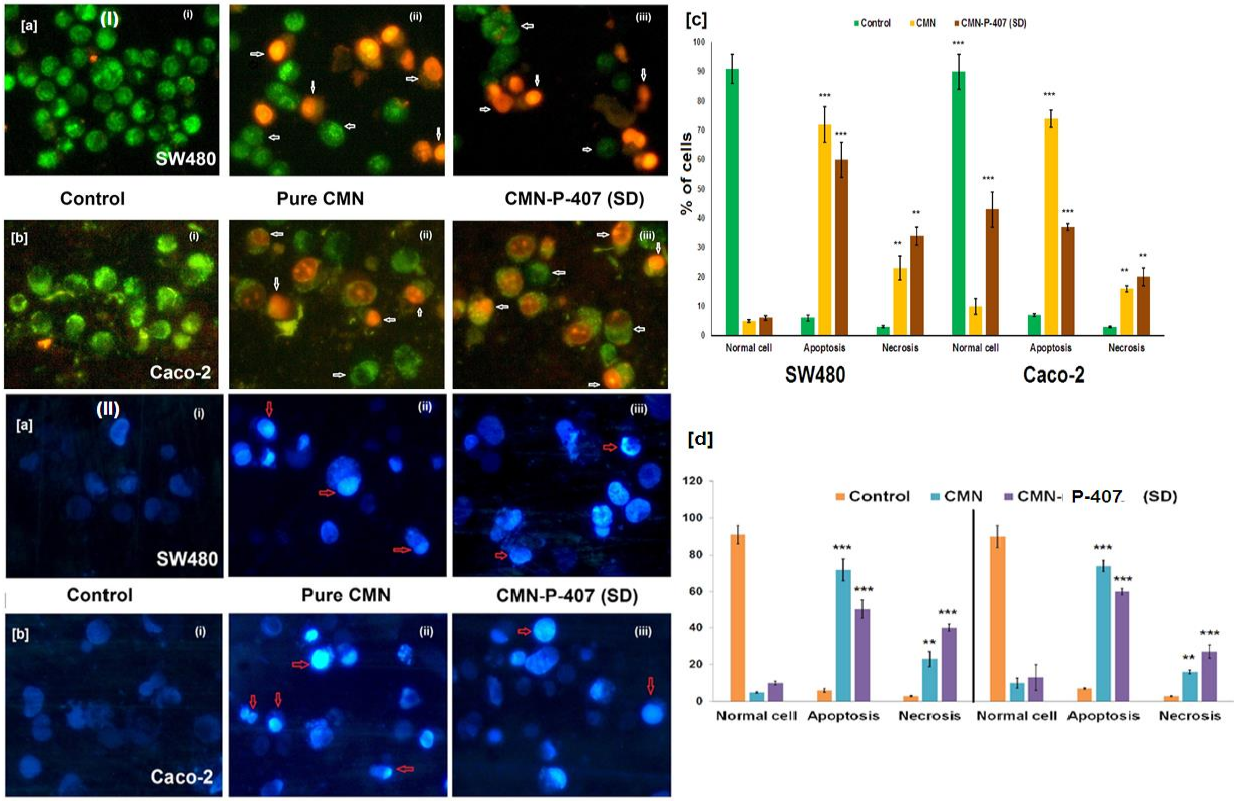

Figure 5: Apoptotic morphology of AO/EB and Hochest staining visualized under fluorescent microscope with (I and II; a) SW480 and (I and II; b) Caco-2 cells; (i) control; (ii) pure CMN; and (iii) CMN-SD and (c and d) percentage of normal, apoptotic and necrotic cells at 24 treatment. Data are expressed as (mean ± SD (%); n=3)

Morphological image changes during cell death are important criteria in apoptosis that can be measured by AO/EB staining. Collectively, if the cells were undergoing the specific form of cell death by AO/EB staining, cytological changes have been observed. As indicated by the fluorescence emission and geologies of the chromatin, cells can be characterized into viable cells; these are very uniform and sorted out structure with green fluorescing cores (Fig. 5; Ia and b; i). Early apoptotic cells, stayed intact membranes yet had quite recently initiated fragmentation of DNA with green fluorescing cores, yet chromatin buildup is perinuclear and visible splendid green pieces or patches (Fig. 5; Ia and b; ii and iii). Late apoptotic cells were divided or condensed chromatin with fluorescing nuclei (orange to red) (Fig. 5I; a and b; ii and iii). Necrotic cells are substantial, or swollen structure had fluorescing nuclei (orange to red) consistently through a nonappearance of chromatin fragmentation (Fig. 5; Ia and b; ii and iii)[26] These results suggest that complex treatment caused cell death through apoptosis and necrosis. Altogether, a higher level of cell death was observed contrasted with necrotic cell death (Fig. 5c and d) of Sw480 and Caco-2 than pure CMN treatment. Hoechst staining revealed the

deviations in cytology of the cell, with particular reference to cytoplasm and nucleus core at the primary level to identify the apoptosis (Fig. 5; IIa and b; ii and iii). The CMN-SD observation was showing that the early apoptotic highlights, such as cell shrinkage, chromatin buildup, and discontinuity had seen in treated cells and small quantities of necrotic cells[27] were observed.

5. CONCLUSION:

This study evolution the trend to examine the solubility and dissolution properties of the curcumin complex with various carriers and evaluated the anticancer potency of soluble curcumin on the colorectal adenocarcinoma cell lines of SW480 and Caco-2. The cytotoxic results of the MTT-reduction assay of pure CMN and CMN-SD-P-407 (1:5) on colorectal adenocarcinoma cells and IC_{50} value was very much lower than pure CMN. The profound efficiency of 1:1 stoichiometry soluble curcumin indicated its potential application for CRC treatment. The research provided an existing and novel method for the implementation of a valuable cancer therapy. We hope the present investigation will inspire further performing along these lines.

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7. CONFLICT OF INTEREST:

The authors declare no conflicts of interests.

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RESEARCH ARTICLE

Estimation of Temperature effect on PVC-Based Ciprofloxacin selective Electrode Response

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ABSTRACT:

In this work we investigated the effect of temperature variance on the response of the ion selective electrode by investigating the ability of using ciprofloxacin ion selective electrode in a temperature range spreading over 5°C to 60°C, and so determined the optimum range of temperature the ion selective electrode can show near NERNSTIAN response, performing good sensitivity, best LOD and LOQ, widest linearity range, shortest response time, and maximum life time. Temperature variance effect on the response of ion selective electrode was studied according to the response of ion selective electrode by Nernst equation, which (when the ion selective electrode express a near NERNSTIAN response) indicate the perfect sensitivity for the ion selective electrode towards the studied ion comparing to the other ions in the sample. For that, a PVC-Based ciprofloxacin selective electrode was used with CFX-phosphotungestic acid (CFX-PTA) as an electro active material. The characterization and analytical properties were determined, and the casting selective membrane of the selective electrodes was plasticized by di-n-butyl phthalate (DBP). The assembled electrode has an internal reference Ag/AgCl electrode, and an external reference Ag/AgCl electrode. The developed sensors showed near NERNSTIAN response for ion pair percentages of 6%. PVC-Based ciprofloxacin selective electrode could work in temperature range from 5°C to 55°C without any significant change in its parameters, with linear range 10-10000µM of ciprofloxacin, and total measuring range (TMR) 3.16-31623µM over a pH range of 2.0-6.0, both when the temperature is similar between the filling solution of the electrode and the sample solution, and when the temperature is unsimilar between the filling solution of the electrode and the sample solution. Also, LOD and LOQ values were determined. Temperature variance between 5°C and 55°C didn't affect the slope of the selective electrode more than 2.3% which indicate a good sensitivity of the selective electrode in this wide range of temperature. Temperature above 55°C has a destructive effect on the response of membrane. In addition, temperature variance between 5°C and 55°C didn't affect the response time of the CFX selective electrode.

KEYWORDS: Drug Selective Electrode, Novel Drug selective membrane, PVC membrane, Ciprofloxacin, Potentiometric Determination, Temperature effect on selective electrode.

INTRODUCTION:

Ciprofloxacin. HCL (CFX) {1-cyclopropyl- 6-fluoro- 4-oxo-7-(piperazin-1-yl) -1,4 dihydroquinoline-3-carboxylic acid hydrochloride} (Fig. 1) is a synthetic bactericidal from the 2nd generation of fluoroquinolones widely used in the treatment of urinary and respiratory tract infections caused by susceptible organisms.

Exerting its bactericidal effect by obstructing the bacterial DNA gyrase, which cause inhibiting of the DNA synthesis and preventing the growth of bacterial cells[1,2].

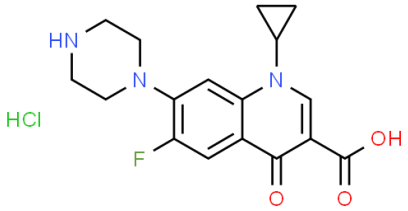

Fig. 1: Chemical Structure of Ciprofloxacin HCl

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Various analytical methods is used for the determination of CFX including HPLC and RP-HPLC[3], UV spectrophotometry[4-8], Derivative UV-Spectrophotometric[9,10], Spectroflourimetric[11], Capillary Zone Electrophoresis[12], Rayleigh light scattering[13], Electrochemical Titrations[14], Electrical Micro-Titration[15], potentiometry[16,17].

Potentiometric method using ion-selective electrodes (ISEs) is simple method to analyses CFX in bulk solutions and pharmaceutical formulations, In previous works we found ISEs which uses PVC as a matrix for ion pair useful for the determination of a single drug providing fast result, simple analysis procedures, and over that offering high selectivity towards the drug in the presence of various pharmaceutical excipients[18-23], also we succeed inventing a selective electrode for the Simultaneous determination of two drugs ciprofloxacin and metronidazole by using a PVC membrane containing two ion pairs[24].

Potential measurement using ion selective electrodes depends on creating electrochemical cell consisting of two electrodes, one is the operating electrode (detecting electrode) which contain known concentration of standard solution, and the other is reference electrode which have constant potential, Nernst equation is used to calculate the potential of an ion of charge Z across the membrane of the selective electrode, this potential is determined using the activity of the ion both inside and outside the cell (both sides of the selective membrane)[25].

$$E = E^{\circ} + \frac{RT}{Z_A F} \ln a_A \qquad (1)$$

E°: Standard potential of the electrochemical cell.
R: Universal Gas Constant, (R = 8.314472 $J.K^{-1}.mol^{-1}$).
T: Temperature in kelvins
Z_A: Ion charge of the relevant ion.
F: Faraday constant (96485 $C.mol^{-1}$).
a_A: Chemical activity for the studied ion.

Equation (1) can be rewritten in the form of $\log_{10}$:

$$E = E^{\circ} + (2.303\frac{RT}{Z_A F}) \log a_A \qquad (2)$$

All measurements with ion selective electrodes are made in constant temperature (25°C, T=298 K), in this case, the measured potential only affected by the activity of the ions outside the electrode (the sample solution), and Nernst equation become as follow:

$$E = E^{O} + \frac{0.059}{Z_A} \log a_A \qquad (3)$$

The quantity $0.059/Z_A$ expressed as Nernstian slope (mV/ΔpC) indicating the perfect sensitivity for the ion selective electrode towards the studied ion comparing to the other ions in the sample.

In this work, we investigated the effect of temperature variance on the response of the ion selective electrode, and so determined the optimum range of temperature the ion selective electrode can perform good sensitivity.

2. MATERIALS AND METHODS:

2.1 Apparatus:

Electrochemical measurements were made with IONcheck-10 pH/mV meter-Radiometer analytical S.A., France, with CFX-phosphotungstic Acid (PTA)–polyvinyl chloride (PVC)–di-n-butyl phthalate (DBP) plasticized membrane electrodes in conjunction with Ag/AgCl wire as an external reference electrode. Crison-GLP 21/EU pH-meter was used for pH adjustment. MS 300 Bante, China hot-plate magnetic stirrer with temperature probe was used to perform constant stirring and temperature adjustment. All weights were taken by analytical balance (BP 221S Sartorius, Germany) with accuracy ±0.1mg. Conductivity meter (inoLab-cond 720, Germany) was used for bi-distill water quality. Oven (WTB binder-78532 Tuttlingen, Germany).

2.2 Reagents and Materials:

Ciprofloxacin (CFX•HCl) 99.0% (Sigma-Aldrich), high molecular weight poly vinyl chloride (PVC) (SABC, KSA), phosphotungstic Acid (PTA) 99% (BDH Laboratory, England), di-n-butyl phthalate (DBP) 99.0%, tetrahydrofuran (THF) 97.0%, hydrochloric acid, sodium hydroxide, potassium chloride (guarantee reagent grade, Merck, Germany) were used. Bi-distilled water (conductivity≤10 µS/cm), silver wire (Φ=1 mm, Swiss, 99.99%) were used.

2.3 Standard Drug Solutions:

Stock standard solutions (0.01 M) CFX•HCl (Mw=367.805 $g.mol^{-1}$) was prepared by dissolving accurate weight of CFX.HCl in 1 M KCL, this solutions were stable for several weeks if kept in the dark at 4°C. Working solutions ranging 0.1-10000 µM were prepared by serial dilution of the previous stock solutions with 1 M KCL. These solutions are stable for 1 week if stored in a cool and dark place. Britton-Robinson universal buffers 0.2 M were used[26,27].

3. Ion Selective Electrode:

3.1 Preparation of Ion Pair:

The ion pair (IP) was prepared by mixing equal volumes of 10 mM CFX solution with 20 mM phosphotungestic acid (PTA) solution to form the ion pair (CFX-PTA). The mixture was stirred for about 30 min, left in the dark for over-night to settling down. The resulting precipitate

was filtered, washed with bi-distilled water several times until the conductivity of the washed water is close to the conductivity of the used bi-distilled water. After that, the precipitate was dried at room temperature over the night away from light and dust. Ion pair was grounded into a fine powder with an agate mortar, then dried in the oven at 60°C until the weight was stable.

Ion pair was stored in will-closed dark glass bottles at 4°C. The molecular ratios of the complexe found to be 1:1 for CFX-PTA.

3.2 Casting of Ion Selective Membrane:
A membrane contain 6% of CFX-PTA was prepared by dissolving 0.031g of matrix PVC and 0.031g of the plasticizer (DBP), and the 0.04g of the ion pair to have the target ion selective membrane. The mixture was dissolved by minimum volume of THF. The resulting solution was poured into a 9cm glass Petri dish and covered with a filter paper, avoided from air movement, dust and direct sunshine. The solvent was allowed to evaporate slowly at room temperature, leaving the casted ion selective membrane that represents the electro-active part of ion selective electrode (ISE). Membranes were stored between two aluminum foils, in will-closed container at 4°C.

3.3 Construction of Ion Selective Electrode (ISE):
Circular cut from casted membrane was glued to a polished polyethylene tube. The result bucket was attached to the end of a suitable glass tube. This body of the ISE was filled with internal reference solution consisting of 1mM of CFX in 1M potassium chloride (KCl) solution. Ag/AgCl wire electrode (lab. assembly) was used as an internal reference electrode[28,29]. The indicator electrode conditioned by soaking it in a 1 mM aqueous CFX solution for 30 min.

3.4 Assembling of Ion Selective Electrode Cell:
Ion Selective Electrode cell was assembled by attaching the above ISE in conjunction with Ag/AgCl wire as an external reference electrode. The circuit was closed by attaching the cell and the outer reference electrode to temp./pH/mV-meter. And so we accomplished The following electro-chemical cell[30]:

$SE_{CFX\text{-}PTA}$: Ag/AgCl-KCl (1M) + CFX (1mM) || CFX–PTA–DBP–PVC membrane || Test solution-KCl (1M) || Ag/AgCl

3.5 Electrode Calibration:
A sample of standard solutions 0.1-10000 µM of CFX in 1 M KCL were transferred into a fit compartment held in fixed temperature chamber, and the cell assembled from membrane electrode in conjunction with Ag/AgCl reference electrode was immersed in the test solution. The measured potential was plotted against the minus logarithm of drug concentration (pC_{CFX}). Between measurements, the electrode was washed with bi-distilled water and wiped with tissue paper.

3.6 Effect of Temperature on Electrode Slope:
The effect of temperature variance on the electrode slope, which (when it is near to Nernst slope) indicates sensitivity for the ion selective electrode towards the studied ion comparing to the other ions in the sample was studied in two lines, the first line is temperature similarity between the selective electrode filling solution and the sample solution, and the second line is non-similarity between the selective electrode filling solution (approximate 25°C) and the sample solution (variance temperature).

The best sensitivity for an ion selective electrode is achieved when the electrode accomplish a near Nernstian response, and doesn't roll away from the Nernst slope.

4. RESULTS AND DISCUSSIONS:

4.1 Calibration Graph, Statistical Data, LOD, and LOQ:
We measured CFX standard solutions of 0.1-10000µM (Pc_{CFX}=1-6) using the suggested ISEs in conjunction with Ag/AgCl reference electrode. Each of the different concentration of standard solution was tested five times. The obtained potentials of the five analyses averaged at each concentration. The average potential was plotted versus Pc_{CFX} according to the straight-line equation: $E = S \times Pc_{CFX} + b$.

The linear part of the calibration graph was taken as the analytical range of the potentiometric sensor (quantitative part), where the total measuring range (TMR) which can be considered as qualitative part and including the linear part of the graph plus the lower curved part of the calibration graph. The suggested sensors exhibited a linear response over the concentration range 10-10000µM, and TMR 3.16-31623 µM over a pH range of 2.0-6.0 (Fig. 2).

The limit of detection (LOD) and the limit of quantification (LOQ) were determined according to the IUPAC recommendation[31]. LOD and LOQ values were 0.130µM, 0.394 µM, respectively when the temperature is similar between the filling solution of the electrode and the sample solution, and 0.128µM, 0.388µM, respectively when the temperature is unsimilar between the filling solution of the electrode and the sample solution. Table 1 sum up response characteristics of CFX sensors at 25˚C.

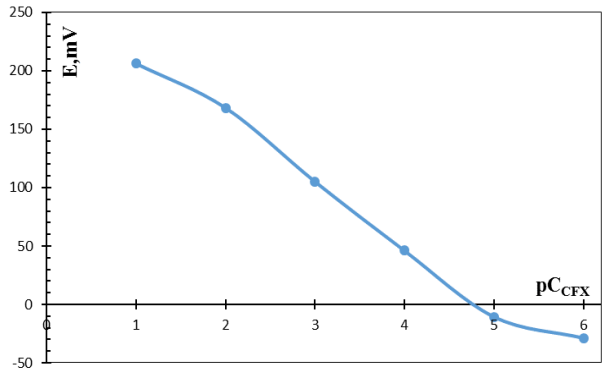

Fig. 2: Calibration curve of CFX selective electrode at 25°C

Table 1: Response characteristics of CFX sensor[a] at 25˚C.

Parameter	Temperature similarity	Temperature un-similarity
IP%	6 %	6 %
Slope, mV.decade^{-1}	59.58 ± 0.11	59.51 ± 0.41
Intercept, mV.decade^{-1}	285.73	285.51
Correlation coefficient (R^2)	0.9994	0.9994
Linear range, µM	10 - 10000	10 - 10000
TMR, µM	3.16 - 31623	3.16 - 31623
LOD, µM	0.130	0.128
LOQ, µM	0.394	0.388
Response time for 1 mM, sec	10 ± 2	10 ± 2
Life time, day	15	15
Working pH range	2 – 6 * 2 – 6.5 **	2 – 6 * 2 – 6.5 **

[a] Five replicate measurement.
* Without buffer.
** Using Britton-Robinson universal buffer.

4.2 Effect of Temperature on Electrode Slope:

When the temperature is similar between the filling solution of the selective electrode and the sample solution the slope values were as in table 2, and we found that temperature variance between 10°C and 55°C didn't affect the slope of the selective electrode more than 1% which indicate the best sensitivity of the selective electrode towards the studied ion comparing to the other ions in this wide range of temperature. At 5°C the slope of ion selective electrode was affected by temperature change by 6% comparing to Nernst slope at 25°C, and although this indicate not the best sensitivity but it indicates a good accepted sensitivity of the selective electrode, in other hand when the temperature reached 60°C the slope of the electrode changed by 7% comparing to Nernst slope at 25°C, but we noticed a migration of the plasticizer from the selective electrode membrane as oil drops in the sample container, and so working with the selective electrode at such high temperature is unacceptable due to altering in selective membrane structure. Fig. 3 shows the effect of temperature on ciprofloxacin selective electrode slope comparing to Nernst slope value.

Table 2: The least squares equations data obtained from the liner equation for variable temperature degrees (Temperature similarity between filling solution and sample solution)

	CFX-PTA		
T(˚C)	S (mV.decade^{-1})	b (mV.decade^{-1})	R^{2*}
5	55.96	267.11	0.9999
10	59.05	282.85	0.9997
15	59.54	285.34	0.9994
20	59.55	285.50	0.9994
25	**59.58**	**285.73**	**0.9994**
30	59.48	285.63	0.9994
35	59.41	285.56	0.9994
40	59.28	285.38	0.9993
45	59.22	285.47	0.9994
50	59.08	285.53	0.9994
55	58.95	285.35	0.9993
60	55.60	285.07	0.9977

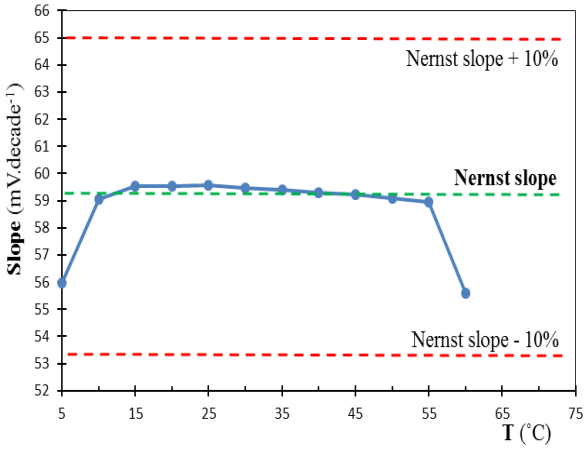

Fig. 3: Effect of temperature on ciprofloxacin selective electrode slope (Temperature similarity between filling solution and sample solution)

Table 3 summarize slope values of the ciprofloxacin selective electrode when the temperature is unsimilar between the filling solution of the electrode and the sample solution, in similar way to the previous discussion we found that changing temperature of the sample solution between 10°C and 55°C didn't affect the slope of the selective electrode more than 2.3% which indicate the best sensitivity of the selective electrode towards the studied ion comparing to the other ions in this wide range of temperature. At 5°C the slope of ion selective electrode was affected by sample temperature change by 3.1% comparing to Nernst slope at 25°C, and this indicates a good accepted sensitivity of the selective electrode, in other hand when the temperature of the sample reached 60°C the slope of the electrode changed by 4.2% comparing to Nernst slope at 25°C, and we started noticing a migration of the plasticizer from the selective electrode membrane as an oil drop in the sample container, and so working with the selective electrode at such high temperature is unacceptable due to altering in selective membrane structure. Fig. 4 shows the effect of temperature of the sample on ciprofloxacin

selective electrode slope comparing to Nernst slope value.

Table 3: The least squares equations data obtained from the liner equation for variable temperature degrees(Temperature un-similarity between filling solution and sample solution)

	CFX-PTA		
T(˚C)	**S**(mV.decade^{-1})	**b**(mV.decade^{-1})	**R^{2}** *
5	57.68	273.88	0.9989
10	59.12	283.02	0.9996
15	59.24	284.04	0.9996
20	59.39	284.61	0.9996
25	**59.51**	**285.51**	**0.9994**
30	59.48	285.73	0.9994
35	59.40	285.80	0.9993
40	59.34	285.89	0.9993
45	59.23	285.93	0.9992
50	59.16	286.26	0.9992
55	58.15	282.40	0.9997
60	57.04	285.99	0.9980

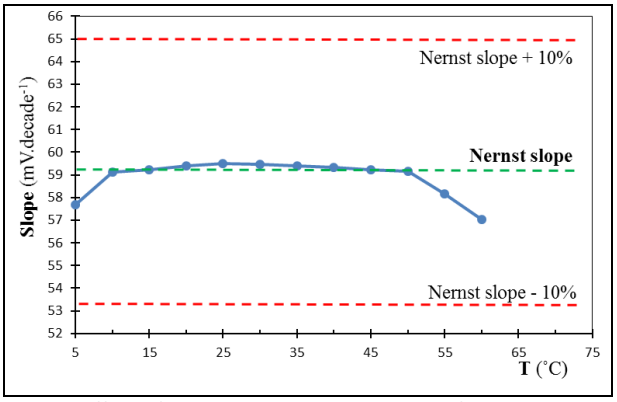

Fig. 4: Effect of sample temperature on ciprofloxacin selective electrode slope
(Temperature un-similarity between filling solution and sample solution)

4.3 Effect of Temperature on Electrode Lifetime

We estimated the lifetime of the electrode from the calibration curves, for that daily-periodical tests of standard CFX solutions (1–10000 µM) were made and its response slopes were calculated. The calibration graphs were plotted after optimum soaking time of 6 hours in 1mM CFX solution. The slope of the calibration curve was -59.58 mV.decade^{-1} at 25°C when the temperature is similar between the filling solution of the electrode and the sample solution, and -59.51 mV.decade^{-1} at 25°C when the temperature is unsimilar between the filling solution of the electrode and the sample solution.

The electrodes were continuously soaked in 1mM solution of CFX for 20 days. The calibration plot slopes declined delicately from day to day for both temperature similarity state and temperature unsimilarity state, respectively after number of days as shown in table 4 for each temperature. This demonstrate that soaking sensors in the drug solution with temperature above 55°C has a destructive effect on the response of membrane. Taking into consideration that Such as this effect appears after working with the sensors for a long time even at temperature 25°C.

Table 4: The Effect of Temperature on Electrode Lifetime

	Temperature similarity		Temperature un-similarity	
Tempera-ture (ºC)	**Slope (mV.decade^{-1})**	**Lifetime (Day)**	**Slope (mV.decade^{-1})**	**Lifetime (Day)**
5	55.96	10	57.68	11
10	59.05	14	59.12	14
15	59.54	15	59.24	15
20	59.55	15	59.39	15
25	**59.58**	**15**	**59.51**	**15**
30	59.48	15	59.48	15
35	59.41	15	59.40	15
40	59.28	15	59.34	15
45	59.22	15	59.23	15
50	59.08	14	59.16	15
55	58.95	12	58.15	12
60	55.60	2	57.04	4

4.4 Effect of Temperature on Electrode Response time:

Response time is the time, which elapses between the moment when an ion-selective electrode and a reference electrode (ISE cell) brought into contact with a sample solution (or at which the activity of the ion of interest in solution is changed) and the first moment at which the potential been readed by ISE is constant.

We found that temperature variance between 10°C and 55°C didn't affect the response time of the CFX selective electrode. Table 5 shows response time for CFX selective electrode when the temperature is similar between the filling solution of the electrode and the sample solution, and when the temperature is unsimilar between the filling solution of the electrode and the sample solution.

Table 5: The Effect of Temperature on Electrode Response time

	Response time Sec	
Temperature (˚C)	**Temperature similarity**	**Temperature un-similarity**
5	16 ± 3	15 ± 2
10	12 ± 2	11 ± 2
15	11 ± 2	11 ± 3
20	10 ± 2	10 ± 3
25	**10** ± 1	**10** ± 1
30	10 ± 1	10 ± 2
35	10 ± 2	9 ± 2
40	9 ± 3	9 ± 2
45	9 ± 2	8 ± 3
50	9 ± 3	8 ± 3
55	9 ± 3	8 ± 2
60	12 ± 3	11 ± 3

5. CONCLUSION:

We concluded that PVC-Based ciprofloxacin selective electrode could work in temperature range of degrees ranging from 10°C to 55°C without any significant change in its parameters.

The PVC-Based Ciprofloxacin Selective Electrode exhibited a good selectivity towards the drug in the presence of various pharmaceutical excipients all over temperature range of 10-55°C.

Temperature variance of the measured sample between 10-55°C didn't affect the linear range of the ciprofloxacin selective electrode, and the electrode response with linearity over the concentration range 10-10000µM of ciprofloxacin, also total measuring range of the selective electrode didn't affect by temperature variance between 10-55°C. Linear range and TMR were 10-10000µM, 3.16-31623µM respectively over a pH range of 2.0-6.0, both when the temperature is similar between the filling solution of the electrode and the sample solution, and when the temperature is unsimilar between the filling solution of the electrode and the sample solution.

LOD and LOQ values were 0.130µM, 0.394µM, respectively when the temperature is similar between the filling solution of the electrode and the sample solution, and 0.128µM, 0.388µM, respectively when the temperature is unsimilar between the filling solution of the electrode and the sample solution.

Temperature variance between 10°C and 55°C didn't affect the slope of the selective electrode more than 2.3% which indicate the best sensitivity of the selective electrode towards the studied ion comparing to the other ions in this wide range of temperature. Temperature above 55°C has a destructive effect on the response of membrane, and so working with the selective electrode at such high temperature is unacceptable due to altering in selective membrane structure.

Temperature variance between 10°C and 55°C didn't affect the response time of the CFX selective electrode.

Ciprofloxacin ion selective electrode can be used as indicator electrodes for direct determination of CFX in its pharmaceutical preparations as well as in pure form solutions over a wide range of temperature degrees lengthened between 5-55°C without any significant change in electrode parameters.

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RESEARCH ARTICLE

Simultaneous determination of Trovafloxacin and Marbofloxacin in equine serum and human plasma samples using previously validated HPLC Method

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ABSTRACT:

A previously validated HPLC method was applied for the simultaneous determination of marbofloxacin (MBX) and trovafloxacin (TVX) in equine serum and human plasma. Samples have been precipitated with 20% $HClO_4$ and drugs were separated using a gradient mobile phase system containing of 17.5mM of Monosodium phosphate (NaH_2PO_4) and 1.5mM aqueous solution of tetrabutylammonium hydroxide ($C_{16}H_{37}NO$), pH 3 (A) and 50% acetonitrile (C_2H_3N) and 50% methanol (B). Antibiotics were determined through UV detection at 293 and 270nm. The quantification limits for TVX and MBX were 10ng/ml and 2ng/ml respectively, and the retention times were 6.63 and 4.93 min respectively for both compounds. Over a concentration range of 10–50,000ng/ml for both antibiotics, the calibration curves were linear. No interacted signals were detected in both horse serum and human plasma. The linearity, precision and accuracy of the assay were estimated from spiked horse serum. The study suggested the use of this method in potential pharmacokinetics studies for selected matrices.

KEYWORDS: Human plasma; Horse serum; HPLC; Marbofloxacin; Trovafloxacin.

1. INTRODUCTION:

Many transferable diseases in horses such as pleuropneumonia and respiratory bacterial infections[1-3] are susceptible to fluoroquinolones, where treatments with traditional antibiotic having inadequate effects. The quinolone antibiotics[4], are wide spectrum, very effective specific antibacterial agents, used in human and animals for treatment of complicated bacterial infections. Marbofloxacin (MBX) is a 3rd generation fluoroquinolone antibiotic (**Figure 1A**), have been presently used only in veterinary medicine. Limited occurrences of resistant pathogens were found after long term treatment of MBX in cattle[5], horses[6-8] and pets[9]. Trovafloxacin (TVX), a 4th generation fluoronaphthyridone (**Figure 1B**) is no longer used in humans, after being withdrawn from pharmacies due to frequent occurrence of idiosyncratic hepatotoxicity in 1999[10,11].

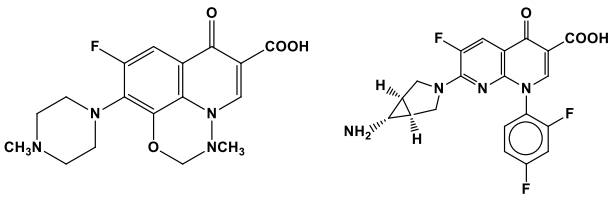

Figure 1: Chemical structures of Marbofloxacin (MBX) and Trovafloxacin (TVX).

Consequently, MBX and TVX might be appropriate antibiotics for treatment of equine bacterial infections. In order to evaluate these candidates, a sequence of *in vivo* pharmacokinetic experiments will be needed, which necessitate the expansion of innocent and sensitive HPLC method for these antibiotics in biological fluids. In the literature, limited data for MBX assays in equine serum and no reports of determination of TVX levels in the same matrix and using HPLC with UV detection.

Since no adequate information about procedures that could be beneficial at the same time for variant animal species and for different tissues. The study defines the expansion and validation of an enhanced HPLC

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systematic method using UV detection for concurrent determination of MBX and TVX which is sensitive, fast, and necessitate very simple sample preparation.

2. MATERIALS AND METHODS:

2.1 Chemicals and materials:

Materials (Marbofloxacin and Trovafloxacin mesylate), were kindly provided by Pfizer Inc. (Eastern Point Rd., Groton CT 06340) as MBX and TVX respectively. The HPLC measurements were carried out using same Shimadzu chromatographic system described in previously published work[12]. Equine serum was purchased from Serum Australia, while human plasma samples were available in the lab from other experiments. The study was performed at the Therapeutic Research Centre (TRC), Translation research Institute (TRI), The University of Queensland, Brisbane, Australia. The mobile phase, gradient mixture flow and retention times were exactly similar to what are found in previously reported method for sheep plasma[12].

2.2 Preparation of standard, quality control samples (QCs) and sample preparation procedure:

All stock solutions and samples were prepared and processed as same as previously described method sheep plasma.

2.3 Validation of method:

The selectivity, linearity, precision and accuracy detection limit and quantification limit were determined for equine serum. The method was also tested for human plasma. According to FDA guidelines, detection limit and quantitation limit were obtained based on the standard deviation of the response and the slope of the calibration curve[13]. The precision and accuracy for both intra-day and inter-day of the control samples were assessed and measured as percentage variations from nominal concentrations of both compounds in horse serum.

50µL MBX and TVX stock solutions (1mg/ml) were spiked in 0.9ml of blank plasma, the calibration curves for both agents were formed to make a high concentration of 50,000ng/ml in plasma. Plasma solutions of 20,000, 10,000, 5,000, 2,000, 1,000, 500, 250, 100, 50, 20, and 10ng/ml were prepared by stepwise dilutions. The Quantitation limit for each analyte was determined using pure standards that were analyzed based on peak areas in the HPLC method. The limit of detection was estimated from a low concentration reference standard through continuous dilution.

The method was validated using a standard statistical calculation was used for 3 groups of calibration curves plus 2 extra samples of lower limit of quantification (LLOQ) for each antibiotic.

The quality control samples (n = 6) were determined by extracting the spiked equine serum samples consisting of both MBX and TVX at 20,000, 4,000, 500 and 50ng/ml. The intra-assay precision was measured at 20,000, 4,000, 500 and 50ng/ml, while inter-assay precision was determined at 4,000, 500 and 50ng/ml (n = 4). The accuracy was measured by relating the calculated concentration to nominal concentrations.

3. RESULT AND DISCUSSION:

Demonstrative chromatograms of equine serum and human plasma are shown in **Figures** 2 and 3 respectively. As seen, no endogenous constituent signals were interfered with either MBX or TVX peaks in the pre-treatment samples.

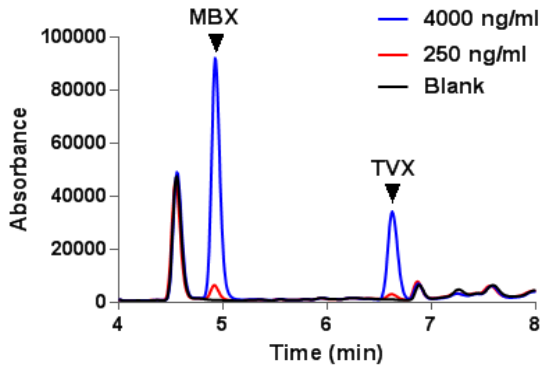

Figure 2: Chromatogram blank horse serum spiked with 4000 ng/ml of MBX and TV, blank horse serum spiked with 250 ng/ml of MBX and TVX, and a blank horse serum.

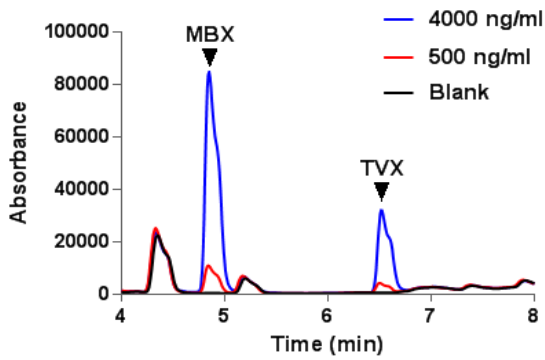

Figure 3: Chromatogram blank human plasma spiked with 4000 ng/ml, blank human plasma spiked with 500 ng/ml of MBX and TVX, and a blank human plasma.

The retention times of MBX and TVX were 4.9 and 6.7 min, respectively, with a calibration range of 10-50000 ng/ml for both antibiotics. The detection and quantitation limit (LLOQ) for MBX were (0.3,0.9), while for TVX were (3.0, 9.4) ng/ml, respectively. The precision of LLOQ was 4.4% for MBX and 9.4% for TVX.

Means of the control samples, precision and accuracy at intra-day and inter-day analysis are presented in **Table 1**. This HPLC method allows simultaneous analysis for MBX and TVX, unlike the assays in[14-16] and is shorter, simpler and the volume of plasma or serum required was slightly lower. than previously reported assays for MBX [17,18] and TVX[19,20]. The detection limit of MBX and TVX was slightly less than that stated by Garcia *et al* and Carretero *et al*[15,18] and Liang *et al*[20], respectively.

Table 1: Means, precision and accuracy ranges of the control samples in horse serum.

Concentration (ng/ml)	Intraday			Interday		
	Mean± SD (ng/ml)	Precision (%)	Accuracy (%)	Mean± SD (ng/ml)	Precision (%)	Accuracy (%)
MBX						
20000	20581±747	4,0	2.9			
4000	4227±195	5	5.7	4592.2±204.5	4.5	14.8
500	510±12	2.6	2	502±12.2	2.4	0.4
50	47±1.7	3,9	-6	48.0±4.6	9.6	-0.4
TVX						
20000	21413±718	3.7	7.1			
4000	4200±207	5.4	5	4130.4±211.2	5.1	3.3
500	505±31	6.7	1	507.8±28.2	5.6	1.6
50	48.4±1.9	4.4	-3.2	48.5±3.0	6.3	-3.1

4. CONCLUSIONS:

The current study examined previously developed HPLC method for simultaneous analysis of both MBX and TVX on different matrices. The precision, accuracy and linearity are proposed to be appropriate clinical procedures for both antibiotics.

This method is simple, time and cost saving, and it was effectively tested in human plasma samples. Additional studies are needed in the future, in order to evaluate it this method in the other matrices.

5. ACKNOWLEDGEMENTS:

The author wishes to thank The Therapeutic Research Centre (TRC) particularly Professor Michael Roberts for providing materials and helping in performing this study.

6. CONFLICT OF INTEREST:

The present work has no conflict of interest.

7. ETHICAL APPROVAL:

No human or animals were participants in this study performed by the author.

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RESEARCH ARTICLE

ADME and Inhibitory Target Molecules Predicition of C*yamopsis tetragonoloba*

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ABSTRACT:

Cyamopsis tetragonoloba is also known as a cluster bean which is an annual legume typically grown in the summer season. Asian and South East Asian countries dominate in the cultivation of this plant and is used as a food source wherein both the seeds as well as the seedpods are consumed. It is also used as feed for cattle and fish. While the immature pods contain a high level of hyaluronic acid, trace amounts of it are seen in the mature pods. It is also the source of the Guar Gum which is made from dried and crushed seeds and chemically, is a neutral polysaccharide containing mannose and galactose units. This is primarily used as a thickening agent and also is known to have certain laxative properties. Previous literature has also suggested its usage in the management of diabetes. The aim of our research is to evaluate the ADME properties and its potential inhibitory target molecule prediction of *Cyamopsis tetragonoloba* through *in-silico* analysis. ADME is Absorption, Distribution, Metabolism and Excretion properties that is essential for drug designing. The active compounds of the plant were obtained from literature survey. The canonical SMILES of the compound were retrieved from PubChem database which is submitted to Swiss ADME to obtain its properties. The inhibitory target molecule was obtained from Swiss target prediction online software. The compound were studied to understand its role in molecular pathway which helps in drug designing.

KEYWORDS: ADME, *Cyamopsis tetragonoloba*, inhibitory target molecule prediction, *in-silico*.

1. INTRODUCTION:

Legumes are plants belonging to the family Fabaceae, and are unique in their nutritional properties. They produce pods that hold the seeds. Importantly these are known for their nitrogen fixing properties by the root nodules. *Cyamopsis tetragonoloba* is one such legume that is commonly known as the cluster bean and is distinctively known as a green manure[1]. It is used as cattle feed and food for fish[2]. It is also the source of guar gum which is a galactomannanpolysaccharide. Chemically, guar gum is an exo-polysaccharide composed of the sugars galactose and mannose[3].

Primarily used as a thickening agent, it is also known to have certain laxative properties. It has also been known for its anti-diabetic properties. Guar gum retards ice crystal growth by slowing mass transfer across the solid/liquid interface. It shows good stability during freeze-thaw cycles. In addition to this, immature pods contain a high level of hyaluronic acid and trace amounts of it are seen in the mature pods[4]. All these properties make it an ideal candidate for checking any inhibitory properties of the constituent molecules for the purpose of drug designing. One of the efficient ways to achieve this is with the help of bioinformatics tools. In order to do this, the role of the specific compounds must be established as their contribution to metabolism. The ADME properties of certain compounds of this plant were predicted. ADME stands for - absorption, distribution, metabolism, and excretion and describes the disposition of a compound within an organism. This is helpful in determining the drug designing and its role in the metabolism of the organism. Active compounds were found from the literature survey which formed the basis of this study. Further bioinformatics based tools were used to then evaluate these compounds[5,6].

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Inhibitory target prediction helps to identify the proteins targeted by certain compounds. These compounds bind to certain proteins thereby inhibiting their metabolic and biological functions. Often these compounds bind to primary target proteins as well as off target proteins. Inhibitory target prediction helps to evaluate the efficiency of the binding between the target proteins and the compounds[7]. Target prediction helps to identify the pharmacokinetics, medical biochemistry and physiochemistry of certain compounds[8]. Pathway analysis predicts the molecules and energies involved in organisms metabolic pathways. It helps to predict and analyse the role and types of metabolic pathways a compound is involved in. Pathway analysis provides a representation of the relevant pathways in which compounds are involved[9].

2. MATERIALS AND METHODS:

2.1 Active Compounds of the Plant *Cyamopsis tetragonoloba*:

All parts of the plant would have certain compounds that react differently. Some of which may be actively involved in the ADME effects of this plant by activating different pathways and initiating different processes in the body. These were found based on a literature survey and their molecular formula and the PubChem IDs were obtained from the PubChem database. The compounds formed the basis of the study thus conducted. The active compounds of *Cyamopsis tetragonoloba* are 1,2-Cyclopentanedione, Isopentyl acetate, 3,5-Dihydroxy-6-methyl-2,3-dihydro-4H-pyran-4-one, 2,3-Dihydro-benzofuran, Acetyl monoglyceride, 1-(p-methoxyphenyl)propene, Ethyl alpha-d-glycopyranoside, Palmitic acid, Ethyl hexadecanoate, Hexopyranosyl hexopyranoside, Phytol, Ethyl (9Z,12Z)-9,12-octadecadienoate, Ethyl (9Z)-9-octadecenoate, Ethyl n-octadecanoate, 2-Hexadecanoyl glycerol, Mono(2-ethylhexyl) phthalate, Ethyl nonadecanoate, Aletamine, Propyleneglycol monoleate, Alpha-Monostearin, Ethyl docosanoate, 3-(2-Hydroxy-3,4-dimethoxyphenyl)-7-chromanol, Nonacosane, beta-Tocopherol, Tetracontane, dl-alpha-Tocopherol, Ergost-5-en-3-ol, Stigmasterol, gamma-Sitosterol, Alpha-amyrin and Lupeol.

2.2 ADME Analysis:

The ADME (Absorption, Distribution, Metabolism and Excretion) analysis of the above compounds was done with the help of the online SwissADME tool that allowed the quantification of the same. SwisADME software helps to predict the pharmacokinetics, medicinal biochemistry, drug likeness and physicochemistry of compounds or molecules. SwissADME helps in drug discovery and medicinal chemistry applications. Here, the analysis was done with the help of the Cannonical Smiles obtained for each compound from the PubChem database. The cannonical smiles obtained are then pasted on the smiles list of SwissADME and then the calculations where run to obtain the ADME properties of each compound.

2.3 Inhibitory Target Prediction of Compounds:

Out of these active compounds, only certain molecules may have any kind of inhibitory properties. An understanding of these, along with the pathways they are involved in, would help in targeted drug designing". Thus, to analyse this, the targets were predicted with the help of the Swiss Target Prediction, Expasy Medicinal Chemistry. Swiss Target Prediction is an online tool to predict the targets of bioactive small molecules in human and other vertebrates. This is useful to understand the molecular mechanisms underlying a given phenotype or bioactivity, to rationalize possible side-effects or to predict off-targets of known molecules. This tool identifies the target molecules based on their Cannonical Smiles of the compounds obtained from the PubChem database. The cannonical smiles obtained are then pasted on the smiles list of Swiss Target Prediction database and then run to predict the target molecules of each compound

2.4 Pathway Analysis of the Target Molecules:

Many target molecules were obtained from the Target Prediction. Three molecules for each of the compounds were selected. Based on this, the pathways in which these are involved in were found. This was done with the help of a literature survey and previously established work.

3. RESULTS:

3.1 ADME Analysis:

The ADME (Absorption, Distribution, Metabolism and Excretion) analysis of compounds of the plant *Cyamopsis tetragonoloba* was done with the help of the online SwissADME tool that allowed the quantification of the same. The following results were obtained from the SwissADME software after running the canonical smiles of each compound. The ADME properties of each compound are enlisted in the table below:

Table 1 ADME properties of the compounds derived using the SwissADME software

SN	Molecule	HA	HBA	HBD	RB	TPSA	GA	LK	LV	BS
1	1,2-Cyclopentanedione	7	2	0	0	34.1	High	-7.2	0	0.55
2	Isopentyl acetate	9	2	0	4	26.3	High	-5.5	0	0.55
3	3,5-Dihydroxy-6-methyl-2,3-dihydro-4H-pyran-4-one	10	4	2	0	66.8	High	-7.4	0	0.56
4	2,3-Dihydro-benzofuran	9	1	0	0	9.23	High	-5.5	0	0.55
5	Acetyl monoglyceride	9	4	2	4	66.8	High	-7.8	0	0.55
6	1-(p-methoxyphenyl)propene	11	1	0	2	9.23	High	-4.9	0	0.55
7	Ethyl alpha-d-glycopyranoside	14	6	4	3	99.4	High	-9.2	0	0.55
8	Palmitic acid	18	2	1	14	37.3	High	-2.8	1	0.56
9	Ethyl hexadecanoate	20	2	0	16	26.3	High	-2.4	1	0.55
10	Hexopyranosyl hexopyranoside	23	11	8	4	190	Low	-11	2	0.17
11	Phytol	21	1	1	13	20.2	Low	-2.3	1	0.55
12	Ethyl (9Z,12Z)-9,12-octadecadienoate	22	2	0	16	26.3	High	-3	1	0.55
13	Ethyl (9Z)-9-octadecenoate	42	10	3	31	133	Low	-6.6	1	0.55
14	Ethyl n-octadecanoate	22	2	0	18	26.3	Low	-1.8	1	0.55
15	2-Hexadecanoyl glycerol	39	5	1	33	72.8	Low	-0.1	2	0.17
16	Mono(2-ethylhexyl) phthalate	24	4	0	11	52.6	High	-4.1	0	0.55
17	Ethyl nonadecanoate	23	2	0	19	26.3	Low	-1.6	1	0.55
18	Aletamine	13	1	1	4	26	High	-5.2	0	0.55
19	Propyleneglycol monoleate	24	3	1	19	46.5	High	-3.3	1	0.55
20	alpha-Monostearin	25	4	2	20	66.8	High	-3.2	0	0.55
21	Ethyl docosanoate	26	2	0	22	26.3	Low	-0.7	1	0.55
22	Isomucronulatol	22	5	2	3	68.2	High	-6.1	0	0.55
23	Nonacosane	29	0	0	26	0	Low	2.1	1	0.55
24	beta-Tocopherol	30	2	1	12	29.5	Low	-1.5	1	0.55
25	Tetracontane	40	0	0	37	0	Low	5.4	2	0.17
26	dl-alpha-Tocopherol	31	2	1	12	29.5	Low	-1.3	1	0.55
27	Ergost-5-en-3-ol	29	1	1	5	20.2	Low	-2.5	1	0.55
28	Stigmasterol	30	1	1	5	20.2	Low	-2.7	1	0.55
29	gamma-Sitosterol	30	1	1	6	20.2	Low	-2.2	1	0.55
30	Alpha-amyrin	31	1	1	0	20.2	Low	-2.5	1	0.55
31	Lupeol	31	1	1	1	20.2	Low	-1.9	1	0.55

Heavy atom: HA, H-bond acceptors:– HBA, H-bond donors:- HBD, Rotable bonds :- RB, GI absorption:- GA, log Kp (cm/s) – LK, Lipinski violations:- LV and Bioavailability Score – BS

3.2 Inhibitory Target Prediction of Compounds:

Out of these active compounds, only certain molecules may have any kind of inhibitory properties. Swiss Target Prediction, Expasy Medicinal Chemistry was used to analyse and predict the targets for which the compounds have an inhibitory action.

The important inhibitory target of 1,2-Cyclopentanedione are Nitric oxide synthase, brain; Nitric oxide synthase, inducible and Nitric oxide synthase, endothelial. The important inhibitory target of Isopentyl acetate are Carbonic anhydrase I, Indoleamine 2,3-dioxygenase and Beta-chymotrypsin. The important inhibitory target of 3,5-Dihydroxy-6-methyl-2,3-dihydro-4H-pyran-4-one are Tyrosinase, D amino-acid oxidase and Thymidine phosphorylase. The important inhibitory target of 2,3-Dihydro-benzofuran are odium channel protein type IV alpha subunit, Serotonin 2a (5-HT2a) receptor and Cannabinoid receptor 1. The important inhibitory target of Acetyl monoglyceride is Protein kinase C alpha, Cytidine deaminase and Beta-glucocerebrosidase. The important inhibitory target of 1-(p-methoxyphenyl) propene are Nuclear factor NF-kappa-B p65 subunit, 2) Quinone reductase 2 and Arachidonate 5-lipoxygenase. The important inhibitory target of Ethyl alpha-d-glycopyranoside are Vascular endothelial growth factor A, Gamma-secretase and Acidic fibroblast growth factor. The important inhibitory target of Palmitic acid are Fatty acid binding protein adipocyte, Peroxisome proliferator-activated receptor alpha and Fatty acid binding protein muscle. The important inhibitory target of Ethyl hexadecanoate are Carbonic anhydrase II, Carbonic anhydrase I and Dual specificity phosphatase Cdc25A.

The important inhibitory target of Hexopyranosyl hexopyranoside is Cyclin-dependent kinase 1, Heat shock protein HSP 90-alpha and Vascular Endothelial growth factor A. The important inhibitory target of Ethyl docosanoate are Vitamin D receptor, Carbonic anhydrase II and Carbonic anhydrase I. The important inhibitory target of Isomucronulatol are Arachidonate 15-lipoxygenase, Arachidonate 1 2-lipoxygenase and Arachidonate 15-lipoxygenase, type II. The important inhibitory target of Nonacosane are Testis-specific androgen -binding protein, Sphingosine kinase 2 and Acetylcholin-esterase. The inhibitory target of Beta-Tocopherol are Serine/threonine- protein kinase AKT, PH domain leucine-rich repeat-containing protein phosphatase 1 and Serine/threonine-protein kinase ILK-1. The inhibitory target of Tetracontane are Testis-specific androgen -binding protein, Acyl coenzyme A:

cholesterol acyltransferase and Carboxylesterase 2. The inhibitory target of dl-alpha-Tocopherol are Serine/threonine- protein kinase AKT, Cannabinoid receptor 1 and Cannabinoid receptor 2. The inhibitory target of Ergost-5-en-3-ol are Androgen Receptor, LXR-alpha and HMG-CoA reductase. The inhibitory target of Stigmasterol are Androgen Receptor, Niemann-Pick C1-like protein 1 and HMG-CoA reductase. The inhibitory target of gamma-Sitosterol are Androgen Receptor, HMG-CoA reductase and Cytochrome P450 51. The inhibitory target of Alpha-amyrin are Androgen Receptor, Protein-tyrosine phosphatase 1B and Estrogen receptor alpha. The inhibitory target of Lupeol are 11-beta-hydroxy steroid dehydrogenase 1, UDP-glucuronosyl transferase 2B7 and Androgen Receptor. The inhibitory target of Phytol is Androgen receptor, Dual specificity phosphatase cdc25a and UDP-glucuronosyltransferase 2b7. The inhibitory target of Ethyl (9Z,12Z)-9,12-octadecadienoate are Anandamide amidohydrolase, Cannabinoid receptor 1 and Cannabinoid receptor 2. The inhibitory target of Ethyl (9Z)-9-octadecenoate are Protein kinase C delta, Protein kinase C theta and 11-beta-hydroxysteroid dehydrogenase 1. The inhibitory target of Ethyl n-octadecanoate are Vitamin D receptor and UDP-glucuronosyltransferase 2B7. The inhibitory target of 2-Hexadecanoyl glycerol are Protein kinase C gamma, Protein kinase C epsilon and Protein kinase C eta. The inhibitory target of Mono (2-ethylhexyl) phthalate are Cystic fibrosis transmembrane conductance regulator, Phosphodiesterase 4B and Cathepsin K. The inhibitory target of Ethyl nonadecanoate is Vitamin D receptor Dual specificity phosphatase Cdc25 and UDP-glucuronosyltransferase 2B7. The inhibitory target of Propyleneglycol monoleate are Protein kinase C alpha, Prostaglandin E synthase and Anandamide amidohydrolase. The inhibitory target of Alpha-Monostearin is Protein kinase C alpha, Protein kinase C gamma and Protein kinase C eta.

3.3 Pathway Analysis of the Target Molecules:

Many target molecules were obtained from the Target Prediction. The pathways in which these are molecules are involved in, were listed below.

- Nitric oxide synthase, brain - Positive regulation of adenylate cyclase-activating adrenergic receptor signaling pathway involved in heart process.
- Nitric oxide synthase, inducible- cytokine mediated signalling pathway
- Nitric oxide synthase, endothelial - liposaccharide mediated signalling pathway, negative regulation of extrinsic apoptotic signalling pathway via death domain receptors, positive regulation of NOTCH pathway.
- Carbonic anhydrase I - Nitrogen metabolism, metabolic pathways
- Indoleamine 2,3-dioxygenase - Kynurenine pathway
- Beta-chymotrypsin - Protein biosynthesis
- Tyrosinase - Melanine biosynthesis
- D amino-acid oxidase - Glyoxylate metabolism and glycine degradation, Peroxisomal protein import.
- Thymidine phosphorylase - dTMP biosynthesis by salvage pathway
- Sodium channel protein type IV alpha subunit - “Interaction between L1 and ankyrins, Phase 0 rapid depolarization.
- Serotonin 2a (5-HT2a) receptor - G-coupled protein receptor signalling pathway, phospholipase C-activating G protein coupled receptor signalling pathway, phospholipase C-activating serotonin receptor signalling pathway.
- Cannabinoid receptor 1 - G protein coupled receptor signalling pathway, adenylate cyclase modulating G protein coupled receptor signalling pathway, cannabinoid receptor pathway.
- Protein kinase C alpha - MAPK1/3 (ERK1/2)-dependent signaling pathway, activates the integrin heterodimer ITGA2B-ITGB3 through the RAP1GAP pathway for adhesion.
- Cytidine deaminase - cell surface receptor signaling pathway.
- Beta-glucocerebrosidase - cholesterol metabolism, Sphingolipid metabolism, Activated by PKC in the salvage pathway of ceramide formation
- Nuclear factor NF-kappa-B p65 subunit - cytokine-mediated signalling pathway, Fc-epsilon receptor signalling pathway, interleukin-1-mediated signalling pathway, negative regulation of extrinsic apoptotic signalling pathway,, negative regulation of insulin receptor signalling pathway, nucleotide-binding oligomerization domain containing 2 signalling pathway, nucleotide-binding oligomerization domain containing 2 signalling pathway, postsynapse to nucleus signalling pathway, stimulatory C-type lectin receptor signaling pathway, T cell receptor signaling pathway, tumor necrosis factor-mediated signaling pathway.
- Quinone reductase 2 - Detoxification pathway.
- Arachidonate 5-lipoxygenase - leukotriene A4 biosynthesis, cytokine-mediated signaling pathway, interleukin-18-mediated signaling pathway, lipoxygenase pathway.
- Vascular endothelial growth factor A - cytokine-mediated signalling pathway, positive regulation of cell proliferation by VEGF-activated platelet derived growth factor receptor signalling pathway, positive regulation of endothelial cell chemotaxis by VEGF-activated vascular endothelial growth factor receptor signalling pathway, positive regulation of vascular endothelial growth factor receptor signalling pathway, vascular endothelial growth factor receptor

signalling pathway

- Gamma-secretase -vascular endothelial growth factor signalling pathway, VEGF-activated neuropilin signalling pathway, VEGFA-VEGFR2 Pathway.
- Acidic fibroblast growth factor - Fibroblast growth factor receptor signaling pathway.
- Fatty acid binding protein adipocyte - Triglyceride catabolism, Transcriptional regulation of white adipocyte differentiation
- Peroxisome proliferator-activated receptor alpha - peroxisomal beta-oxidation pathway of fatty acids, Nuclear Receptor transcription pathway.
- Fatty acid binding protein muscle - Triglyceride catabolism.
- Carbonic anhydrase II - angiotensin-activated signaling pathway
- Dual specificity phosphatase Cdc25A - Deregulated CDK5 triggers multiple neurodegenerative pathways in Alzheimer's disease models
- Heat shock protein HSP 90-alpha - Cytokine-mediated signalling pathway, ERBB2 signalling pathway, Fc-gamma receptor signalling pathway involved in phagocytosis, vascular endothelial growth factor receptor signalling pathway, VEGFA-VEGFR2 Pathway.
- Vascular endothelial growth factor A - Cytokine-mediated signaling pathway, positive regulation of cell proliferation by VEGF-activated platelet derived growth factor receptor signalling pathway, positive regulation of endothelial cell chemotaxis by VEGF-activated vascular endothelial growth factor receptor signalling pathway, VEGFA-VEGFR2 Pathway, VEGF-activated neuropilin signalling pathway.
- Dual specificity phosphatase cdc25a - Deregulated CDK5 triggers multiple neurodegenerative pathways in Alzheimer's disease models.
- UDP-glucuronosyltransferase 2b7 - Artemether Metabolism Pathway, Codeine Action Pathway, Codeine and Morphine Metabolism
- Anandamide amidohydrolase - Arachidonic acid metabolism, Fatty acid metabolism, anandamide degradation.
- Cannabinoid receptor 1 - “adenylate cyclase-modulating G protein-coupled receptor signalling pathway, cannabinoid signalling pathway, G protein-coupled receptor signalling pathway.
- Cannabinoid receptor 2 - G protein-coupled receptor signalling pathway
- Protein kinase C delta - Fc-gamma receptor signalling pathway involved in phagocytosis, interferon-gamma-mediated signalling pathway, intrinsic apoptotic signalling pathway in response to oxidative stress, negative regulation of insulin receptor signalling pathway, positive regulation of apoptotic signalling pathway, stimulatory C-type lectin receptor signalling pathway.
- Protein kinase C theta - Fc-epsilon receptor signalling. Pathway, negative regulation of insulin receptor signalling pathway, T cell receptor signalling pathway.
- 11-beta-hydroxysteroid dehydrogenase 1 - 11-beta-Hydroxylase Deficiency, 17-alpha-Hydroxylase Deficiency, 21-Hydroxylase Deficiency.
- Vitamin D receptor - Bile acid signalling pathway, vitamin D receptor signalling pathway, Nuclear Receptor transcription pathway.
- Dual specificity phosphatase Cdc25A - Deregulated CDK5 triggers multiple neurodegenerative pathways in Alzheimer's disease models.
- UDP-glucuronosyltransferase 2B7 - Artemether Metabolism Pathway, Codeine Action Pathway, Codeine and Morphine Metabolism.
- Protein kinase C gamma - Cellular signalling pathways, Beta-catenin independent WNT signalling, G Protein Signalling Pathways, ErbB Signalling Pathway.
- Protein kinase C epsilon - RHOA pathway, Fc-gamma receptor signalling pathway involved in phagocytosis, lipopolysaccharide-mediated signalling pathway, TRAM-dependent toll-like receptor 4 signalling pathway.
- Protein kinase C eta - mTOR pathway, PI3K/AKT pathway, positive regulation of B cell receptor signalling pathway.
- Cystic fibrosis transmembrane conductance regulator - ABC transporter disorders, ABC-family proteins mediated transport, Defective CFTR causes cystic fibrosis, Disorders of trans-membrane transporters.
- Phosphodiesterase 4B - G Protein Signalling Pathways, GPCR downstream signalling, Myometrial Relaxation and Contraction Pathways.
- Cathepsin K - Toll-like receptor signalling pathway, Generic Transcription Pathway, RNA Polymerase II Transcription.
- Vitamin D receptor - Bile acid signalling pathway, vitamin D receptor signalling pathway, Nuclear Receptor transcription pathway.
- Dual specificity phosphatase Cdc25A - Deregulated CDK5 triggers multiple neurodegenerative pathways in Alzheimer's disease models.
- UDP-glucuronosyltransferase 2B7 - Artemether Metabolism Pathway, Codeine Action Pathway, Codeine and Morphine Metabolism.
- Protein kinase C alpha - MAPK1/3 (ERK1/2)-dependent signalling pathway,RAP1GAP pathway, apoptotic signalling pathway,ERBB2 signalling pathway, positive regulation of adenylate, cyclase-activating G protein-coupled receptor signalling pathway, positive regulation of lipopolysaccharide-mediated signalling pathway.

- Prostaglandin E synthase - Cyclooxygenase pathway, Acetaminophen Action Pathway, Acetylsalicylic Acid Action Pathway.
- Anandamide amidohydrolase - Arachidonic acid metabolism, Fatty acid metabolism, anandamide degradation.
- Protein kinase C alpha - MAPK1/3 (ERK1/2)-dependent signalling pathway, RAP1GAP pathway, apoptotic signalling pathway,ERBB2 signalling pathway, positive regulation of adenylate, cyclase-activating G protein-coupled receptor signalling pathway, positive regulation of lipopolysaccharide-mediated signalling pathway.
- Protein kinase C gamma - Cellular signalling pathways, Beta-catenin independent WNT signalling, G Protein Signaling Pathways, ErbB Signaling Pathway.
- Androgen Receptor - activation of prostate induction by androgen receptor signalling pathway, androgen receptor signalling pathway, intracellular receptor signalling pathway, negative regulation of extrinsic apoptotic signalling pathway, positive regulation of insulin-like growth factor receptor signalling pathway, Positive regulation of intracellular oestrogen receptor signalling pathway.
- HMG-CoA reductase - Involved in sub-pathway that synthesizes (R)-mevalonate from acetyl-CoA".
- Cytochrome P450 51 - Involved in the sub-pathway that synthesizes zymosterol from lanosterol.
- Protein-tyrosine phosphatase 1B - growth hormone receptor signalling pathway, insulin receptor signalling pathway, negative regulation of insulin receptor signalling pathway, negative regulation of vascular endothelial growth factor receptor signalling pathway, platelet-derived growth factor receptor-beta signalling pathway, regulation of hepatocyte growth factor receptor signalling pathway, regulation of type I interferon-mediated signalling pathway.
- Estrogen receptor alpha - intracellular oestrogen receptor signalling pathway, intracellular steroid hormone receptor signalling, pathway, phospholipase C-activating G protein-coupled receptor signalling pathway, regulation of intracellular oestrogen receptor signalling pathway, regulation of toll-like receptor signalling pathway, regulation of Wnt signalling pathway.
- 11-beta-hydroxy steroid dehydrogenase 1 - Glucocorticoid biosynthetic process".
- UDP-glucuronosyl transferase 2B7 - Androgen metabolic process, lipid metabolic process.

4. DISCUSSION:

The ADME properties of all the active compounds of *Cyamopsis tetragonoloba* were checked using the online tool SwissADME. "This tool gives a comparison between multiple parameters that help one judge the biochemistry and the pharmacokinetics of a molecule thus providing a basis for drug designing. "It uses the canonical SMILES of the compounds to interpret many different aspects such as hydrogen bond donors and acceptors as well as the bioavailability of a particular molecule[5]. "Another important parameter is the gastro-intestinal absorption which is important to understand the absorption effects of the drug. In addition to this, cell membrane penetration of a drug is determined by the TPSA which stands for the Topological Polar Surface Area, is also estimated by the SwissADME tool[10]. It is in consideration with the sulphur and the phosphorous atoms[11,12]. Generally, molecules with a TPSA of over 140Å are not as efficient in entering the cell whereas one with less than 70Å is required for a molecule to cross over the blood-brain barrier[12]. The Lipinski violations are one of the primary criteria to check the drug likeness of any compound if it were to be administered orally. The five rules suggest that a molecule with ideal characteristics to be used as a drug must have no more than 5 and 10 hydrogen bond donors and acceptors receptively. It must have a molecular weight of less than 500 Da and a lower than 5 octanol-water partition coefficient. Thus, it is imperative that a compound considered for drug development, should have least violations to this rule[13,14].

Thus, based on all these factors and our study of the active compounds *Cyamopsis tetragonoloba*, we understand that the compounds – Isomucronulatol, 3,5-Dihydroxy-6-methyl-2,3-dihydro-4H-pyran-4-one, 1,2-Cyclopentanedione, Isopentyl acetate as well as 2,3-Dihydro-benzofuran seem ideal candidates for further investigation. With the help of the tabulated information, we were able to compare the probability of binding of our compounds with their target proteins. After analysing carefully it was observed that the highest probability of binding is between the compound palmitic acid and its targets which was 0.935895 compared to the rest of the compounds and target proteins. The probability of binding is important so that we can specifically choose and increase the specificity of our compound to certain target molecules and eliminate the other less specific ones, this will play a major role during drug development. Certain target proteins or receptors were seen more frequently than others[15]. These include Vitamin D receptor, Androgen receptor and Protein kinase C alpha. This shows us that these targets are of much importance further into effective drug development and its specificity to the host being administered. Two pathways namely G protein signalling pathway and Cytokine mediated signalling pathway were the most common pathways that our targets were involved in. Cytokine mediated signalling pathway is involved in processes such as immunity, cell

division, cell death and tumour formation by activating genes through a transcription. Proper functioning of G Protein signalling pathway is necessary to prevent diseases like diabetes, blindness, allergies, depression, and certain cancers[16].

5. CONCLUSION:

Cyamopsis tetragonoloba is an important candidate for the study of active and inhibitory targets owing to its many ideal qualities. Based on our study, in *Cyamopsis tetragonoloba* we suggest the active compounds - Isomucronulatol, 3,5-Dihydroxy-6-methyl-2,3-dihydro-4H-pyran-4-one,1,2-Cyclopentanedione, Isopentyl acetate as well as 2,3-Dihydro-benzofuran for further investigation whereas palmitic acid and its targets could be processed in the future for their inhibitory properties.

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RESEARCH ARTICLE

Enhanced Production of an Anti-Cancer Pigment from *Bacillus endophyticus* JUPR15: Single Factor System Vs RSM

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ABSTRACT:
As a pink coloured bioactive fraction, B5, produced by *B. endophyticus* JUPR15 was showing promising anti-cancer properties, response surface methodology (RSM) was employed to enhance its yield. Various carbon, nitrogen, amino acids, vitamins and metal ions along with varying pH, temperature and incubation time were screened independently for enhancing pigment and biomass yield. Central Composite Design (CCD) of RSM was applied to evaluate the optimal conditions for the chosen factors – glycerol, mannitol and malt extract, which were studied at five levels. The R^2 value for biomass was 0.9874 and that for pigment yield was 0.9931. The observed responses were in close agreement with the predicted responses. Through CCD, a 14.31-fold (12.02 g/L) increase in biomass and 7.77-fold (202.2 mg/L) increase in pigment yields were achieved as opposed to the 0.84 g/L and 26.03 mg/L of biomass and pigment yields obtained through control conditions. The model was found to be significant ($P<0.01$) for both the responses. Besides this, the addition of mannitol alone gave 191.36 mg/L of pigment, closer to that obtained via optimized conditions. It can be concluded that both single factor and RSM can be employed to increase the anti-cancer pigment production.

KEYWORDS: *B. endophyticus*, pink pigment, anti-cancer, single factor, central composite design.

INTRODUCTION:
Microbial diversity is one of the major resources for biotechnological products as well as processes because of its important role in agricultural, industrial and medicinal applications. One of the most dominant group of micro-organisms is bacteria because of their presence in diverse ecological niches which allows them to produce compounds with wide structural variability that exhibit several biological activities such as antimicrobial, immunosuppressive, antiviral and antitumor activities, therefore enabling the bioactive compounds to become important lead molecules for drug development[1].

The compounds produced from *Bacillus* sp. are well known vitamins such as riboflavin[2], carotenoids[3], antibiotics from *B. cereus* – cerexin and zwittermicin; *B. circulans* – circulin; *B. subtilis* – polymixin, difficidin, subtilin, myobacillin; *B. pumilus* – pumulin[4]. Antimicrobial peptides have been reported to exhibit a mechanism of immune defense with low antigenicity[5].

The secondary metabolites produced by microbes may not be very important for their growth but essential for nutrition, health and economics of our societies[6,7]. Amongst the secondary metabolites, microbial pigments possess antioxidant, antimicrobial and anticancer properties[8]. One of the advantages of pigment production from microorganisms over plants is their easy cultivation that is independent of weather conditions, availability, stability, yield, labor, downstream processing and cost efficiency[9,10].

More importantly, bacterial pigments are receiving increased attention due to their biodegradability[11] and lesser toxicity[12]. The yield of pigment is affected by culture conditions such as pH and temperature along with nutrients supplied such as the carbon, nitrogen and

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inorganic salts[13]. Designing a fermentation medium is of critical importance because the medium components affect mainly the product concentration, yield and volumetric productivity[14]. Medium composition also eases the cost of downstream processing. Statistical methods such as central composite design (CCD) and response surface methodology (RSM) for microbial fermentation processes are mainly used to determine the main effects and interaction between factors that play a basic role in product output[15] because considering a one-factor approach will be disadvantageous as it is time consuming and more importantly, the possible interactions between factors will go overlooked whereas in reality, the interactions between factors may play a very important role in product development[16,17]. RSM is most commonly used to explore the nonlinear relationships between the studied factors and dependent variables. It provides information about the optimal values of factors to determine the expected (largest or smallest) values for the dependent variables of interest [18]. RSM is a very significant platform to identify the optimum conditions and thereby establish correlations for the target responses.

The main objective of this study was to optimize the culture conditions and media components so as to enhance the production of an anti-cancer pigment B5 from *B. endophyticus* JUPR15 by employing RSM factorial design. As this is the first report of an anti-cancer pigment production from *B. endophyticus*, it was found necessary to enhance its yield for future drug developmental studies.

MATERIALS AND METHODS:

Isolation and identification of the micro-organism:

The microorganism under the current study was isolated from a soil sample obtained from Belgaum district, Karnataka by serial dilution method[19]. The microorganism was identified as *Bacillus endophyticus* by 16s rRNA sequencing (Chromous Biotech Pvt. Ltd., Bangalore) and the sequence was submitted to Genbank. The accession number obtained was KX010971.

Inoculum preparation and culture conditions:

The pure culture of *B. endophyticus* JUPR15 was maintained on nutrient agar plates at 37°C for 72 h. The plates were stored at 4°C for further studies. A primary inoculum of $1x10^8$ CFU/ml in nutrient broth was used for all the optimization experiments.

Cytotoxicity of the metabolite:

The metabolites from *B. endophyticus* JUPR15 extracted by methanol demonstrated anticancer potentials on three cancer cell lines (HeLa, HepG2 and MCF-7). Promising results led to the partial purification of the crude extract by thin layer chromatography (TLC) that gave five fractions out of which the pink coloured fraction, B5 (bioactive metabolite), had maximum cytotoxic effects to the cancer cell lines which was confirmed by various *in-vitro* assays[20].

λ – max of the pigment extract:

The absorption maxima (λ – max) for the TLC purified B5 was determined by spectral scanning using a UV/Vis spectrophotometer (Shimadzu, Japan) and thereafter by plotting wavelength versus optical density. The pigment was quantified by checking its optical density at the λ – max during the optimization studies.

Extraction of metabolites and determination of dry cell mass:

The primary inoculum was spread on nutrient agar plates and the plates were incubated at 37°C for 72 h. After the incubation period, the bacterial colony was carefully scraped from the petri dish and was homogenized with 10 ml methanol using a mortar and pestle. The bacterial colony along with methanol was transferred to sterile beakers and was left undisturbed overnight at 4°C to enhance pigment extraction. The contents were centrifuged at 8000 rpm for 10 min at 4°C. The supernatant was collected and the cell pellet was subjected to a second extraction until the cell pellet was completely colourless, thereby ensuring complete extraction of the pigment. Supernatants from both the extractions were pooled and the absorbance was recorded at λ – max wavelength to determine the concentration of the metabolite. The cell pellet obtained was allowed to dry in a pre-weighed vial at 70°C overnight after which the dry weight of biomass was determined. All experiments were performed in triplicates and the results were expressed as mean ± SE.

Effect of varying culture conditions:

A single component system was employed at first to screen for highest pigment as well as biomass production. Nutrient agar medium consisting of 2.0% agar concentration was used as control for all experiments. A list of varying sources of nutrients used in the single factor system along with the basal media (nutrient agar) for dry weight of biomass (response 1) and pigment yield (response 2) are listed in Table 1. Varying culture conditions such as incubation period (24, 48 and 72 h), pH (5 to 11; control pH at 6.85) and temperature (4°C, RT at 28-30°C, 37°C, 50°C and 70°C) were also considered for biomass production and enhanced pigment yield. Determination of pigment yield and biomass were performed as previously mentioned. All experiments were conducted in triplicates.

Table 1: List of nutrients used in the single factor system

Sl. No.	Nutrient Source	Concentration	Varying factors
1	Carbon	1.0%	Glycerol, Starch, Glucose, Lactose, Maltose, Mannitol, Sucrose, Dextrose, Galactose, Fructose
2	Nitrogen	1.0%	Malt extract, Yeast extract, Beef extract, Peptone, Urea, Ammonium ortho phosphate, Ammonium sulphate, Sodium nitrate
3	Amino acids	0.5X, 1.0X, 1.5X	(MEM Amino acids solution – 100X, Himedia)
4	Vitamins	0.001 mg/ml	B1 (Thiamine hydrochloride), B2 (Riboflavin), B3 (Nicotinic acid), B5 (Pantothenic acid), B6 (Pyridoxal 5'- phosphate), B12 (Cobalamin), Folic acid
5	Metal ions	0.05%	Magnesium sulphate, Ferrous sulphate, Calcium carbonate, Manganese sulphate, Barium sulphate, Nickel chloride, Lead acetate

Optimization by response surface methodology:

Three factors affecting the pigment production as well as biomass were chosen based on the single factor system that was found to be statistically significant (GraphPad Prism 6.0). The optimal conditions for enhanced pigment and cell mass production were investigated through CCD. Statistical analysis was conducted using the Design Expert version 11 (Stat-Ease Inc., Minneapolis, Minnesota, USA). The three factors chosen were studied at five different levels (-α, -1, 0, +1 and +α). Statistical terms were well understood [21]. Total number of experiments were run based on the following equation,

$$N = 2^k + 2k + n_0$$

where, N is the total number of experimental runs, k is the number of factors used and n_0 is the number of replicates at centre points. For the ease of statistical calculations, the relation between the coded and real values were described by the following equation.

$$X_i = (A_i - A_0)/ \delta A_i$$

where, X_i represents the coded value of the variable, A_i represents the actual value of the variable, A_0 represents the actual value of A_i at the central point and δA_i is the step change of variables.

The data obtained was subjected to a second order multiple regression analysis to study the effect of the variables on the response.

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{12} AB + \beta_{13} AC + \beta_{23} BC \quad \text{- (Eq. A)}$$

where, Y is the predicted response, β_0 is the intercept co-efficient; β_1, β_2 and β_3 are the coefficients of linear terms; β_{11}, β_{22} and β_{33} are the coefficients of quadratic terms; β_{12}, β_{13} and β_{23} are the interaction coefficients; A, B and C are the variables under the study.

RESULTS:

λ – max of B5:

The spectral scanning across the UV/Vis range for the TLC purified pink coloured B5 bioactive fraction revealed the absorption maxima (λ – max) at 516 nm (Fig. 1).

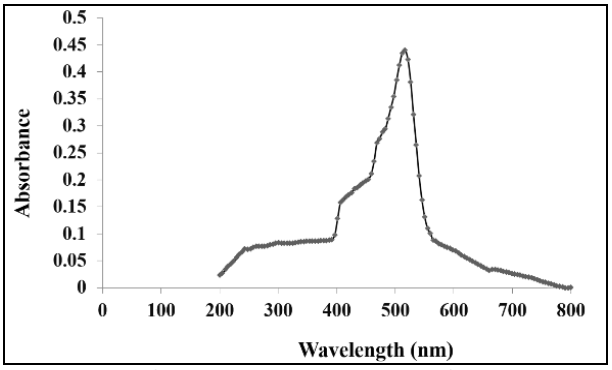

Fig. 1: λ – max of the pink coloured B5 bioactive fraction

Effect of single factor systems:

Parameters such as incubation period of 72 h with 0.975 g/L biomass and 15.22 mg/L pigment yield, pH 7.0 with 1.94 g/L biomass and 46.82 mg/L pigment yield and at 37°C temperature with 0.995 g/L biomass and 18.83 mg/L pigment yield were found to be the best (Fig. 2A-C) when observed during the single factor screening process.

The effect of the supplementation of additional factors to the nutrient media was evaluated on biomass and pigment yield. The presence of carbon in the culture medium plays a very important role in microbial growth and metabolite production. Amongst the carbon sources used (Fig. 2D) at 1.0% concentration, mannitol showed the highest pigment yield of 191.36 mg/L with 2.95 g/L of biomass. The highest biomass was observed when glycerol was an additional factor in the medium with 6.97 g/L but this diminished the pigment yield that was observed to be only 149.79 mg/L in comparison to the control where the biomass was recorded to be 0.697 g/L and the pigment yield to be only 30.37 mg/L. Glucose, maltose, sucrose and dextrose also resulted in a biomass between 2.74 – 3.17 g/L and the pigment yield between 167.22–186.94 mg/L which were statistically significant.

Amongst the varying nitrogen sources (1.0%) tested for their effect on biomass and pigment yield, the presence of malt extract at 1% concentration gave a highly significant yield of 2.469 g/L and 148.72 mg/L in terms of biomass and pigment yield respectively. In the control conditions, 0.847 g/L of biomass of and 27.26 mg/L of pigment yield were observed (Fig. 2E). The rest of the

tested nitrogen sources except yeast extract, did not result in inducing significant changes to the yields of biomass and pigment.

A concentrated mix of amino acids solution (100X) from Himedia was used to check for the effect of amino acids for both the responses at 3 different concentrations (0.5X, 1X and 1.5X). The biomass was found to be slightly higher in the bacterial culture supplemented with 0.5X of amino acids solution with 0.627 g/L of biomass as compared to that of control with 0.609 g/L. The pigment yield was lower than 32.66 mg/L as observed in control (Fig. 2F). Using amino acids as a factor was found was not effective in increasing the yield of the pigment or biomass.

Vitamins in the current study were examined for their effect on biomass production and pigment yield at 0.001 mg/ml concentration when used as a single factor in the growth medium. Vitamin B5 was found to have increased the biomass as well as the pigment yield with 0.719 g/L and 40.11 mg/L respectively, in comparison to the control. Only Vitamin B6 was found to give a slightly lower yield of pigment (31.02 mg/L) in comparison to the other vitamins used. Vitamins did influence cell growth with increase in biomass (Fig. 2G) as compared to the control but the results were found to be not significant.

The effect of metal ions at 0.05% concentration on the biomass and pigment yield when added as a single factor into the medium were checked. We found that most of the metal ions did not have much effects except for calcium carbonate which had resulted in slightly higher pigment yield of 39.53 mg/L in comparison to 32.58 mg/L of pigment in the control conditions. The weight of biomass was found to be lower in the presence of all metal ions in comparison to the control when used as a single factor, thereby inhibiting cell growth and pigment yield (Fig. 2H). Manganese sulphate and nickel chloride were found to hinder the pigment production with only 6.06 mg/L and 8.02 mg/L of pigment yield respectively concomitant with decreased biomass.

Based on the single factor system and their statistical significance, glycerol, mannitol and malt extract were chosen as the nutrients to be used in CCD and thereby to check for their combined effect on biomass and pigment yield along with the fixed parameters of pH, temperature and incubation period.

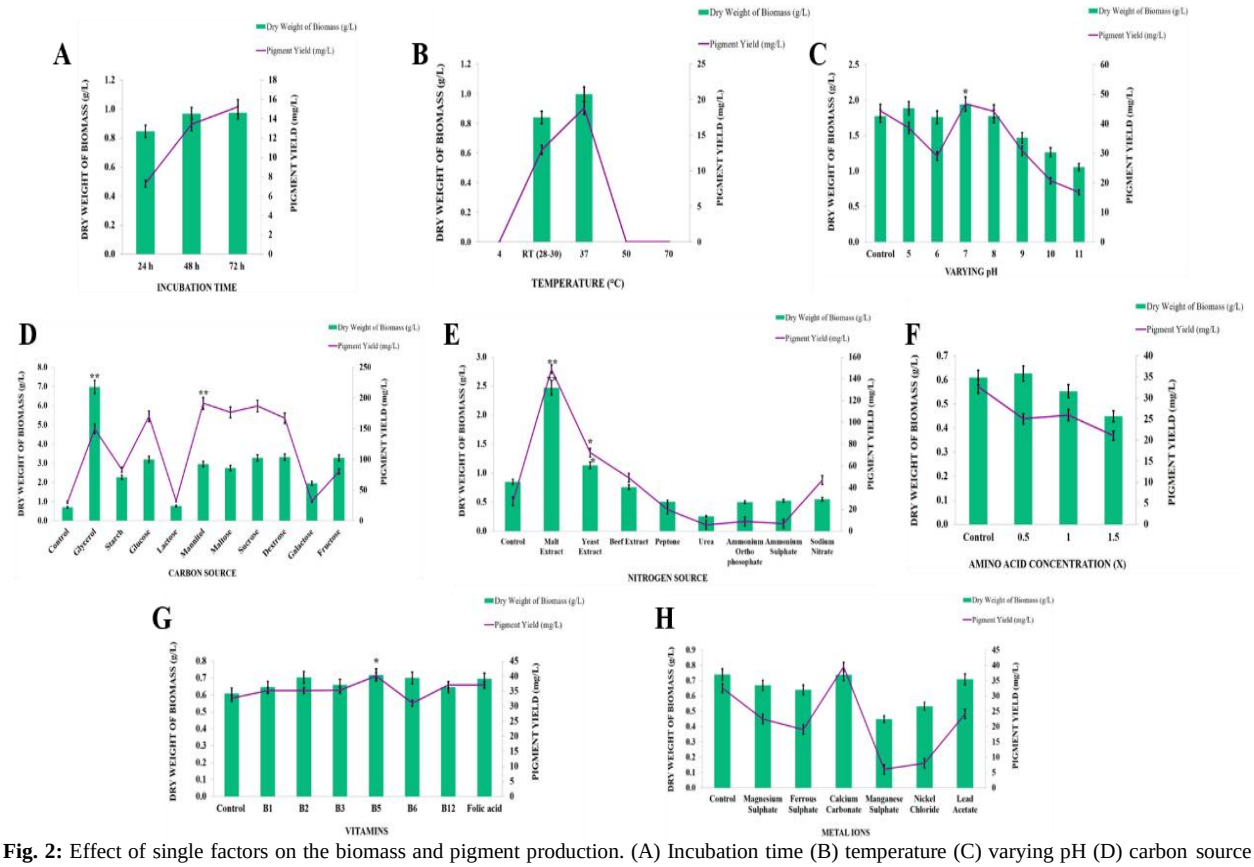

Fig. 2: Effect of single factors on the biomass and pigment production. (A) Incubation time (B) temperature (C) varying pH (D) carbon source (E) nitrogen source (F) amino acid concentration (G) vitamins and (H) Metal ions. The level of significance is indicated as $*p < 0.05$ and $**p < 0.01$ in comparison to the control

Optimization by response surface methodology:

RSM, as discussed earlier, is an efficient strategic experimental tool by which the optimal conditions of a multivariable system can be determined. Based on the results obtained from the single factor experimental system, three variables that exerted a greater response in terms of biomass and pigment yield were chosen for CCD experimentation. The factors chosen were glycerol, mannitol (carbon sources) and malt extract (nitrogen source) along with parameters like as pH (7.0), temperature (37°C) and incubation time (72 h) that promoted maximal biomass and pigment yield during the single factor screening process. Altogether, the three factors along with the culture conditions were investigated for optimal conditions for the responses under study.

In the current study, the range and levels of independent factors were varied as per Table 2, and a total of 20 experimental runs consisting of varying media components were performed. The results of the experiments for studying the effect of the three independent variables on biomass production and pigment yield are presented in Table 3 along with the predicted response. According to the actual experimentation results, the dry weight of biomass varied from 5.14 g/L to 12.017 g/L whereas the pigment yield varied from 106.6 mg/L to 202.2 mg/L. The highest biomass production was observed in run 6 and that of pigment yield in run 2. It was observed that the correlation (R=0.5031) between the two responses were moderately positive which indicates that there is a tendency for higher biomass which can result in a higher pigment yield and a higher pigment yield may be due to a higher biomass.

Table 2: Levels of independent factors used in CCD

Factors	Levels				
	-α	**-1**	**0**	**1**	**+α**
A: Glycerol (%)	0.24	0.75	1.50	2.25	2.76
B: Mannitol (g/L)	0.79	2.50	5.00	7.50	9.20
C: Malt extract (g/L)	1.54	7.00	15.00	23.00	28.45

*α = 1.68

Table 3: Impact of medium components on the dry weight of biomass and pigment yield as per CCD

Run	Factors			Response 1		Response 2	
				Dry weight of biomass (g/L)		Pigment Yield(mg/L)	
	A: Glycerol (%)	B: Mannitol (g/L)	C: Malt Extract (g/L)	Observed	Predicted	Observed	Predicted
1	0.75	2.5	23.0	8.745	8.67	110.80	111.7
2	2.25	7.5	23.0	9.576	9.62	202.20	199.4
3	1.5	5.0	15.0	8.530	8.29	134.20	133.4
4	1.5	5.0	15.0	8.247	8.29	135.80	133.4
5	1.5	5.0	15.0	8.167	8.29	129.50	133.4
6	1.5	5.0	28.45	12.017	11.81	164.00	166.3
7	1.5	5.0	1.54	7.065	7.00	127.50	125.7
8	0.75	2.5	7.0	7.025	7.17	155.30	157.7
9	1.5	9.2	15.0	7.893	7.64	180.20	181.5
10	0.75	7.5	7.0	7.790	7.75	177.80	179.6
11	2.25	2.5	23.0	9.233	9.46	151.40	149.2
12	2.25	2.5	7.0	5.179	5.07	109.90	109.7
13	2.76	5.0	15.0	6.200	6.03	131.10	134.7
14	2.25	7.5	7.0	5.144	5.41	106.60	105.3
15	1.5	5.0	15.0	8.147	8.29	132.40	133.4
16	0.75	7.5	23.0	8.769	9.07	188.50	188.3
17	0.24	5.0	15.0	7.448	7.34	168.80	165.7
18	1.5	0.79	15.0	7.049	7.02	121.70	120.9
19	1.5	5.0	15.0	8.140	8.29	137.20	133.4
20	1.5	5.0	15.0	8.473	8.29	131.50	133.4

The observed experimental results in each run for both the responses were subjected to multiple regression analysis and the two regression equations obtained in terms of coded factors are as follows:

Final equation in terms of coded factors for the estimation of dry weight of biomass:

Dry weight of biomass =
$8.29 - 0.39A + 0.18B + 1.43C - 0.06AB + 0.72AC - 0.045BC - 0.57A^2 - 0.34B^2 + 0.39C^2 -$ (Eq. B)

Final equation in terms of coded factors for the estimation of pigment yield:

Pigment =
$133.42 – 9.2A + 18.02B + 12.06C – 6.59AB + 21.36AC + 13.66BC + 5.94A^2 + 6.3B^2 + 4.46C^2 -$ (Eq. C)

The statistical significance for Eq. (B) was evaluated by F-test and analysis of variance (ANOVA) for the response surface quadratic model for dry weight of biomass is given in Table 4. An F-value of 86.98 and P<0.0001 indicates that the model terms are highly significant. The R^2 value of 0.9874 and adjusted R^2 value

of 0.976 is indicative of the model accuracy. The predicted R^2 of 0.9198 points to a good agreement between the observed and predicted values for biomass production. The predicted R^2 is also in reasonable agreement with the adjusted R^2. An adequate precision of 40.301 for the response indicates an adequate signal. Non-significant lack of fit is good which indicates that the model equation was adequate for predicting the response under the given combination of values of the three factors. The interaction between factors A and C are significant with P<0.0001 which indicates that these two factors are mainly responsible for biomass production. But when single factors are considered, malt extract, with a higher F-value of 498.96, has a greater effect on biomass production.

Table 4: Analysis of variance (ANOVA) for dry weight of biomass

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	43.74	9	4.86	86.98	< 0.0001	significant
A-Glycerol	2.05	1	2.05	36.77	0.0001	
B-Mannitol	0.4638	1	0.4638	8.3	0.0164	
C-Malt extract	27.88	1	27.88	498.96	< 0.0001	
AB	0.0288	1	0.0288	0.5156	0.4892	
AC	4.19	1	4.19	74.91	< 0.0001	
BC	0.0164	1	0.0164	0.2943	0.5994	
A^2	4.64	1	4.64	82.99	< 0.0001	
B^2	1.65	1	1.65	29.54	0.0003	
C^2	2.23	1	2.23	39.92	< 0.0001	
Residual	0.5588	10	0.0559			
Lack of Fit	0.4078	5	0.0816	2.7	0.1497	not significant
Pure Error	0.151	5	0.0302			
Cor Total	44.3	19				

$R^2 = 0.9874$, Adj. $R^2 = 0.976$, Pred. $R^2 = 0.9198$

Table 5: Analysis of variance (ANOVA) for pigment yield

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	14216.5	9	1579.61	160.82	< 0.0001	significant
A-Glycerol	1157.03	1	1157.03	117.79	< 0.0001	
B-Mannitol	4434.24	1	4434.24	451.43	< 0.0001	
C-Malt extract	1985.91	1	1985.91	202.18	< 0.0001	
AB	347.16	1	347.16	35.34	0.0001	
AC	3650.85	1	3650.85	371.68	< 0.0001	
BC	1493.31	1	1493.31	152.03	< 0.0001	
A^2	509.05	1	509.05	51.83	< 0.0001	
B^2	571.42	1	571.42	58.17	< 0.0001	
C^2	286.46	1	286.46	29.16	0.0003	
Residual	98.23	10	9.82			
Lack of Fit	57.57	5	11.51	1.42	0.356	not significant
Pure Error	40.65	5	8.13			
Cor Total	14314.8	19				

$R^2 = 0.9931$, Adj. $R^2 = 0.987$, Pred. $R^2 = 0.9653$

The statistical significance for Eq. (C) was evaluated by F-test and ANOVA for the response surface quadratic model for pigment yield is given in Table 5. A higher F-value of 160.82 and P<0.0001 indicates that the model terms are highly significant. The R^2 value of 0.9931 and adjusted R^2 value of 0.987 is indicative of the model accuracy. The predicted R^2 of 0.9653 indicates a good agreement between the observed and predicted values for pigment yield. The predicted R^2 was also found to be in reasonable agreement with the adjusted R^2. An adequate precision of 42.4917 for the response indicates an adequate signal. Non-significant lack of fit is good which indicates that the model equation was adequate for predicting the response under the given three factor combination. The interaction between all factors (A and B, A and C, B and C) are significant with P<0.0001 which indicates that all factor combinations contribute towards pigment yield. When single factors are considered, mannitol, with a higher F-value of 451.43, has a larger effect on pigment yield.

3D response surface plots were constructed for each pair of factors to illustrate the interaction between them while keeping the third factor at the middle level for biomass production (Fig. 3) and pigment yield (Fig. 4) from *Bacillus endophyticus* JUPR15. Three response surface plots for biomass production and three response surface plots for pigment yield were generated. The interaction of malt extract and glycerol was found to be significant with respect to both the responses where lower glycerol concentration and a higher malt extract led to an increase in biomass production (Fig. 3A) and an increase in pigment yield (Fig. 4A). Interactive effects between mannitol and malt extract were found to be insignificant in case of biomass production where only malt extract had played a role in increasing the biomass (Fig. 3B).

Figure 4B represents the significant interaction between malt extract and mannitol in improving the pigment yield. In case of glycerol and mannitol interaction, a plateau surface was obtained (Fig. 3C) which explains the non-significant interaction of factors with respect to biomass production. Over a wide experimental range of interaction between mannitol and glycerol, increase in concentrations of both factors yielded higher pigment (Fig. 4C) and hence was statistically significant. A moderately positive correlation was observed during the pairing of these two factors and thereby with respect to their responses.

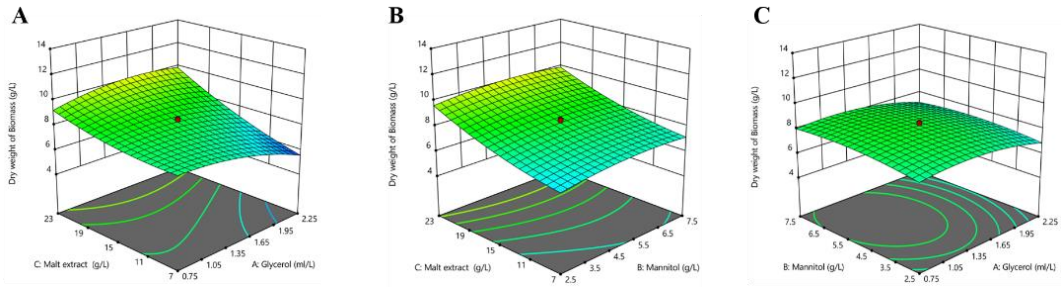

Fig. 3: Response surface plots representing the interaction between (A) malt extract and glycerol (B) malt extract and mannitol (C) mannitol and glycerol for the estimation of dry weight of biomass (response 1). The third factor was kept constant at the middle level

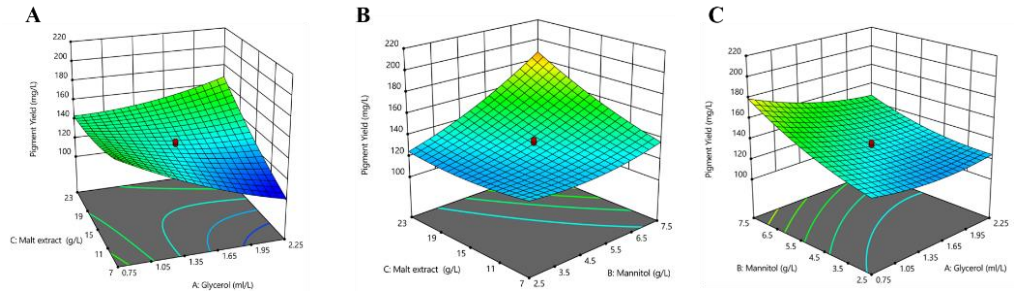

Fig. 4: Response surface plots representing the interaction between (A) malt extract and glycerol (B) malt extract and mannitol (C) mannitol and glycerol for the pigment yield (response 2). The third factor was kept constant at the middle level

Validation of optimized conditions:

From the CCD matrix, the optimum values of factors for increased biomass production were 1.5% of glycerol, 5.0 g/L of mannitol and 28.45 g/L of malt extract. The optimum values of factors for increased pigment yield were 2.25% of glycerol, 7.5 g/L of mannitol and 23.0 g/L of malt extract. The validation experiments were carried out in triplicates. The observed and predicted values closely match which indicates that the generated model adequately predicts the response. When the basal media was set up parallel to the validation experiment, the observed dry weight of biomass was 0.84 g/L and that of pigment yield was 26.03 mg/L.

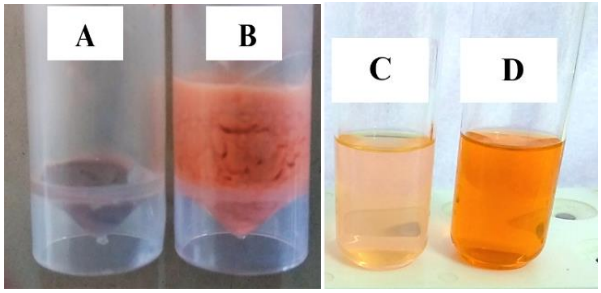

Fig. 5: Pictorial representation of (A) biomass produced under control conditions (B) biomass produced under optimized conditions (C) pigment yield under control conditions and (D) pigment yield under optimized conditions

With respect to basal media conditions, a 14.31-fold increase in biomass production and 7.77-fold increase in pigment yield was observed as per optimum values obtained through CCD (Fig. 5). Therefore, the optimized values could be considered suitable for increased dry weight of biomass and pigment yield.

DISCUSSION:

A number of pigments of industrial relevance are available in sufficient quantities but mostly, they are of plant origin. It is advantageous to obtain pigments from microbial sources as they are more stable and soluble than those obtained from plant or animal sources. Their rapid growth, high productivity throughout the year adds on to their benefits. Many natural pigments from bacteria have important pharmacological properties[11,22].

There are many reports of anticancer properties of microbial pigments such as prodigiosin, violacein, astaxanthin, pyocyanin, β-carotene among many others [23,24]. Pigments produced by *Bacillus* sp. belong to the carotenoid group with the bacterium producing predominantly three colour pigments – yellow, orange and pink. In the current study, the bacterium was isolated from soil and was identified as *B. endophyticus* which produced a light pink coloured pigment only in the

presence of agar. The promising anti-cancer potential of the pigment prompted us to continue our research towards enhancing the yield through statistical method i.e. RSM.

The increase in pigment yield under specific experimental conditions was monitored by recording the absorbance at its λ-max and thereby reporting its concentration. The pink pigment reported by us in this study could possibly belong to the carotenoid group as the λ-max of the pink coloured pigment is 516 nm which is slightly higher than previously reported value[25] of 492 nm for carotenoids. Carotenoids have been reported to have an absorption range between 460-550 nm[26,27]. We initially checked for the two responses under study (biomass production and pigment yield) by the single factor screening approach where only one factor at a time along with the basal nutrient media was supplied for the bacterium as growth conditions. The best growth for the bacterium was observed at 37°C at an incubation time of 72 h which maximized the biomass production as well as pigment yield. The pH of the culture medium affects the functioning of the cell membrane, cell structure, uptake of various nutritional sources and also the biosynthesis of metabolites[28,29], and in our study, neutral pH (pH 7.0) exhibited promising responses. We observed that the bacterium produces pigment only in the presence of agar unlike another strain of *B. endophyticus* (AVP-9(Kf527823) which produced an orange fluorescent pigment in broth conditions[30], with the highest pigment production observed in the presence of glucose and peptone, both at 1.0% concentration when used as a single additional factor in the basal medium. The pigment production is affected by the presence of agar which might be due to the presence of certain compounds in agar that promotes microbial growth and enhance metabolic activities[31]. Hence, we kept 2.0% agar, 37°C temperature, pH of 7.0 along with 72 h incubation period as constants during the statistical optimization studies.

Effect of carbon and nitrogen sources as a single additional factor in the growth medium largely influences pigment production in different microbial strains. Most of the carbon sources under the study except for lactose and galactose yielded promising results with respect to the desired response and in the case of nitrogen sources, only malt extract and yeast extract gave significantly enhanced responses. Amino acids are essential for bacterial growth but previous reports suggest that there are several amino acids that inhibit microbial growth wherein when they are present in a mixture, there are some inhibitory members that diminish the benefits provided other members[32,33]. In the current study, a mixture of amino acids at varying concentrations were used which resulted in the inhibition of growth as well as pigment yield. Vitamins play a very important role[34] in bacterial metabolism as they act as coenzymes or precursors of coenzymes which make them a crucial element for growth and enzyme activities at very less concentrations. Just as important vitamins are for bacterial metabolism, certain metal ions serve as excellent sources of minerals for their well-known roles in bacterial metabolism and as a co-factor for metabolic enzymes[35]. But both the vitamins and metal ion sources used in study have not given statistically significant results with only a marginal increase in both biomass and pigment yield.

Therefore, three factors namely glycerol, mannitol and malt extract were selected for RSM based on their response and statistical significance. In the CCD, the interaction between these factors were investigated. With respect to the 3D surface models generated and through statistical analysis, it was found that the factor that had a greater influence on biomass production (malt extract) was different from the factor that exerted a greater effect on the pigment yield (mannitol) with statistically significant effects. One of the reasons for this discrepancy could be the metabolism of the micro-organisms being very complicated and they respond in a complex manner to different environmental conditions. It is reasonable that the conditions that favour the best growth are different from those that favour pigment production, because the microorganism has to first compete for the available nutrients within the culture media and second, the available electron donors within the cell[29].

CONCLUSION:

The pink pigment produced by *B. endophyticus* JUPR15 has proven to have anti-cancer properties and the main objective of this study was to optimize the culture conditions to maximize the biomass production as well as pigment yield. Single factor screening was initially performed and the best factors were chosen for RSM. The optimal conditions obtained for the two responses under study were validated and the model was found to be statistically significant. The optimized condition obtained as per CCD matrix is more suitable rather than single factor approach to enhance biomass with 14.31-fold increased production and pigment with 7.77-fold increase. But in the case of pigment production, as mannitol addition also resulted in a yield of 191.36 mg/L, which is close to the value we got by RSM approach, it can be assumed that even single factor approach can be adopted to get the desired effect.

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CONFLICT OF INTEREST:

The authors declare that they have no conflict of interest.

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RESEARCH ARTICLE

Comparative study of several Analytical methods for determination of Manganese content in some dietary supplements in Syrian market

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ABSTRACT:

In this study, three different analytical methods for manganese determination (volumetric, spectrophotometric, FAAS) were compared regarding sensitivity, accuracy, specificity and cost. The spectrophotometric method was chosen to analyze pharmaceutical formulations which are available in Syrian markets as Dietary supplements containing many minerals including manganese. Manganese was determined first quantitatively by complexometric direct titration with EDTA at pH 10 using Eriochrome black T as an indicator, the linearity range was 16-26µg/ml (R^2=0.9963). The precision as RSD was <2%, and accuracy was determined as recovery which ranged between 100.18 – 105.31%. The spectrophotometric method depends on manganese oxidation to permanganate. The absorbance of the resulting permanganate solution is measured by visible-spectrophotometer at the maximum absorption wavelength (λ max) at 525nm. Beer's law is obeyed over the concentration ranges of 5-30µg/ml (R^2=0.9971). The precision as RSD was <2%, and accuracy was determined as recovery which ranged between 93.94 – 103.13%. Previous methods were compared to one of the most sensitive but costly processes to measure manganese by Atomic Absorption Spectroscopy (AAS) using a manganese bulb with the maximum absorption wavelength (λ max) at 279.5nm. Beer's law is obeyed over the concentration ranges of (0.5-3µg/ml) and the correlation coefficient value (R2 = 0.9993). The precision as RSD was <2% and accuracy was determined as recovery which ranged between 97.24 and 100.04%. Manganese content in a number of dietary supplements samples was estimated, and the results did not coincide with the addressed limits on the labelled cards and the authorized global values.

KEYWORDS: Manganese, Dietary supplements, EDTA, permanganate, Spectrophotometer, Atomic Absorption Spectroscopy (AAS).

INTRODUCTION:

The use of Dietary supplements in general and multivitamins-multiminerals in particular is prevalent and growing randomly in Syria and wild world in the last few years. Although multivitamins, multiminerals (MVM) and similar terms (eg, multis or multiples) are commonly used, they have no standard scientific, regulatory, or marketplace definitions. MVM composition databases use label values as surrogates for analyzed values. However, actual vitamin and mineral amounts often deviate from label values[1].

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FDA reports defines Dietary supplements as orally taken products that contain a "dietary ingredient including vitamins, minerals, amino acids, and herbs or botanicals, as well as other substances that can be used to supplement the diet[2]., meanwhile the Agency for Healthcare Research and Quality in USA defined multivitamin/mineral supplements as any supplements containing 3 or more vitamins and/or minerals without herbs, hormones, or drugs, each at a dose less than the UL determined by the Food and Nutrition Board[3].

Manganese is a nutritionally essential mineral but it is potentially toxic[4]. It is involved in many physiological processes in the body. It is located largely in the mitochondria[5] and plays an important role in a number of physiologic processes as a constituent of multiple

enzymes and an activator of other enzymes[6]. Also, it is involved in neurological function[7,8]. and essentially required for normal thyroid function and thyroxin formation[9].

Because of the rising consumption of this kind of products in Syria and the high cost of most modern devices that provide the selectivity required for the element to be analyzed in this type of preparations. The objective of this study was to compare between three different analytical methods for manganese determination (spectrophotometric, volumetric, AAS) to study the possibility of analyzing it in alternative methods, As well as comparing the analysis results of several samples with the labelled values, especially that Dietary supplements do not undergo drug control in many countries around the world.

MATERIALS AND METHODS:

INSTRUMENTATION:

UV/VIS Spectrophotometer (single beam – Scinco S-3100)
Atomic Absorption Spectroscopy (AAS) (ZEE nit7 700P)
Manganese hollow cathode lamp
Magnetic stirrer
A SHIMADZU analytical balance with sensitivity 0.01 mg.

Chemicals and Reagents:

Manganese ion Standard Solution 1000ppm (Merck), Distilled water, Eriochrome Black (HIMEDIA), Ascorbic Acid (Shenzhen China CO- LTD), Disodium Ethylene Diamine Tetra Acetic Acid (EDTA-Na) (Sigma Aldrich), Deionized water, Phosphoric Acid 85% (Merck), Potassium Periodate (M&B Laboratory Chemicals), Nitric Acid 65% (Panereac), Ammonia (Panereac), Ammonium chloride (Merck), Dietary supplements samples from Syrian markets for three local companies A, B, C

Company A: Each tablet contains: vitamin D3 200 IU, Calcium (as carbonate) 600mg, magnesium (as oxide) 40mg, copper (as sulfate) 1mg, zinc (as oxide) 7.5mg, manganese (as sulfate) 1.8mg, Lot: 2620, 2627

Company B: Each tablet contains: vitamin D3 200 IU, Calcium (as carbonate) 600mg, magnesium (as oxide) 40mg, copper (as sulfate) 1mg, zinc (as oxide) 7.5mg, manganese (as sulfate) 1.8mg, Boron (as sodium borate) 250mcg, Lot: 6089 ،6088 ،6087

Company C: Each tablet contains: vitamin D3 200 IU, Calcium (as carbonate) 600mg, magnesium (as oxide) 40mg, copper (as sulfate) 1mg, zinc (as oxide) 7.5mg, manganese (as sulfate) 1.8mg, Boron (as sodium borate) 250mcg, Lot: 867 ،806 ،869

- **Preparation of chemicals for Volumetric method:[10]**

Preparation of Buffer Solution (Ammonia-Ammonium Chloride) pH=10:

67.5g of Ammonium Chloride were dissolved in 57ml of Ammonia in a 1000ml volumetric flask and the volume was diluted with distilled water.

Preparation of 0.01M EDTA:

3.72g of EDTA were dissolved in 900ml of distilled water in 1000ml volumetric flask and the volume was diluted with distilled water.

Preparation of 0.05M Zinc Ion Solution for EDTA Standardization:

0.68g of zinc chloride were dissolved with distilled water in 100ml volumetric flask.

Standardization of an EDTA Solution with Zinc Ion Solution:

5ml of zinc ion solution were put in a beaker and 1ml of buffer solution (ammonia-ammonium chloride) pH=10 was added with few drops of Eriochrome Black T, the mix was titrated with pre-prepared EDTA

Preparation of Eriochrome Black T:

0.5g of Eriochrome Black and 4.5g hydroxylamine hydrochloride were dissolved in 90ml ethanol 95% in 100ml volumetric flask and the volume was diluted with distilled water.

- **Procedure for volumetric method:**

A suitable volume (1.6, 1.8, 2, 2.2, 2.4, 2.6ml) of manganese ion standard solution (1000ppm) were transferred in a series of 100ml volumetric flask and diluted with distilled water to the volume.

Each flask content was titrated with 0.1M Na_2EDTA after adding 1mg Ascorbic Acid and 10ml of Buffer Solution (Ammonia- Ammonium Chloride) with few drops of Eriochrome Black T as an indicator.

- **Preparation of chemicals for spectrophotometric method:[11,12]**

Preparation of phosphoric acid 25% :

30ml of 85% commercial phosphoric acid were transferred in a 100ml volumetric flask and diluted with distilled water to the volume.

Preparation of 500ppm manganese-working solution:

25ml of manganese standard solution were transferred in a 50ml volumetric flask and diluted with distilled water to the volume.

- **Procedure for spectrophotometric method:**

A suitable volumes (1, 2, 3, 4, 5, 6ml) of manganese -working solution (500ppm) were transferred in a series

of 100ml volumetric flask, 10 ml of distilled water and 10ml of phosphoric acid 25% were added with 20cg of potassium periodate.

The flasks content were boiled for 2 minutes and were cooled to room temperature, then each flask was diluted with distilled water to the volume.

The absorbance of resulting colored solutions was measured by visible-spectrophotometer at the maximum absorption wavelength λ max = 525nm.

- **Preparation of chemicals for FAAS method:**[13]

Preparation of nitric acid / water solution 1%:
1.5ml of nitric acid were transferred in a 100ml volumetric flask and diluted with de-electrolyte water (drip distillation) to the volume.

Preparation of 10 ppm Manganese working solution:
0.5ml of manganese standard solution were transferred in a 50ml volumetric flask and the volume was supplemented with nitric acid / water solution 1%.

- **Procedure for FAAS method:**

A suitable volume (2.5, 5, 7.5, 10, 12.5, 15ml) of manganese -working solution (10ppm) were transferred in a series of 50ml volumetric flask and diluted with distilled water to the volume. Then they were measured by Atomic Absorption Spectroscopy (AAS) using a manganese bulb according to the conditions in table 1.

Table 1: work conditions on FAAS

279.5	Main line
C_2H_2/Air	Flame
0.2 nm	Slit Width
Current HCL	7 Ma
C/O Stoichiometric	0.1555
Fuel flow	60 NL/h
Usable burner height	5-9 mm

- **Procedure for commercial tablets:**

Ten tablets of labeled claim 2mg of manganese were weighed precisely. An average weight of each tablet was determined. An accurately weighed quantity of powder equivalent to 2mg of manganese was dissolved with 10ml distilled water and 10ml phosphoric acid 25%. The samples were mixed well with Ultrasonic then filtered and 20cg of potassium periodate were added to be boiled for 2 minutes. The samples were cooled and diluted with distilled water to the volume.

The absorbance was measured with visible spectrophotometer λ max = 525nm and manganese concentration was calculated using calibration curve.

RESULTS AND DISCUSSION:

Absorption spectra for Permnganate in Spectrophotometer:
Permnganate (which resulted from manganese oxidation with potassium periodate and phosphoric acid 25%) showed maximum absorption at 525nm.

Validation of the methods[14]:
Linearity:
In the volumetric method, the correlation coefficient (R^2) was 0.9963 over the concentration ranges of 16-26µg/ml and in spectrophotometric method Beer's law was obeyed over the concentration ranges of 5-30µg/ml (R2=0.9971) while in AAS, linearity was shown between 0.5-3µg/ml (R^2=0.9993)

The three analytical methods indicated good linearity (Figures 2,3,4). The limit of detection (LOD) and limit of Quantification (LOQ) were calculated using the following equations:

$LOD = 3.3\ \sigma / S$, $LOQ = 10\ \sigma / S$

Where σ is the standard deviation of intercept. S is the slope of calibration curve. The results are summarized in table1.

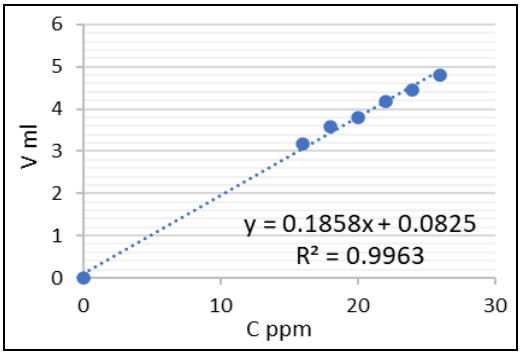

Figure1: Calibration graph for Manganese determination in volumetric method

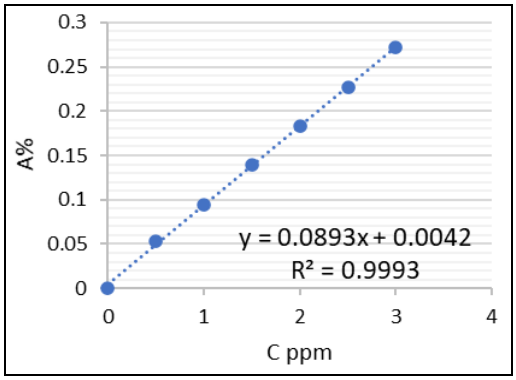

Figure 3: Calibration graph for Manganese determination in AAS

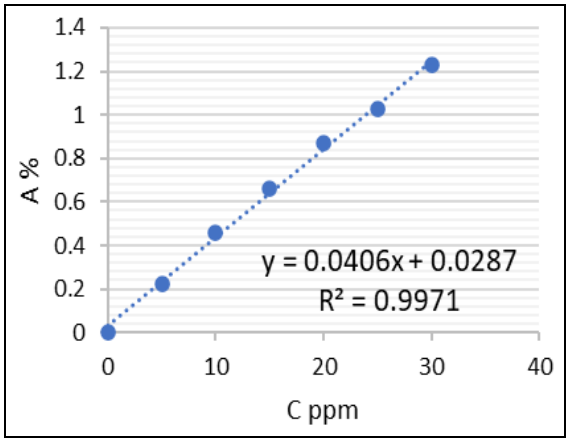

Figure 2: Calibration graph for Manganese determination in Spectrophotometric method

Table 2: Quantitative parameters of the three analytical methods for Manganese determination

Parameter	Volumetric method	Spectro photometric method	AAS
λ_{max} (nm)	-	525	279.5
Linearity limits (µg/mL)	16-26	5-30	0.5-3
Regression equation (y=b+ac)*	y = 0.1858x + 0.0825	y = 0.0406x + 0.0287	y = 0.0893x + 0.0042
Correlation coefficient (R^2)	0.9963	0.9971	0.9993
Slope, *a*	0.1858	0.0406	0.0893
Intercept, *b*	0.0825	0.0287	0.0042
Standard deviation of intercept	0.034646	0.011569	0.00173
LOD (µg/ml)	0.61534	0.940303	0.063939
LOQ (µg/ml)	1.8643	2.849403	0.193754

*$Y = aX + b$, where X is the concentration of Manganese µg ml^{-1}

Table 3: Accuracy Comparison for the three analytical methods for Manganese determination

Analytical Method	Concentration	Recovery %*	Average Recovery %
Volumetric method	16	105.3168	103.2736
	18	104.3205	
	20	100.1835	
Spectrophotometric method	12	103.267	103.7178
	15	104.2852	
	18	103.6012	
Atomic Absorption Spectroscopy	1.6	99	99
	2	100.04	
	2.4	97.24	

*n=3

Accuracy and Precision:

In the three analytical methods, Accuracy was determined for three concentration levels, three replicates and the results were recorded as percent recovery.

Precision was estimated at one concentration level, 6 replicates and the results were recorded as relative standard deviation. The calculated relative standard deviations were below 2% indicating good precision of the results are summarized in table 3, 4.

Table 4: Precision comparison for the three analytical methods for Manganese determination

Analytical Method	Concentration	Recovery %	RSD
Volumetric method	24	97.8628	0.4621
Spectrophotometric method	10	106.45	0.0026
Atomic Absorption Spectroscopy	1	100.61	0.0034

*RSD=Relative standard deviation

Specificity and effect of excipients:

The specificity of the methods was investigated by observing any interferences encountered from the common tablet excipients, such as Talc, sodium loryl sulfate, methyl cellulose, starch, magnesium stearate. These excipients did not interfere with the compared methods.

Cations presence effect on the spectrophotometric method:

To make sure there is no overlap and to avoid any interference with other associated cations in the pharmaceutical formulations, the spectrophotometric method was applied on standard concentrations of manganese-associated minerals within the pharmaceutical forms (zinc, magnesium, calcium and copper) (each cation standard separately) and mixtures from (cation standard -manganese standard) and no color was shown. The change of manganese concentration was negligible indicating the selectivity of the analytical method.

Assay of tablets:

Samples were analyzed by visible spectrophotometer, the manganese content of company A, B, C tablets was 95.61%, 53.62%, 2.012 % respectively of the label claim, and there were no significant differences when the previous samples were analyzed by the atomic absorption spectroscopy. The results are summarized in Table (5).

Table 5: Analyzed samples results with two methods (Colorimetric, FAAS)

Company	Labeled amount (mg/tab)	Amount obtained by FAAS method	Percentage recovery*	Amount obtained by spectrophotometric method	Percentage recovery*
A	1.8	1.74	97.14	1.72	95.61
B		0.97	54.06	0.96	53.62
C		0.036	2.04	0.036	2.012

n=24 (8 for each company)

CONCLUSION:

The validity of the three analytical methods was verified for manganese determination. However, the spectrophotometric method was favorable to estimate manganese in dietary supplements tablets because of the wide range of cation-manganese interactions in the complexometric titration which require multi-step titration using masking agents to mask other cations that could interact with manganese determination. The determination of manganese in commercial tablets was performed by spectrophotometer comparing with atomic absorption spectroscopy. The results showed no significant differences between the two methods even in the presence of other cations in commercial formulations, and therefore the atomic absorption spectroscopy method could be replaced by the spectrophotometric method using visible spectrophotometer, which has wide availability in quality control laboratories and could be a valid method for routine manganese determination.

Stricter quality control is required over Dietary supplements in the Syrian market to ensure their compliance with label claims.

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RESEARCH ARTICLE

Formulation and Evaluation of Herbal Cream for Treating Psoriasis

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ABSTRACT:
Psoriasis is the most common chronic autoimmune disease. The objective of the present study was to formulate herbal cream that consists of *Azadirachta indica* (Neem) extract used in treating Psoriasis. Various phytochemical identification tests were carried out using reagents. DPPH (2,2- diphenyl-1-picrylhydrazyl) free radical scavenging assay was carried out to confirm antioxidant activity. Coconut oil, Olive oil and Vitamin E oil were used in the formulation which provides different pharmaceutical activities. Phytoconstituents present in the herbal extract were identified by Liquid Chromatography-Mass Spectroscopy (LC-MS) studies. Evaluation studies were carried out for prepared cream. LC-MS studies concluded that various herbal constituents were identified and it concludes that active constituents responsible for treating psoriasis were present in the obtained extract and also possessed antioxidant activity.

KEYWORDS: *Azadirachta indica*; Psoriasis; LC-MS; Phytochemicals; Antioxidant activity.

INTRODUCTION:
Psoriasis is a skin disease which is distinguished by massive proliferation, thick inflammatory cell infiltrates, generation of new blood vessels, modifications in lymphatic structure and impaired differentiation of epidermis. It is an autoimmune disorder where environment and genetic components have a major function. The immune system releases proinflammatory cytokines and growth factors that accelerate the growth of skin cells which accumulate and form thick red patches of skin on various parts of the body[1,2].

Azadirachta indica (Neem) belongs to the family Meliaceae. Different parts of this tree have various uses and medicinal properties.

Neem leaves are useful in treating chickenpox, increase immunity of the body, reduce fever caused by malaria, treating various foot fungi, useful against termites, used in curing neuromuscular pains and in treatment of skin diseases like leprosy, psoriasis etc. Neem seed cake is used as a natural fertilizer and insecticide. Neem bark and roots helps in controlling fleas and ticks on pets, fights against skin infections such as acne, psoriasis, scabies, eczema, etc, treats diabetes, AIDS, cancer, heart disease, herpes, allergies, ulcers, hepatitis and several other diseases[3].

Pharmacological actions of Neem are Abortifacient, analgesic, anthelminthic, antibacterial, antiyeast, antiulcer, antifilarial, antifungal, antihyperglycemic, anti-inflammatory, antiviral, antimalarial, diuretic, antipyretic, antispasmodic, insecticidal, antispermatogenic, antitumor, hypercholesteremic, hypoglycaemic, immunomodulator.

Chemical composition of neem leaves:
The most important bioactive principal are Nimbin, Nimbidin and Azadirachtin. Other compounds present are Nimbidol, Sodium nimbinate, Quercetin, Gedunin, Salannin. In present study methanolic extract of neem leaves is used[4].

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MATERIALS AND METHODS:

Azadirachta indica leaves were obtained from Govinda Raj Shetty stores, Mysuru, India. All other reagents used were of analytical grade.

Preparation of extracts:

Total Methanolic Neem Extract (MeNE) was prepared by maceration technique. For this purpose, dry leaves (500gm) of Neem were extracted with 1.5 liter methanol and then evaporated by rotary evaporator. The total methanol extract was preserved at 4°C until being analyzed[5].

- **Qualitative analysis of phytochemicals:**

Herbal extract was subjected to preliminary phytochemical screening. Presence of alkaloids (Mayer's test), flavonoids (alkaline reagent test), tannins (Braymer's test) carbohydrates (Molischs test), glycosides (Liebermann's test), saponins (Salkowski test), triterpenoids (Liebermann Burchard test), proteins and amino acids (Ninhydrin test) were tested[5-7].

- **LC-MS studies:**

The herbal extract was analyzed by LC-MS method in order to identify different constituents present in them[8]. Specifications of Instrument used in the study was as below:

Specifications:

LC column: ACQUITY UPLC BEH C18 1.7µm, Solvent selection A: 0.1% Formic acid in water, B: Acetonitrile, Mobile Phase: Water: Methanol, Ionization Mode: ES+, Mode: Positive, Injection Volume: 2 microlitre, Column Dimension: 25cm×2.5mm, Mass range: 50-1500, Software: 1.40.2532.

- **DPPH free radical scavenging assay:[9]**

The free radical scavenging activity (antioxidant capacity) of extracts on stable radical 1, 1-diphenyl -2-picrylhydrazyl (DPPH) was estimated. Briefly, 2ml of extract at varying concentrations (50µg/ml to 250µg/ml) was mixed with 2.0ml of DPPH solution in methanol (0.004% w/v). The mixture was allowed to stand at room temperature in dark for 20 min. Then the mixture was vortexed and absorbance was recorded at 517 for neem using spectrophotometer. Ascorbic acid was used as a reference standard and control consisted of DPPH solution without extract. The test was performed in triplicate and percentage scavenging of DPPH free radical by extract was calculated using the equation:

$$(A_{control} - A_{test})/A_{control} \times 100$$

where $A_{control}$ is the absorbance of control and A_{test} is the absorbance in presence of extract or standard.

Preparation of Herbal Cream:[10]

Oil in water (O/W) emulsion-based cream (semisolid formulation) was formulated. The emulsifier (stearic acid) and other oil soluble components (cocoa butter, cetyl alcohol, coconut oil, olive oil, Vit E oil) were dissolved in the oil phase (Part A) and heated to 75°C. The preservatives and other water-soluble components (methyl paraban, triethanolamine, propylene glycol and ethanol extract of neem) were dissolved in the aqueous phase (Part B) and heated to 75°C. After heating, the aqueous phase was added in portions to the oil phase with continuous stirring and perfume was added. The formula for the cream is given in Table 1.

Table 1: Composition of Herbal cream preparation

Ingredients	Quantity
Neem extract (mg)	500
Cocoa butter (g)	3
Stearic acid (%)	3
Cetyl alcohol (%)	2
Coconut oil (ml)	2
Olive oil (ml)	1
Vit E oil (ml)	1
Methyl paraben (%)	1
TEA (ml)	0.5

Evaluation of creams:[11]

- **Type of emulsion under dye test:**

The scarlet red dye is mixed with the cream. A drop of the cream was placed on a microscopic slide, then it was covered with a cover slip and examined under a microscope. If the disperse globules appear red and the ground is colorless, the cream is O/W type. The reverse condition occurs in W/O type cream i.e. the disperse globules appear colorless in the red ground.

- **Appearance:**

The appearance of the cream was judged by its color, pearlscence and roughness and graded.

- **Homogeneity:**

The formulations were tested for the homogeneity by visual appearance and by touch.

- **pH of the Cream:**

The pH meter was calibrated using standard buffer solution. About 0.5 g of the cream was weighed and dissolved in 50.0 ml of distilled water and its pH was measured.

- **Viscosity:**

Viscosity of the formulation was determined by Brookfield Viscometer at 100rpm, using spindle no 7.

RESULTS AND DISCUSSION:

Qualitative analysis of phytochemicals:

Different phytochemicals present in both the extracts were identified using methods mentioned in Table 2. Results obtained were also given in Table 2: The results state that prepared herbal extract contained alkaloids, flavonoids, tannins, carbohydrates, glycosides, saponins, triterpenoids.

Table 2: Phytochemicals present in Methanolic extracts of Azadirachta

S No	Constituents	Test	Obesrvation	Results
1	Alkaloids	Mayer's test	Formation of creamy precipitate.	Positive
2	Flavanoids	Lead acetate test	Formation of yellow precipitate	Positive
3	Carbohydrates	Molish's test	Formation of violet ring at the junction.	Positive
4	Triterpenoids and steroids	Salwonski test	If lower layer turns red indicates presence of steroids.Golden yellow layer at bottom indicates presence of triterpenoids	Positive
5	Deoxy sugars	Killer kiliani's test	Formation of blue color in the acetic acid layer	Positive
6	Glycosides	Legal's test	Formation of pink to blood red color	Positive
7	Reducing sugars	Benedict's test	Solution appears green or yellow or red depending on the amount of reducing sugar present in the test solution	Positive
8	Amino acids	Ninhydrin's test	Formation of blue color	Negative

LC-MS studies:

Base peak Ionization (BPI) Chromatogram of methanolic Neem extract was obtained as shown in Fig. 1 which states that retention time of constituent Nimbin (Molecular weight-540.601g/mol) present in the methanolic extract was around 1.62. Fig.2 shows the Mass spectrum related to this RT. Similarly RT of another constituent Azadirachtin (Molecular weight-720.721g/mol) was around 3.16. Its Mass spectrum is shown in Fig. 3.

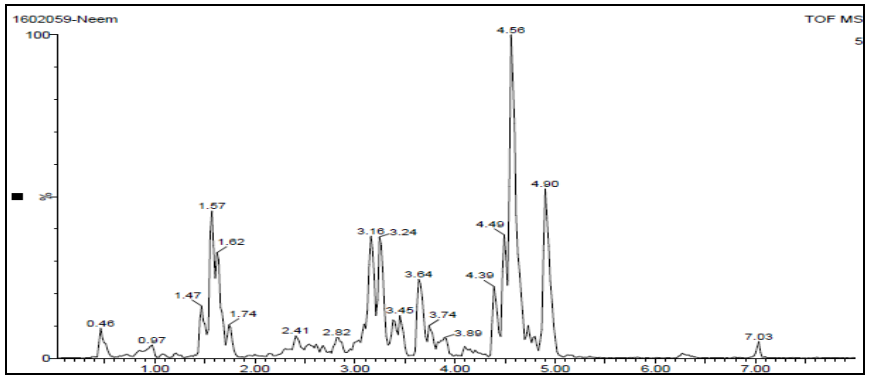

Figure 1: Base Peak Ionization (BPI) Chromatogram of Neem

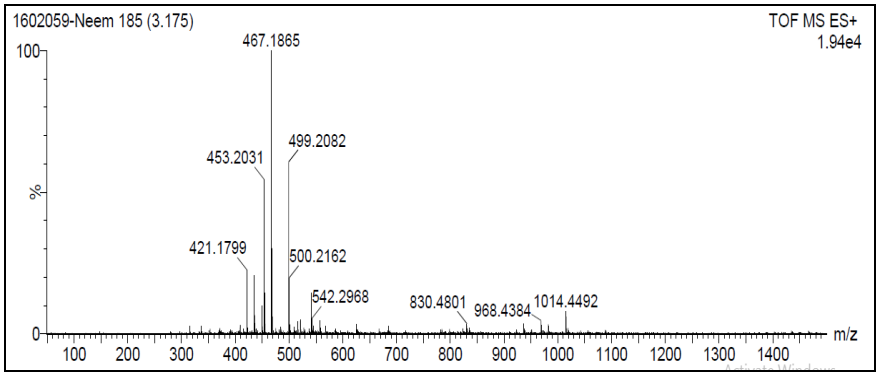

Figure 2: Mass spectrum of Nimbin

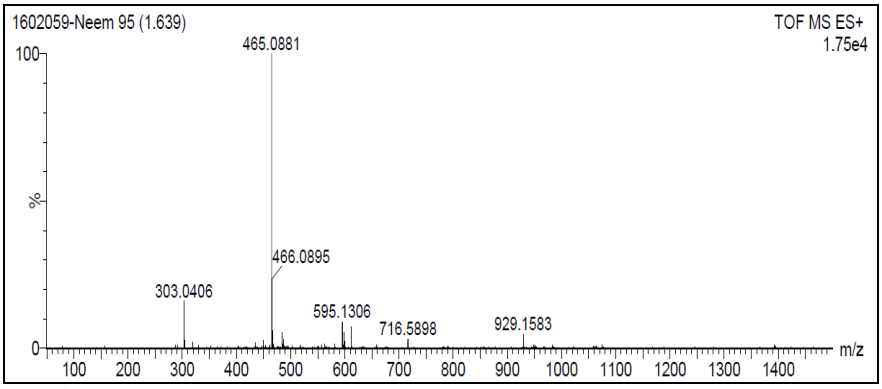

Figure 3: Mass spectrum of Azadirachtin

DPPH free radical scavenging assay:

The antioxidant activity is mainly depends on phenolic compounds, alkaloids, terpenoids and their derivatives. All these compounds and their derivative were present in the herbal extracts. These compounds produce the free radical and then react with DPPH and gradually changed its colour. In this present study, all these herbal extracts were able to decolorize with DPPH. The DPPH scavenging (%) of Methanolic neem extract was 12.04±1.62%.

Evaluation of creams:

- **Dye test:**

It confirmed that formulation prepared was o/w type emulsion cream

- **Appearance:**

There is no change in colour of cream

- **Homogeneity:**

By visual appearance and by touch, it is confirmed that the formulation is homogenous

- **pH of the Cream:**

The formulation had shown pH nearer to skin,i.e., 6.4

- **Viscosity:**

The viscosity of cream was 649 cps which indicates that the cream is easily spreadable by small amounts of shear.

Table 3: Evaluation parameters of formulated cream

Parameters	Results
Dye test	o/w type emulsion
Appearance	No change
Homogeneity	Homogenous
pH	6.4
Viscosity (cps)	649

CONCLUSION:

From the results obtained it was confirmed that herbal extract of *Azadirachta indica* prepared, contains various phytoconstituents that provide several medicinal properties. The DPPH free radical scavenging assay performed during the current study confirm the antioxidant property of herbal extracts. LC-MS studies confirm various constituents present in the extract.

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RESEARCH ARTICLE

Combination Effect of Natural and Synthetic Polymers in Extending the Release of Tolperisone HCl from its Effervescent Floating Tablets

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ABSTRACT:

Aim and Objectives: The aim of the current study is to study the effect of combination of synthetic and natural polymers in extending the release of Tolperisone HCl (TH) from its effervescent Floating Tablets (FT), which can extend its release up to 12 h. **Methods:** The drug- excipient compatibility studies of TH and the polymers used in the study were carried by FTIR studies. THFT were prepared by direct compression method. All batches were evaluated for pre-compression, post-compression and *in vitro* buoyancy studies. Accelerated stability studies were performed for the optimized formulation THFT11 as per ICH guidelines. **Results and Discussion:** The drug-excipient compatibility studies reveal that TH and the polymers used for the study are compatible. Pre- and post-compression parameters were within the acceptable limits for all formulations. *In vitro* dissolution studies showed the formulation THFT11 (6.25% w/w sodium alginate and 18.75% w/w HPMC K100M) had extended the release of TH up to 12 h, with a floating lag time (FLT) of 58 ± 0.71 sec, total floating time (TFT) and matrix integrity (MI) maintained up to 12 h, hence it is selected as an optimized one. *In vitro* drug release kinetics of optimized THFT11; suggests the drug release follows zero order profile (r^2=0.988), drug release is predominantly by diffusion and the release mechanism is by super case-II transport. DSC and FT-IR studies of TH and accelerated stability samples of F11 further confirmed the drug is in the same state as pure TH. Accelerated stability studies of optimized THFT11; indicates it passes the test for stability as per ICH guidelines. **Conclusion:** Finally, it was concluded that an optimized effervescent THFT was formulated and evaluated with the combination of synthetic and natural polymers.

KEYWORDS: Tolperisone HCl (TH), floating tablets (FT), hydroxy propyl methyl cellulose (HPMC), sodium alginate (SA), guar gum (GG), *in vitro* buoyancy studies.

INTRODUCTION:

Oral route is one of the most extensively utilized routes for administration of dosage forms. Drugs that have an absorption window in upper GIT, low solubility and stability at alkaline pH were suitable to convert as gastro retentive dosage forms (GRDFs)[1]. Floating Drug Delivery System (FDDS) are one of the promising approaches of GRDFs[2-4].

FDDS has a bulk density lower than gastric fluids and thus remain buoyant in the stomach for a prolonged period of time, without affected by gastric emptying rate[5].

When the system is floating on the gastric fluids, the drug is released slowly at a desired rate from the system. Based on the mechanism of buoyancy, two different technologies for FDDS are effervescent and non-effervescent systems[6]. Effervescent systems contains carbonates (sodium bicarbonate) and/ or organic acids (citric acid/tartaric acid) in their formulation to produce carbon dioxide (CO_2) gas, which is entrapped in the matrix system, reduces the density and makes it buoyant[7]. The non-effervescent systems are based on the mechanism of swelling of polymer or bio-adhesion to mucosal layer in GI tract[8]. Tolperisone HCl (TH), a centrally acting muscle relaxant agent, is rapidly and completely absorbed from the entire GIT and unstable at alkaline pH. Its elimination half-life ($t_{1/2}$) ranges from 1.5 to 2.5 h. Conventional TH tablets are unable to ensure constant plasma conc.; they require multiple

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administrations of divided doses daily; leading to patient's incompliance. It is overcome by extending the release of TH in the stomach up to 12 h[9]. The objective of the present study is formulation and evaluation of effervescent THFTs with combination of natural and synthetic polymers to extend its release up to 12 h.

MATERIALS AND METHODS:

Materials:

Tolperisone HCl (TH) is a gift sample received from Amanath Pharmaceuticals, Pondicherry, India. Hydroxy Propyl Methyl Cellulose (HPMC K100M), Micro crystalline cellulose (Avicel PH 101) and Hydroxy propyl cellulose (HPC EXF) are received as gift samples from Colorcon Asia Pvt. Ltd., Goa, India. Sodium alginateand guar gum is purchased from Arihant trading Co., Bangalore. Magnesium stearate, sodium bicarbonate and talc were purchased from S.D. Fine-Chemicals Ltd., India. All the excipients used in study are of pharmaceutical grade.

Methods:

Drug-excipient compatibility/FTIR studies:

In order to evaluate the integrity and compatibility of the drug with various polymers used in the study, FTIR spectra of drug and drug: polymer (1:1) physical mixtures were recorded, in the region of 400-4000 cm^{-1} at a spectral resolution of 2 cm^{-1}, by the direct sampling method; with isopropyl alcohol as solvent, using FTIR instrument (Cary 630 FTIR, Agilent Technologies, Japan) and the comparative spectra were studied[10].

Analytical method development:

Standard calibration curve of TH in 0.1 N HCl in the conc. range of 2-20µg/mL; at the λ_{max} 260nm; was established using an UV-Visible spectrophotometer (UV-Vis 3000+, Labindia, India)[10].

Preparation of THFT tablets:

All the formulations were prepared by direct compression method, by keeping the amount of TH constant as 50mg per tablet. The composition of other excipients is varied as mentioned in formulation table (Table.1). In these formulations, HPMC K100M is a synthetic controlled release (CR) polymer, sodium alginate (SA) and guar gum (GG) are natural CR polymers, HPC_{EXF} is a solid binder for direct compression, micro crystalline cellulose (Avicel PH 101) is a directly compressible diluent, magnesium stearate is lubricant and talc is glident. TH and all the other excipients excluding magnesium stearate and talc were Passed through #40 ASTM sieve, Co shifted through 60 ASTM sieve, blended uniformly in a poly bag for 10 min and lubricated with # 60 ASTM sieve; passed magnesium stearate and talc and mixed for additional 3 min. Tablets were compressed on a tabletting machine (Minipress -10 stations, Chamunda Pharma Machinary Pvt. Ltd., India) fitted with a 9 mm standard flat circular punches with a avg. hardness of 6.0 kg/cm^2 and avg. wt. of 500mg[10].

Pre -compression studies:

The flow properties of the directly compressible blends of THFTs; angle of repose (θ)**,** bulk density (BD), tapped density (TD), Carr's index (CI) and Hausner's ratio (HR) was determined by standard methods by using the formulae below:[11, 12]

$\theta = \tan^{-1} h / r$ Eq. No. (1)

$BD = \text{wt of blend} / BV$ Eq. No. (2)

$TD = \text{wt of blend} / TV$ Eq. No. (3)

$CI = (TD-BD)\times 100 / TD$ Eq. No. (4)

$HR = TD/BD$ Eq. No. (5)

Where, θ = angle of repose, h = height of heap, r = radius of base of heap circle, BD = bulk density, BV= bulk volume, TD = tapped density, TV= tapped volume, CI = Carr's index and HR = Hausner's ratio.

Post-compression studies:[13,14]

Weight variation test:

An electronic balance (3-MS-S/MS-L, Mettler Toledo, Switzerland) was used to accurately weigh 20 tablets of each formulation which were randomly selected and weighed.

Table 1: Formulation table of THFT:

INGREDIENTS*	THFT1	THFT2	THFT3	THFT4	THFT5	THFT6	THFT7	THFT8	THFT9	THFT10	THFT11
Tolperisone HCl	50	50	50	50	50	50	50	50	50	50	50
Sodium alginate	75	125	175	--	--	--	--	--	--	--	31.25
Guar gum	--	--	--	75	125	175	--	--	--	31.25	--
HPMC K100M	--	--	--	--	--	--	75	125	175	93.75	93.75
Sodium bicarbonate	50	50	50	50	50	50	50	50	50	50	50
HPC_{EXF}	20	20	20	20	20	20	20	20	20	20	20
MCC	245	195	145	245	195	145	245	195	145	195	195
Lactose	50	50	50	50	50	50	50	50	50	50	50
Mg. stearate	5	5	5	5	5	5	5	5	5	5	5
Talc	5	5	5	5	5	5	5	5	5	5	5
Total:	500	500	500	500	500	500	500	500	500	500	500

*Quantities per each tablet expressed in mg,

Thickness of tablet:
Thickness of 3 tablets of each formulation was determined using a Venire calipers (VC320, Mitutoyo corporation, Japan).

Hardness test:
To evaluate tablet hardness, 3 tablets of each formulation were tested for diametrical crushing strength using a hardness tester (MHT-20, Campbell Electronics, India)

Friability test:
The friability of the 10 tablets (n=10) was tested by a friabilator (TAR-120, Erweka, Germany), at a speed of 25 rpm for 4 min. The % friability was calculated by the equation.

$$\% \text{ Friability} = \frac{\text{initial wt.- wt. after friability}}{\text{initial wt.}} \times 100 \qquad \text{Eq. No. (6)}$$

Drug content:
To evaluate the drug content uniformity, 10 tablets of each formulation were crushed; the quantity of tablet powder equivalent to 100mg of TH was suspended in 100mL of 0.1N HCl and ultrasonicated for 2 min to extract the TH from the tablet blend and filtered through 0.45µ poly tetra fluoro ethylene (PTFE) filter disc to filter the dispersed matter, the filtrate was suitably diluted if necessary and its absorbance was measured by UV-visible spectrophotometer.

In vitro dissolution study:
A dissolution test was performed for 12 h using the dissolution apparatus (Disso 2000, Labindia, India) according to USP**.** Each dissolution flask contains 900 mL of 0.1N HCl; speed of paddle was maintained at 50 rpm, the temperature was kept stable at 37^0C±0.5^0C. At every time interval, 5mL of dissolution media was withdrawn through 0.45µ poly tetra fluoro ethylene (PTFE) filter disc, suitably diluted if necessary and its absorbance was measured by UV-visible spectrophotometer at 260nm. Furthermore, 5mL of fresh 0.1N HCL was replaced to the dissolution flask to keep the volume of dissolution medium constant[15].

***In vitro* buoyancy studies:**
The *in vitro* buoyancy studies were determined as per the method described by (Rosa et al., 1994)[16]. A tablet was placed in a beaker with 100mL of 0.1N HCl, the time taken for a tablet to rise on medium surface is noted as floating lag time (FLT). The duration that a tablet remained on medium surface is noted as total floating time (TFT). During the period of floating the swelled tablets were observed for their matrix integrity (MI); if disintegrated within 12 h indicated as '-' and if not disintegrated upto12 h. indicated as '+'.

***In vitro* drug release kinetics:**
The *in vitro* drug release data of all batches were fitted into zero order, first order, Higuchi and Korsemeyer-Peppas models to ascertain the drug release kinetics. The regression coefficient (r^2) of the kinetic model plots were analyzed using MS EXCEL 2007. The drug release from the matrix tablets whether depends on drug's concentration or not was explained by zero and first order[17]. Higuchi model describes whether the drug release is predominantly by diffusion or not[18]. The Korsemeyer- Peppas model further explains the mechanism of diffusion[19]. The respective models were defined by the equations below.

Zero order: $Q_t = Q_0 + K_0 t$ Eq. No. (7)
First order: $\text{LogQ} = \log Q_0 - K_1 t / 2.303$ Eq. No. (8)
Higuchi model: $Q_t = K_H t^{1/2}$ Eq. No. (9)
Korsemeyer-Peppas model: $M_t / M_\infty = K t^n$ Eq. No.(10)

Where Q_t is the amount of drug dissolved at time, t; Q_0 is the initial amount of drug in the solution at time $t=0$, Q is the amount of drug remaining at time, t; M_t/M_∞ is the fraction of drug released at time, t and n is diffusion exponent. K_0, K_1, K_H and K refer to the rate constants of respective kinetic models.

***In vivo* x-ray imaging studies:**
The study was conducted as per the protocol (SVCOP/IAEC/018/2019-20) dated on 09/11/2019) for *in vivo* study was approved by the institutional animal ethical Committee (IAEC) of Sri Venkateswara College of Pharmacy, Chittoor, A.P., India and is in accordance with guidance of committee for the purpose of control and supervision of experiments on animals (CPCSEA), Ministry of Social Justice and Empowerment, Govt. of India. *In vivo* residence time of $BaSO_4$ loaded placebo of optimized THFT11 was studied by x-ray imaging studies in a rabbit model[20,21]. An adult male New Zealand white strain, rabbit of one-year old age and weighing approximately 2-2.5kg was used for this study. The rabbit was fasted overnight before the start of the study. The tablets were administered through plastic tubing followed by flushing of 25-30mL of water. During the entire study, the rabbits had free access to water only. X-ray images were taken using (Wipro Ge Dx300, Tirumala diagnostic centre, Tirupati) at before administration and at 1st, 3rd, 6th, 9th and 12th h after the administration of tablet.

Accelerated stability studies:
Accelerated Stability Studies for 3 months were carried out according to International Conference on Harmonization (ICH) guidelines[31], to study the quality of the optimized THFT11, 20 tablets were packed, properly labelled and sealed in 10 CC HDPE containers and placed in a humidity chamber (NSW-175, Narang Scientific work, India) maintained at 45°C±2°C and 75%

RH. At the end of every month the, samples were withdrawn and evaluated for avg. wt., thickness, hardness, friability, assay, floating characteristics (FLT, TFT and MI) and *in vitro* dissolution studies. Physical and chemical stability of the drug was accessed by comparing pure drug with drug in the 3M accelerated stability sample of optimized THFT11 by performing FTIR (FTIR 8400s, Shimadzu, Japan) and DSC (DSC 60, Shimadzu, Japan) studies[22].

RESULTS AND DISCUSSION:

Drug-excipient compatibility Studies:

The FTIR spectra of drug- polymer (1:1) blends were compared with that of the Pure TH as shown in (Fig.1). In the FT-IR spectra of pure TH, Functional groups are observed at the corresponding frequencies : C=O amide : 1676.20 cm^{-1} , C-N stretch : 1450.52 cm^{-1}, C-N stretch: 1327.07 cm^{-1} , Alkenes C-H bending: 972.16 cm^{-1}, Alkenes C-H bending: 837.13 cm^{-1} and Aromatic C-H bending: 750.33 cm^{-1}. FTIR spectra of drug- polymer (1:1) blends, show same functional groups at the corresponding frequencies as that of pure drug. Thus, indicates no significant chemical interaction and change in functional groups of TH occurred due the combination of drug and polymers used in the study and they are compatible[23].

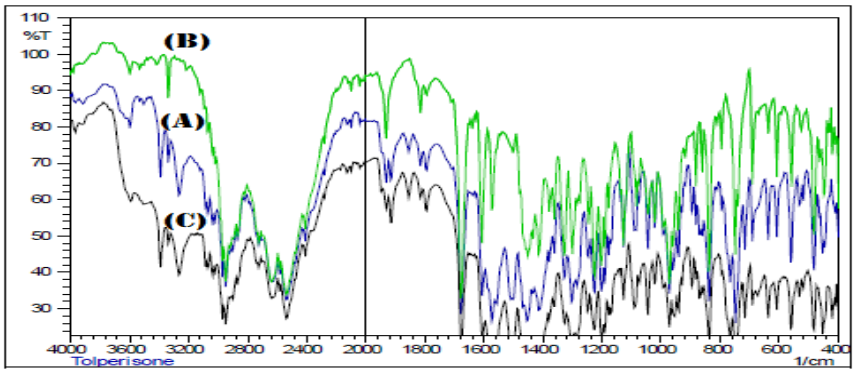

Fig.1. FTIR spectra of A) TH (Pure drug), B) TH+SA and C) TH+GG

Analytical Method:

A spectrophotometric method for estimation of TH, based on the measurement of absorbance at 260nm in 0.1N HCl, gives a straight line; y = 0.064 x + 0.002 with a regression coefficient (r^2): 0.999 as shown in (Fig.2)[24].

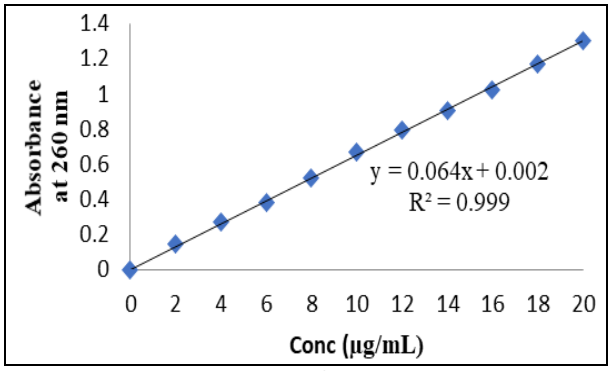

Fig.2. Standard calibration curve of Tolperisone HCl in 0.1 N HCl

Pre-compression studies:

Pre compression studies on directly compressible blends of all formulations reveals that the angle of repose was found between 22°.17'±0.15 to 26.21°±0.11', CI between 12.36 to 18.09% and HR from 1.07 to 1.21. Flow properties of all the formulations were carried out in triplicate (n=3), the consolidated results (mean ±SD) were tabulated in (Table.2), CI and HR are calculated from the mean values of BD and TD of respective batches. The micromertic studies indicate better flow and compression characteristics of all formulations[25].

Table 2: Pre compression studies of THFT:

F. Code	AR (º) (n=3)	BD (g/cc) (n=3)	TD (g/cc) (n=3)	CI (%)	HR
THFT1	22.17 ± 0.15	0.515 ± 0.15	0.522 ± 0.08	13.15	1.10
THFT2	26.11 ± 0.12	0.471 ± 0.11	0.476 ± 0.12	16.23	1.21
THFT3	25.31 ± 0.23	0.505 ± 0.05	0.527 ± 0.15	14.26	1.15
THFT4	23.31 ± 0.14	0.522 ± 0.13	0.519 ± 0.02	12.36	1.09
THFT5	24.27 ± 0.22	0.496 ± 0.21	0.497 ± 0.03	17.42	1.12
THFT6	24.67 ± 0.15	0.481 ± 0.16	0.511 ± 0.14	18.09	1.07
THFT7	25.71 ± 0.13	0.515 ± 0.14	0.522 ± 0.06	13.15	1.10
THFT8	23.31 ± 0.16	0.522 ± 0.13	0.519 ± 0.02	12.36	1.09
THFT9	26.21 ± 0.11	0.496 ± 0.16	0.499 ± 0.03	17.42	1.12
THFT10	25.71 ± 0.03	0.481 ± 0.12	0.511 ± 0.14	18.09	1.07
THFT11	24.21 ± 0.20	0.496 ± 0.15	0.498 ± 0.11	17.42	1.12

AR=Angle of repose, BD=Bulk density, TD= Tapped density, CI=Carr's index and HR=Hausner's ratio. * CI and HR are calculated from the mean values of BD and TD of respective batches.

Post-compression studies:

The avg. wt. of tablets of all the formulations was found to be 500.9±0.3mg. The average thickness of tablets was found to be 5.91±0.23mm. The average hardness of the tablets was 6.3±0.13Kg/cm^2, indicating satisfactory mechanical strength. The average % wt. loss in the friability test ranges from 0.59 to 0.68%, which indicates good mechanical resistance of the tablets. Tablets of all the prepared batches contain TH within 99.98±0.18% of

the labelled content, indicating passing of test for assay. The consolidated results of post compression studies are tabulated in (Table.3), except friability test, all other tests were performed as (n=3), but friability test was conducted on 10 tablets (n=1) from each batch[25].

In vitro buoyancy studies:

The results of *in vitro* buoyancy studies shows the order of FLT with respect to the polymers and their combinations are: SA > GG > HPMC K100M +SA > HPMC K100M + GG > HPMC K100M, which indicates the ease of swelling of individual polymers or their combination with HPMC K100M when contacted with 0.1 N HCl. HPMC K100M being more hydrophilic swells rapidly forms a buoyant matrix and had lesser FLT when compared to others. MI and TFT up to 12 h were not maintained by the matrices formed by SA at all concentrations, but they were maintained at higher concentrations of SA, all concentrations of HPMC K100M and in the combination of HPMC K100M with XG and GG. Maintenance of buoyancy mainly depends upon the concentration of effervescent sodium bicarbonate, which is maintained constant at 10% w/w in all the formulations. The consolidated results of *in vitro* buoyancy studies were tabulated in (Table.3) and the images showing the floating characteristics of optimized THFT11 are seen in (Fig.3) [26].

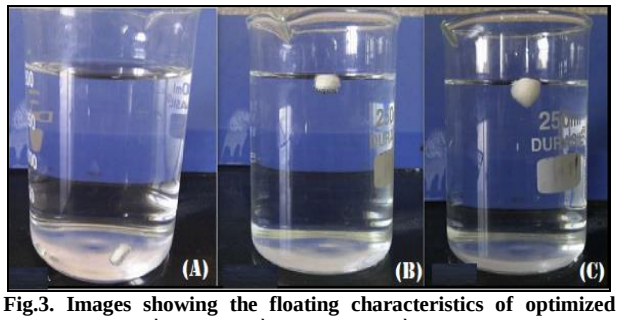

Fig.3. Images showing the floating characteristics of optimized THFT11 at A) 0th Sec, B) 58th Sec and C) 12th h

Table 3: Post compression and *in vitro* buoyancy studies of THFT:

F. Code	Post compression parameters					Floating characteristics		
	Avg. Wt (mg) (n=20)	Thickness (mm) (n=3)	Hardness (kg/cm^2) (n=3)	Friability* (%) (n=1)	Assay (%) (n=3)	FLT (Sec) (n=3)	TFT (h) (n=3)	MI up to 12 h (n=3)
THFT1	500.4 ± 0.12	5.82 ± 0.34	5.9 ± 0.26	0.59	99.98 ± 0.18	49 ± 0.51	> 8	_
THFT2	500.2 ± 0.22	5.91 ± 0.23	6.2 ± 0.25	0.68	99.21 ± 0.20	55 ± 0.22	> 10	_
THFT3	499.6 ± 0.24	5.84 ± 0.14	6.3 ± 0.21	0.58	99.67 ± 0.12	53 ± 0.63	> 12	+
THFT4	500.3 ± 0.31	5.88 ± 0.21	5.9 ± 0.23	0.59	99.32 ± 0.14	150 ± 0.70	> 6	_
THFT5	500.6 ± 0.21	5.87 ± 0.21	6.3 ± 0.13	0.62	99.65 ± 0.18	135 ± 0.83	> 9	_
THFT6	500.9 ± 0.23	5.34 ± 0.14	6.1 ± 0.20	0.59	99.89 ± 0.22	140 ± 0.52	> 10	+
THFT7	500.2 ± 0.26	5.91 ± 0.23	6.2 ± 0.25	0.68	99.21 ± 0.20	75 ± 0.24	> 12	+
THFT8	499.6 ± 0.18	5.84 ± 0.13	6.3 ± 0.21	0.58	99.67 ± 0.12	80 ± 0.85	>12	+
THFT9	500.2 ± 0.21	5.91 ± 0.23	6.2 ± 0.25	0.68	99.21 ± 0.20	64 ± 0.32	> 12	+
THFT10	499.6 ± 0.16	5.84 ± 0.12	6.3 ± 0.21	0.58	99.67 ± 0.12	61± 0.64	> 12	+
THFT11	500.2 ± 0.12	5.88 ± 0.11	5.9 ± 0.23	0.59	99.32 ± 0.14	58 ± 0.71	> 12	+

*Except friability test all other tests were performed as (n=3), but friability test was conducted on 10 tablets (n=1) from each batch.

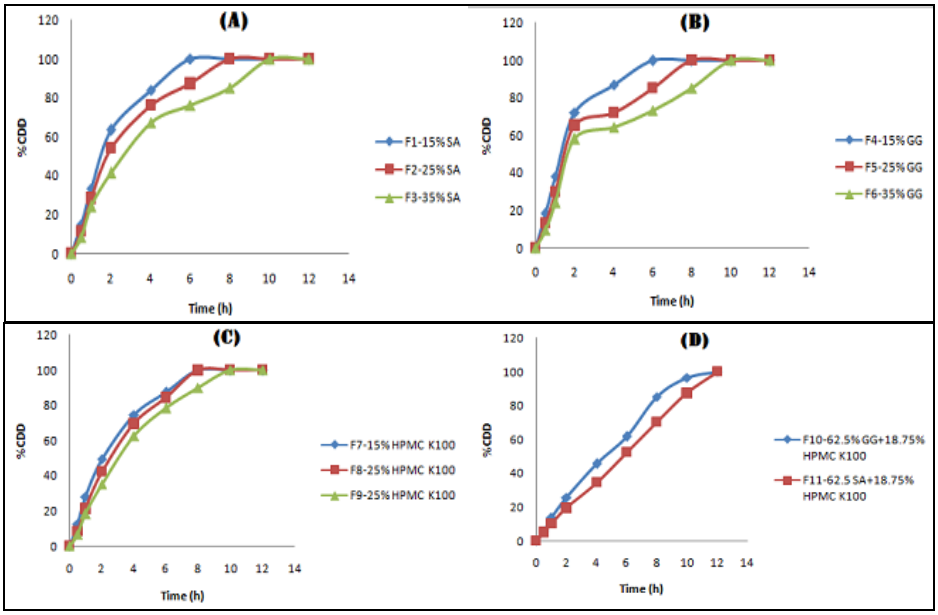

Fig.4. *In vitro* dissolution profiles of THFTs; A) with SA, B) with GG, C) with HPMC and D) with SA/GG + HPMC

In vitro* dissolution studies*:

The *in vitro* dissolution profiles was conducted on 6 tablets (n=6) from each formulation and the dissolution profiles are represented graphically in (Fig.4) indicates that the release rate decreased as the conc. of the CR polymers (HPMC K100M, SA and GG) increased. At higher polymer concentrations, the viscosity of the gel matrix is increased which results in a decrease in the effective diffusion coefficient of the drug and is more likely to be resistant to drug diffusion and erosion.

Table 4: *In vitro* drug release kinetics of THFTs:

Kinetic Model	Parameter	THFT1	THFT2	THFT3	THFT4	THFT5	THFT6	THFT7	THFT8	THFT9	THFT10	THFT11
Zero order	r^2	0.743	0.822	0.901	0.731	0.801	0.865	0.839	0.872	0.917	0.975	0.988
First order	r^2	0.837	0.865	0.805	0.841	0.881	0.810	0.900	0.91	0.852	0.719	0.631
Higuchi	r^2	0.908	0.95	0.979	0.902	0.937	0.959	0.958	0.961	0.975	0.965	0.943
Krosmeyer-Peppas	r^2	0.893	0.916	0.941	0.895	0.899	0.905	0.941	0.947	0.957	0.993	0.999
	n	0.569	0.654	0.729	0.525	0.599	0.680	0.639	0.766	0.839	0.881	0.928

This indicates that drug: polymer ratio is important factor affecting the rate of drug release from the matrix formulations. Factors that may contribute to differences in drug dissolution profile include differences in water penetration rate, water absorption capacity and polymer swelling. The pH independent zero order release profile of hydrophilic drugs like TH can be attained from the matrix systems, by combining the synthetic polymer HPMC K100M with natural polymers like SA and GG than HPMC alone. The combined matrix when exposed to an acidic environment, the HPMC hydrates to form a gel layer at the surface of the tablet while the natural gums (SA and GG), due to lesser hydration rate than HPMC remains insoluble. The resulting matrix acts as a barrier to diffusion of the freely soluble drugs and extends drug release. The proportion of HPMC K100M: Natural gums had significant effect on extending the release profiles of drug, which has to be optimized[27]. Formulation THFT11 with (6.25% w/w SA and 18.75% w/w HPMC K100M) extend the release of TH up to 12 h with zero order kinetics (r^2=0.988), with a FLT of 58 ± 0.71 sec, TFT and a better MI up to 12 h. Hence, formulation THFT11 was the best formulation with good floating parameters and desirable *In vitro* drug release profile when compared to other formulations.

***In vitro* drug release kinetics:**

The drug release kinetics of optimized formulation F11 fitted best to the zero-order kinetics (r^2 = 0.988). The (r^2 = 0.934) value in case of Higuchi release was found to be $r^2 > 0.9$, suggesting that the drug release process is predominantly by diffusion. The (n=0.928) value for the case of cylindrical shape in Korsmeyer-Peppas model, suggested the mechanism of the drug release is Super Case II transport ($0.45 < n < 0.89$). The consolidated drug release kinetic parameters of THFTs were tabulated in (Table.4)[27].

In vivo x-ray imaging studies:

X-ray images of a rabbit taken before and after the administration of $BaSO_4$ loaded placebo of optimized THFT11 as shown in (Fig.5); indicates its floating ability, was strong enough in withstanding repetitive gastric contractions and able to retain in the gastric region up to 12 h[28].

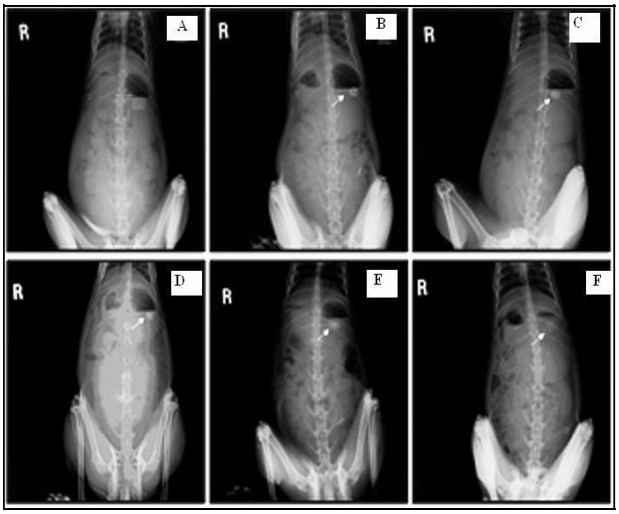

Fig.5. X-ray images of $BaSO_4$ loaded placebo of optimized THFT11 in a rabbit model at A) Before administration, B) 1st h, C) 3nd h, D) 6th h E) 9th h and F) 12th h.

Accelerated stability studies:

The consolidated results of post compression studies of accelerated stability studies data for optimized THFT11 are tabulated in (Table.5). Comparative FTIR spectra of pure TH and 3M accelerated stability sample of optimized THFT11 as shown in [Fig. 6(I)] indicates no significant shifts or reduction in intensity of the FTIR bands of TH were observed. Hence there was no compatibility problem between the drug and excipients used. Comparative DSC thermograms of pure TH and 3M accelerated stability sample of optimized THFT11 as shown in [Fig. 6(II)], reveals that the melting point of TH is 186.16 C and that of TH in the formulation F11 is 189.6 C.

Table 5: Accelerated stability data of optimized THFT11

Time Interval	Post compression parameters					Floating characteristics		
	Avg. Wt (mg)(n=20)	Thickness (mm)(n=3)	Hardness (kg/cm^2)(n=3)	Friability (%)(n=1)	Assay (%)(n=3)	FLT (S)(n=3)	TFT (h)(n=3)	MI up to 12 h. (n=3)
Initial	500.2±0.12	5.88 ± 0.11	5.9 ± 0.23	0.59	99.82±0.14	58±0.71	> 12	+
1 M	501.1±0.11	5.88±0.21	5.8±0.12	0.61	99.52±0.08	60±0.09	> 12	+
2 M	501.2±0.21	5.88±0.22	5.9±0.13	0.64	99.43±0.21	63±0.11	> 12	+
3 M	501.2±0.13	5.88±0.12	5.9±0.21	0.66	99.41±0.11	64±0.12	> 12	+

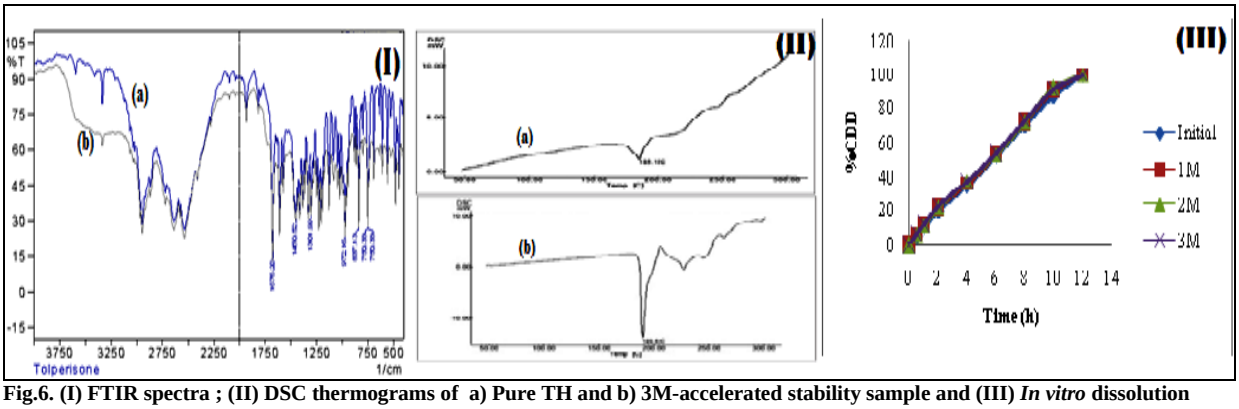

Fig.6. (I) FTIR spectra ; (II) DSC thermograms of a) Pure TH and b) 3M-accelerated stability sample and (III) *In vitro* dissolution profiles of accelerated stability samples of optimized THFT11

As there is no much difference in the melting points, it indicates that the drug is in same state even in the optimized THFT11 during the stability study period; without interacting with the polymers and excipients. Comparative *in vitro* dissolution profiles of initial and accelerated stability samples of optimized THFT11 as shown in [Fig. 6(III)], reveals there were no significant differences. Hence it passes the test for stability as per ICH guidelines[28].

CONCLUSION:

In the above view of findings the effect of combination of synthetic polymer (HPMC K100M) with natural gums (XG and GG) in extending the release of TH from its GRFT is better understood. The formulation F11 (18.75% HPMC K100M and 6.25% SA) is the optimized formulation. It was further concluded that the optimization of the proportion of HPMC K100M: natural gums, had significant effect on extending the release profiles of TH. Among the two natural gums SA in combination with HPMC K100M in the ratio 1:3 respectively forms a better matrix for the extending the release of TH in gastric pH for 12 h. A matrix design of this kind can serve as an alternative strategy for extending the release of other BCS class I drugs and their salts, which are having shorter half-life ($t_{1/2}$ < 5 h).

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CONFLICT OF INTEREST:

Authors have declared no conflict of interest to declare.

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RESEARCH ARTICLE

Fluticasone and Cyclophosphamide towards the track of personalized Medicine

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ABSTRACT:

Personalized medicine or precision medicine is a preventive standard that splits people into distant groups with pharmaceutical accord, method, mediation and products are being made to measure to the entity case based on their forecast response or hazard of infection. The Promise of Personalized Medicine, "therapy with the right drug at the right dose in the right patient" is a description of how personalized medicine will affect the future of treatment. A form of a patient's heredity abnormality can guide the choice of drugs or treatment custom to physicians that will diminish the harmful side effects or ensure the more successful outcomes. Personalized medicine reduces the cost of drugs, adverse drug reactions to patients and Increasing patient compliance with treatment. As Fluticasone and cyclophosphamide has high inter-individual variability, genetic polymorphism and population pharmacokinetics was studied, in-order to provide optimum dose for all individuals. Genetic Polymorphism associated with fluticasone was ABCB1 and CYP3A4*22, whereas genetic polymorphism associated with cyclophosphamide was CYP2B6 and GSTP1. Population Pharmacokinetics of fluticasone was done in different places using the co-factors such as genetic polymorphism, Alcohol consumption, weight, Height, Age and Smoking, whereas population pharmacokinetics of cyclophosphamide was done using co-factors such as Genetic polymorphism, Age, Body weight, Liver dysfunction. From the information obtained from population pharmacokinetics, a personalized chart for fluticasone and cyclophosphamide was constructed. In this study, genetic polymorphism and population pharmacokinetics of Fluticasone and Cyclophosphamide towards Personalized medicine was carried out.

KEYWORDS: High-individual variability, Fluticasone, Cyclophosphamide Genetic polymorphism, Population pharmacokinetics.

1. INTRODUCTION:

Personalized medicine or precision medicine is a pharmaceutical standard with medical decision that separates people into different groups, practices, interventions and products are being made to measure to the individual patient based on their conclude response or risk of disease[1].

The terms personalized medicine or the precision medicine are utilized to depict this idea however a few creators and associations utilize these expressions independently to show particular nuances. Personalized medicine is fitting of medical therapy to the individual attributes of each medicine[2]. The treatment should me safe and effective. The research for PM is increasing in our ability to predict which type of medical treatments will be safe and effective to each patients and which one's will not be Genomics in a lowering costs and by facilitate each victim to accept earlier diagnoses, danger estimate, and excellent medication, personalized medicine holds obligation for developing well-being responsibility[3]. The affiliation of all subject with a certain circumstance, common dosage from that is **'one**

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size fits all' approach to the therapy and to one which it uses a new approaches to accomplish the conquer conclusion in the authority of a victim disease and manage the patients health target therapies[4]. The Promise of Personalized Medicine, **"therapy with the right drug at the right dose in the right patient"** is a description of how personalized medicine will affect the prospect analysis[5]. Personalized medicine has improved in recent years, on basis of it provides the variation in human genome to efficacy in drug treatment, to the affected persons a form of medicine that custom the data about the person's genes, proteins, ADME and habitat to inhibit, diagnose[6]. In inter-individual genetic variability the calculating of the individual gene has exposed that while human kind gene make-up is 99.1% are exact, where the other 0.9% is non-identical. The concept of personalized health care is increasing in acceptance to treat on individual response.

2.HISTORY:

The personalized medicine is initally came in the year 1999. The concepts have been in existence since 1960's. The new technology has now bulit personalized medicine in more phenomenon and welfare to the patients to provide the drug in proper dosage form for the effective treatment and also to provide the drug in cheaper cost.[7]

The two discover keys are:

- **Single nucleotide polymorphism:**

SNP's are single nucleotide changes in the DNA sequences that are to be frequent in the population that contributes about 90% of all known polymorphisms. SNP's are proven to be an invaluable tool in segregating patients in clinical trials.

- **Microarray biochip:**

The ability to analyze and store the patient's genome; it also helps in conducting the SNP genotype(8).

3. INTRODUCTION TO DRUGS:

3.1 FLUTICASONE:

Fluticasone is a synthetic glucocorticoid which is worn in some people to treat nasal syndrome. It is a normal efficacy corticosteroid worn to relax inflammatory and pruritic syndrome and also used to treat allergic and non-allergic rhinitis and orally for the cure of asthma.

3.1.1. DOSAGE

Adult: Initially 2 sprays one by one nostril once often. Maximum of overall doses 2 sprays in each nostril of 200mcg/day.

Children:

First 1 spray in particular nostril once regularly, it may raise to 2 sprays in each nostril once daily Maximum of total dose in each nostril of 200mcg/day[9]

Table 1 Use and adverse effect

Use	Adverse Effect
Fluticasone is a corticosteroid that prevents substances from the body that cause inflammation. Used in treatment of asthma	Throat infection, nasal irritation, menstrual problems, sneezing, cough, nausea, headache, vomiting, back pain, sore throat.

3.2 CYCLOPHOSPHAMIDE:

Cyclophosphamide is determined for the treatment of malignant lymphomas, various myeloma, leukemias, mycosis fungoides, neuroblastoma (disseminated disease), adenocarcinoma of the ovary, retinoblastoma, and carcinoma of the breast. It is also recorded for the regimen of biopsy-proven minimal change nephrotic syndrome in pediatric patients.

3.2.1 DOSAGE:

Adult and Pediatric:

Powder injection: 500mg, 1g, 2g.

Tablet: 25mg, 50mg.

Table 2 Use and adverse effect

Uses	Adverse Effect
Cyclophosphamide is used for the different type of tumor therapy. It is a chemotherapy drug that works by block or retard the cell growth, erasing your immune system's return to many diseases.	Nausea and vomiting, bone marrow suppression, stomach ache, hemorrhagic cystitis, diarrhea, darkening of the skin/nails, alopecia changes in color and texture of the hair and lethargy.

4. SCOPE AND NEEDS:

Each human being has a one of a kind variation in their human genome. Albeit a large portion of the gene variation in humans has no impact on health, a person's energy stems from genetic variation with the practices and impacts from nature (10).

Present day propels in customized medication has improved in innovation that affirms a subject major biology, deoxyribonucleic acid, ribonucleic acid, or protein, which at last prompts affirming infection

As quoted from the article Pharmacogenomics: The Pledge of Personalized Medicine, **"therapy with the right drug at the right dose in the right patient"** is a description of how personalized medicine will affect the destiny of medication[11]

Personalized medicine might be viewed as an augmentation of customary ways to deal with understanding and treating the disease yet with more prominent accuracy[12]. A contour of a subject heredity variations can deal with the selection of drugs or treatment conventions to doctors that will confine the destructive reactions or certification the more useful outcome. Personalized medicine can also hint an specific sensitivity to certain diseases before they become

disclosed, allowing physicians and patients to design a plan for monitoring and prevention of diseases[13]. Physicians can now go beyond the **one-size-fits-all** to **"therapy with the right drug at the right dose in the right patient"** model of prescribing to make more effective clinical decisions for each patient.

The need for personalized medicine is not only improved in drugs it also improved in device manufactures, it's used to reduce the cost of drug and device manufactures and also to easily applicable to all patients at a right dose to improve the public health[14].

5. PROS AND CONS:

5.1 PROS:

a) Decrease the cost of drugs
b) Improve quality of life
c) It customizes the disease prevention.
d) Less adverse drug reactions to patients.[15]

5.2 CONS:

a) Base Requirements
b) Legitimate Problems
c) Important data
d) Cost of healthcare(16)

6. FACTORS STRIVES TOWARDS: PERSONALIZED MEDICINE:

1. Patients as customer
2. The Shift to Value-established responsibility
3. Option of High-performance, memorial Computing
4. One Size DOES NOT Fit All (17)

7. DATABASE DEVELOPMENT OF FLUTICASONE:

7.1 POPULATION PHARMAOKINETICS OF FLUTICASONE:

Based upon the population pharmacokinetic data the possible parameters of personalized medicine on various populations that have the significant changes to pass on the track of personalized medicine. To pass on the track some factors are to be categorized[18]

The tests are conducted in different countries like China, USA, Canada, and Germany.

Table 3 Population and Factors

Sl. No	Population	Factors
1.	China	Genetic polymorphism, Alcohol consumption, Weight, Height, Age, Smoking.
2.	USA	Genetic polymorphism.
3.	Canada	Genetic polymorphism.
4.	Germany	Gender.

7.1.1 IMPACT OF GENETIC POLYMORPHISM FLUTICASONE:

7.1.2 ABCB1:

The genotype and allele frequencies of the ABCB1 rs1045642C>T polymorphic arrangement were accordingly particular between the active group and the non-active group. Haplotype analysis of ABCB1 determined that CTA (rs1045642C– rs1128503T– rs1202184A) haplotype frequencies in the effective group were significantly decreased than the ineffective group (p=0.022), but TCG (rs1045642T– rs1128503C– rs1202184G) haplotype frequencies in the effective group were significantly higher than the ineffective group (p=0.048). CTA/TCG haplotypes are the genetic sequences have the variation in the serine molecules. The constancy of TCG haplotype in the viable gathering are less, hence more examinations on haplotype analysis are expected to growth a superior awareness of SFC therapy reactions in COPD subjects. This genetic polymorphism variation has happened in Chinese population.[19,20,21]

7.1.3 CYP3A4*:*

The nearness of CYP3A4*22 which is related with diminished hepatic CYP3A4 articulation and movement, was accomplished by improved asthma control in the FP-treated children. Diminished of CYP3A4 movement may improve the asthma control with breathed in FP (Fluticasone propionate). This genetic polymorphism variety has happened in USA and Canada population.

7.2 IMPACT OF CIGARETTE SMOKING IN FLUTICASONE:

Another interesting factor was found to be outcome in COPD patients in the involve SFC therapy[22]. it is a noteworthy hazard factor for COPD and expanded danger of respiratory side effects like lung damage, and higher COPD death rates[23]. In this manner, in view of our outcomes, we recommend that transpire might be allied with suffering SFC therapy results in Chronic obstructive pulmonary disease patients because of its extreme effect on the general wellbeing status[24]

7.3 IMPACT OF GENDER IN FLUTICASONE:

Gender was found to be a significant variation on clearance, when compared to the men's women's have the higher clearance on the Germany population, in Chinese population there is about 0.610 of regression coefficient[25].

7.4 IMPACT OF BODY WEIGHT IN FLUTICASONE:

The weight has varied in people and it has the significant variation of about 0.014 of regression coefficient in the Chinese population. If the body weight is raises it affects the significant clearance and the variation is differed[26].

7.5 IMPACT OF HEIGHT IN FLUTICASONE:

The height has varied in people and it has peoples have the significant variation of about 0.027 of regression coefficient in Chinese population[27].

7.6 IMPACT OF ALCOHOL CONSUMPTION IN FLUTICASONE:

Another factor is considered to be the alcohol consumption peoples have the significant variation of about 0.819 of regression coefficient in Chinese population[28].

7.7 IMPACT OF AGE FACTOR IN FLUTICASONE:

Another factor is considered to be the age, the age factor being varied in people and it have the significant variation of about 0.013 of regression coefficient in Chinese population[29].

7.8 PERSONALIZED PRESCREPTION CHART FOR FLUTICASONE:

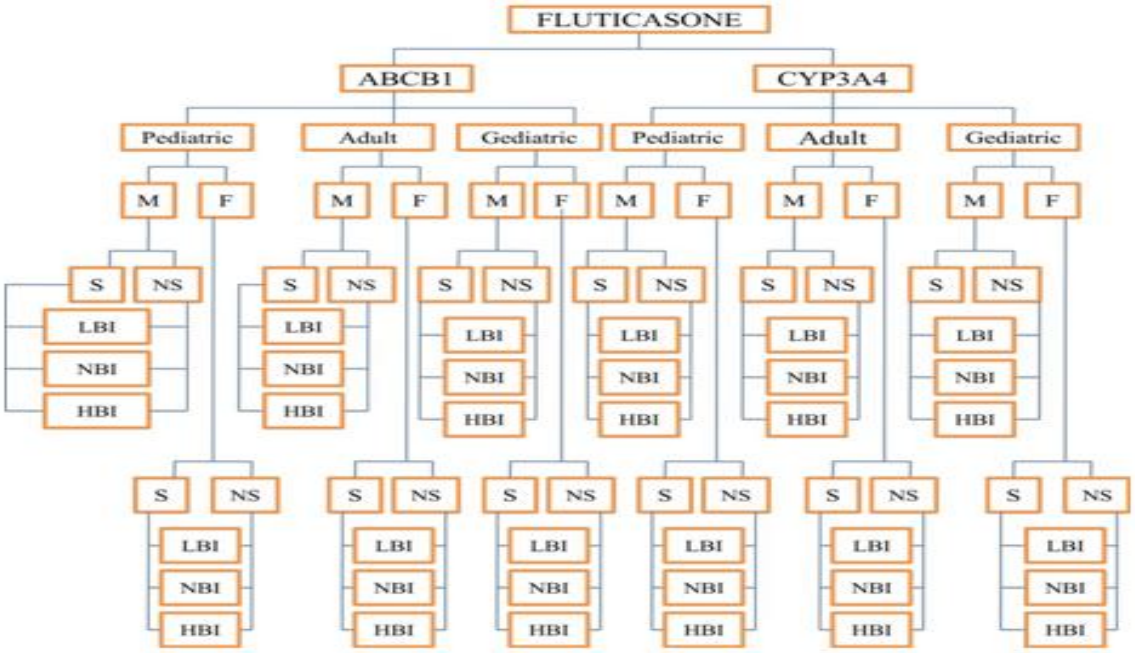

Fig.1 Personalized Prescription Chart for Fluticasone

NOTE:

M- Male, F-Female, S-Smoking, NS- Non- Smoking peoples, Non- Smoking peoples, NBI- Normal Body Mass Index, HBI- Higher Body Mass Index

8. CYCLOPHOSPHAMIDE:

8.1. POPULATION PHARMACOKINETICS IN CYCLOPHOSPHAMIDE:

Based upon the population pharmacokinetic data the possible parameters of personalized medicine on various populations that have the significant changes to pass on the track of personalized medicine. To pass on the track some factors are to be categorized (30)

The tests are conducted in different countries like India, Germany, Paris, China, Bangladesh, and Canada

Table 4 Population and Factors

S. No	Population	Factors
1.	India	Genetic polymorphism, Age, Body weight.
2.	Germany	Genetic polymorphism,
3.	China	Genetic polymorphism.
4.	Canada	Genetic polymorphism.
5.	Bangladesh	Genetic polymorphism.
6.	Paris	Age, Body weight.
7.	General considerations	Liver dysfunction

8.1.1 IMPACT OF GENETIC POLYMORHISM IN CYCLOPHOSPHAMIDE:

8.1.1.1 CYP2B6*:*

A large interindividual change was noticed in the area under the curve ratio of cyclophosphamide computed as the metabolic ratio. They verified that leukocytopenia and neutropenia were significantly ($P<0.01$) resulted to the area under the curve of 4-hydroxycyclophosphamide (31). It was proven to be that the homozygotes of CYP2B6*6 (Q172H and K262R) showed significantly ($P<0.05$) greater clearance and shorter half-life of cyclophosphamide than heterozygotes and homozygotes of CYP2B6*1. The narrow pattern size, however, the collide(32)(33).On the other hand, it was certainly establish that the victim acquire the single nucleotide polymorphisms of the CYP2B6 gene, g.-2320T>C, g.-750T>C (5'-flanking region), g.15582C>T, or g.18492T>C had significantly lower AUC ratios of 4-cyclophosphamide,pinpoint a weaken cyclophosphamide 4-hydroxylation. Of precise concern was the data that leukocytopenia was significantly related to the single nucleotide polymorphisms g.-2320T>C, g.-750T>C, and

g.18492T>C in CYP2B6 gene, which are greatly linked. No relationship was detected between the pharmacokinetics of cyclophosphamide or 4 - hydroxycyclophosphamide and genetic polymorphisms of the other enzymes. They analyze that the single nucleotide polymorphisms is the promoter region in the CYP2B6 change the efficacy of cyclophosphamide stimulation to 4-hydroxycyclophosphamide. This type of genetic polymorphism has occurred in Japanese population (34)(35)(36).

8.1.1.2 GSTP1*:*

The enzyme GSTP1 (Glutothione- S- Transferase Pi1) is associated to produce toxicity if the expression of GSTP1is higher in patients. This type of genetic polymorphism has occurred in India, China, Bangladesh, Canada population (37)(38).

8.1.2. IMPACT OF AGE AND BODY WEIGHT IN CYCLOPHOSPHAMIDE:

Age and body weight of patients directly related to the cyclophosphamide drug efficacy in patients. It clearly shows that children and adult patients showed the variation in action and biotransformation of the drug. Hence the children showed the increased onset of action and biotransformation of the drug when compared to the adults. This type of age and body weight factor has occurred in India, Paris population(39)(40)(41).

8.1.3 IMPACT OF LIVER DYSFUNCTION IN CYCLOPHOSPHAMIDE:

Liver dysfunction in cancer patients may have a chance of decreased effect of cyclophosphamide within patients. This is due to polymorphism in liver ALDH1A1*2 alleles. The liver dysfunction is the general considerations among the patients.

8.2 PERSONALIZED PRESCREPTION CHART FOR CYCLOPHOSPHAMIDE:

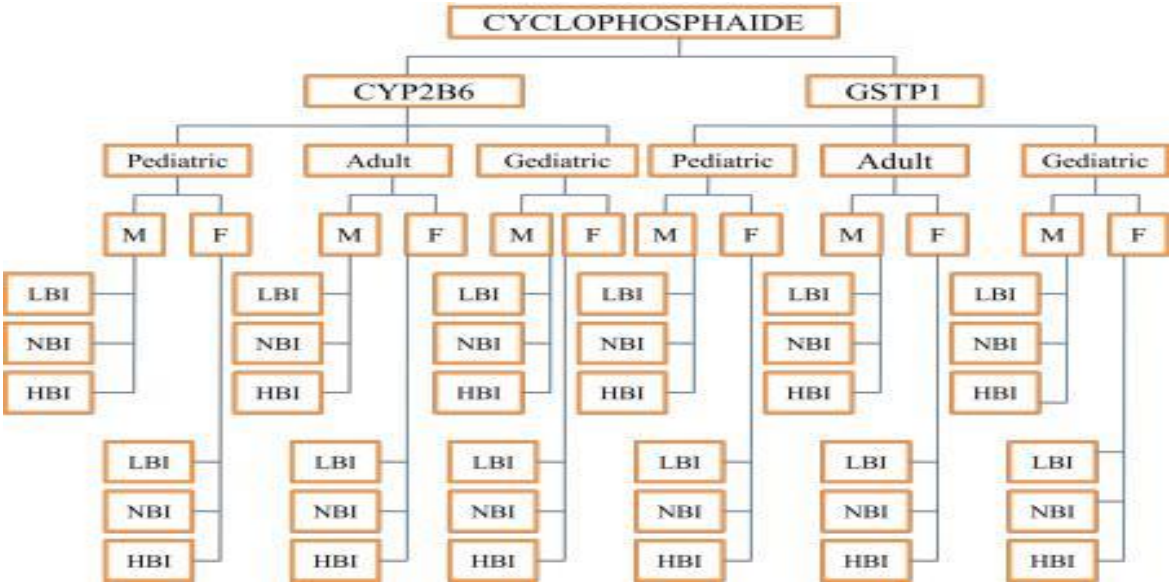

Fig 2 - Personalized Prescription Chart For Cyclophosphamide

NOTE:

M-Male; F-Female; LBI- Lower Body Mass Index; NBI- Normal Body Mass Index; HBI- Higher Body Mass Index

8.3 NEW APPLICATION:

If a new dose of available drug molecule is prepared for clinical use that can either be increasing or decreasing in dose that should be filled under New Drug Application (NDA) by proving the safety and efficacy of the drug.

8.4 FUTURE OF REGULATORY TOWARDS PERSONALIZED MEDICINE:

The research towards personalized medicine is recently increasing nowadays. The regulations of personalized medicine are in budding stage and in countries like US and UK have started implementing the use of personalized medicine regulations. Since we are in budding stage on developing the personalized medicine research, it may take some more years to implement a strong guideline about personalized medicine in India

9. CONCLUSION:

There should be a strong co-ordination between manufacturer, researcher and clinicians for real-time implementation of personalized medicine. So that the research will strive from bench to bed-side for the maximized therapeutic benefit of population with minimal expenses and side effects. Based on this, the review of genetic polymorphism and population pharmacokinetics of Fluticasone and Cyclophosphamide

towards Personalized medicine and its regulatory feasibility have carried out and completed successfully.

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11. CONFLICT OF INTEREST:

The authors have no conflict of interest.

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RESEARCH ARTICLE

Method development and Validation for the Estimation of 5-Fluorouracil by using Reverse phase high-performance liquid chromatography

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ABSTRACT:
An analytical method was developed to quantify 5-Fluorouracil (5-FU) using reverse phase high performance liquid chromatograph (RP-HPLC). A C-18 reverse-phase column was used as stationary phase for the separation of 5-FU. Potassium dihydrogen orthophosphate buffer (0.05 M) containing 0.1% of Triethanolamine (TEA) was use as a mobile phase to facilitate the elution. The flow rate was 1.2mL min^{-1} and the chromatogram of 5-FU was detected at wavelength of 266nm. 5-Bromouracil (5-BU) was used as internal standard. Method was validated as per ICH Q2 (R1) guidelines. The retention time of 5-FU was found to be 7.568 min and 5-BU was 20.067 min. The developed method was found to be linear at a concentration range of 2-10µg/mL with r^2 of 0.9996. The mean percentage recovery of 5-FU was found within 95-105 % at all the levels which indicated that the method was accurate. The percentage relative standard deviation was found less than 2% which indicated that method was satisfactorily précised. The LOD and LOQ for 5-FU were found to be 3.3 and 0.83 respectively. The method was found to be robust as there was no significant change in response with variation in the flow rate and wavelength. It was concluded that the developed method has passed all the validation tests and can be successfully applied to estimate the presence of 5-FU in bulk as well as in pharmaceutical formulations.

KEYWORDS: 5-Fluorouracil, RP-HPLC, Linearity, Validation, Precision.

INTRODUCTION:
5-Fluorouracil (5-FU) is chemically 5-fluoro-1,2,3,4-tetrahydropyrimidine-2,4-dione[1-2]. It is an analogue of uracil which is a component of ribonucleic acid. 5-FU is believed to function as an antimetabolite. During the cell division, it interferes in the process of DNA synthesis by blocking the conversion of deoxyuridylic acid to thymidylic acid with the cellular enzyme thymidylate synthetase[3]. It is also believed that, 5-FU may interfere with synthesis of RNA. 5-FU may be used alone or in combination for the management and treatment of common malignancies specifically in the cancer of colon and breast[3-4].

A simple and novel analytical method is required to quantify 5-FU, which can be utilized successfully during the quality control test of dosage forms. Till date there is no such simple, cheaper and sensitive method is available to estimate the amount of 5-FU. In this work a simple and sensitive method was developed and validated to estimate 5-FU.

MATERIALS AND METHODS:
Materials:
5-FU was procured from Molychem, Pvt. Ltd., India. All other chemicals and reagents used were of analytical grade and HPLC grade solvents were employed for the study. Triple distilled water was used throughout the study.

Method Development for the estimation of 5-FU using RP-HPLC:
The RP-HPLC system consisted of a mobile phase delivery pump (LC-20 AD; Shimadzu, Japan), a photodiode array detector (SPDM20A; Shimadzu, Japan), a 20µL loop fitted with manual Rheodyne injector and LC Solution software. A C-18 reverse-phase

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column (Nucleodur C18, 250mm × 4.6mm i.d., 5µ) was utilised as stationary phase for the estimation of 5-Fluorouracil, using 0.05M potassium dihydrogen orthophosphate buffer containing 0.1% Triethanolamine (TEA), as mobile phase. 5-Bromouracil (5-BU) was used as internal standard. The flow rate was 1.2 mL min^{-1} and detection wavelength was 266nm. Standard dilutions (2, 4, 6, 8 and 10µg/mL) were prepared in mobile phase and analysed. The developed method was validated as per ICH Q2 (R1) guidelines[5].

Method validation:
The standard analytical method validation parameters i.e., linearity and range, accuracy, precision, robustness, LOD and LOQ, tailing factor, peak purity index and system suitability parameters were performed.

Preparation of quality control standards:
The quality control standards were prepared at three different levels i.e., Lower Quality Control standard (LQC), Medium Quality Control standard (MQC) and Higher Quality Control standard (HQC) of calibration curve. Hence, 6µg/mL was kept as 100 % (MQC) level, 80 % of 6µg/mL (i.e., 4.8µg/mL) was used as LQC and 120 % of 6µg/mL (i.e., 7.2µg/mL) was kept as HQC levels. All the three concentrations were prepared in mobile phase.

Linearity and range:
The calibration curve was developed by plotting the graph between mean peak area of five replicates versus corresponding concentrations of 5-FU, and the regression equation was obtained.

Accuracy:
The accuracy of the method was determined through calculation of recovery of the drug from the quality control standard solutions prepared in mobile phase. The LQC, MQC and HQC standard solutions were injected five times to HPLC and its mean of response was recorded. Percentage recovery was calculated by dividing the actual recovery of drug with their respective theoretical concentrations and multiplying them by hundred (Eq.1). The mean of response was recorded and percentage relative standard deviation was calculated.

$$\text{Percent recovery} = \frac{\text{Actual concentration recovered}}{\text{Theoretical concentration}} \text{X } 100$$

Precision:
Precision of the method was evaluated in terms of repeatability and intermediate precision. Repeatability was tested by injecting five times the samples of LQC, MQC and HQC on the same day and under same experimental conditions. The intermediate precision was evaluated by determining LQC, MQC and HQC samples five times on each of three different days (inter-day) as well as by the three different analysts (inter-analyst) under the same experimental conditions. The mean of response was recorded and percentage relative standard deviation was calculated.

Robustness:
In order to check the effect of small changes on robustness of the developed method, the study was carried out by varying the flow rate (0.8, 1.0 and 1.2 mL/min) and wavelength (264, 266 and, 268nm), respectively. Five replicates of medium concentration (6 µg/mL) were injected and their effect on area of the peak, recovery and retention time was observed and mean of response was recorded.

Estimation of LOD and LOQ:
LOD and LOQ were determined by standard deviation of response (sigma) and slope of calibration curve (S). Standard deviation of Y intercepts of regression line was used as standard deviation.

$LOD = 3.3\ \sigma/S$
$LOQ = 10\ \sigma/S$

System suitability:
In order to check the system suitability, 5 replicate injections of standard solution (6µg/mL) of 5-FU were injected to the HPLC and the system suitability parameters were calculated.

RESULTS AND DISCUSSION:

Selection of mobile phase for the estimation of 5-FU:
For the estimation of 5-FU, different trials by changing the mobile phase and its composition were performed such as acetonitrile-water, acetonitrile-potassium dihydrogen orthophosphate buffer, methanol-potassium dihydrogen orthophosphate buffer and potassium dihydrogen orthophosphate buffer containing 0.1 % TEA.

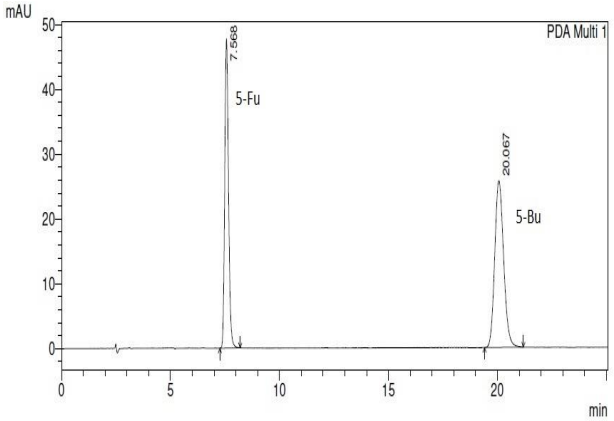

Fig. 1: Optimised chromatogram of 5-FU and 5-BU in potassium dihydrogen orthophosphate buffer containing 0.1 % TEA

Out of the performed trials, the trial conducted with the mobile phase potassium dihydrogen orthophosphate buffer (0.05) containing 0.1% TEA showed better results in terms of resolution, sharpness of peak and, separation between the peak of 5-FU and the internal standard 5-BU. Since, there is a significant difference in the retention time of 5-FU (7.595 min) and 5-BU (20.238 min) peaks (Fig.1); the mobile phase composition was selected for validation.

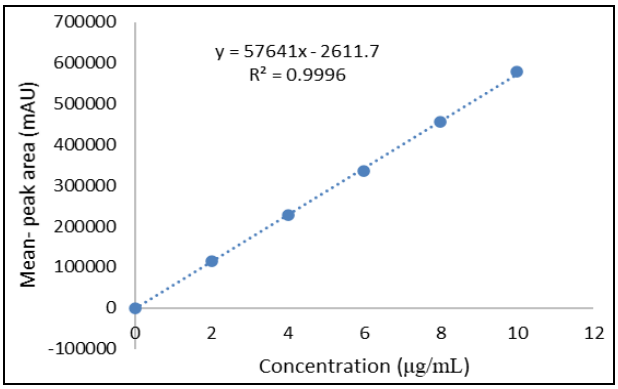

Fig.2: Calibration curve of 5-FU

Linearity and Range:

The calibration curve was developed by plotting the graph between concentration and mean area. The curves were found to be linear in the range of 2-10 µg/mL with a correlation co-efficient (r^2) of 0.999 (Fig.2).

Accuracy:

The accuracy of the developed method was accessed by determining the mean percentage recovery of the LOQ, MQC and HQC solutions in mobile phase. The data revealed that for all the three levels, the mean percentage recovery in mobile phase was within the fixed limits of 95-105 % (Table 1). The accuracy of developed method was verified by percentage relative standard deviation which was less than 2 %. The results of accuracy study are summarised in Table 1.

Precision:

The precision of developed method was evaluated by calculating the percentage relative standard deviation for the five determinations of LQC, MQC and HQC solutions at interday, intraday and interanalyst level under the same experimental conditions. The observed percentage relative deviation was less than 2 % for all the samples (Table 2). These results clearly indicated that the developed method was satisfactorily précised. The results of precision study are summarised in Table 2.

Table 1: Results of accuracy studies

Levels	Concentration of standard solution (µg/mL)	Acutal mean concentration (µg/mL)	% Recovery	SD	% RSD	Mean % recovey ± SD
LQC	4.8	4.65	96.87	0.018	0.390	97.38 ± 1.07
MQC	6	5.8	96.67	0.016	0.283	
HQC	7.2	7.1	98.61	0.017	0.251	

Table2: Results of precision studies

Parameters	Levels	Conc. (µg/mL)	Analytical response (area, injections)					Mean (N = 5)	SD	% RSD
			1	2	3	4	5			
Repeatability (Intraday precision)	LQC	4.8	261537	269160	260333	264533	261091	263330.8	3627.9	1.4
	MQC	6	329074	322689	321821	321669	320028	323056.2	3498.89	1.09
	HQC	7.2	403341	403793	403368	405443	411993	405587.6	3682.54	0.91
Intermediate precision (Interday)										
Day 1	LQC	4.8	263767	268581	266419	265093	268901	266552.2	2210.05	0.83
	MQC	6	335937	338629	334677	337235	334089	336113.4	1855.99	0.55
	HQC	7.2	409092	403711	401537	400572	402485	403479.4	3345.51	0.83
Day 2	LQC	4.8	268166	263043	267974	267588	262304	265815	2887.2	1.09
	MQC	6	331842	332900	341562	332230	339908	335688.4	4569.26	1.36
	HQC	7.2	405432	407958	408082	411012	405213	407539.4	2365.47	0.58
Day 3	LQC	4.8	261537	269160	260333	264533	261091	263330.8	3627.9	1.4
	MQC	6	329074	322689	321821	321669	320028	323056.2	3498.89	1.09
	HQC	7.2	403341	403793	403368	405443	411993	405587.6	3682.54	0.91
Intermediate precision (Intraday)										
Analyst 1	LQC	4.8	264231	262121	263120	265317	258360	262629.8	2669.98	1.02
	MQC	6	334927	333972	333671	334237	325104	332382.2	4095.03	1.23
	HQC	7.2	378130	383741	390211	391042	392584	387141.6	6062.73	1.56
Analyst 2	LQC	4.8	255146	263023	257104	257082	255344	257539.8	3202.22	1.24
	MQC	6	341942	332101	342171	332180	331894	336057.6	5477.81	1.63
	HQC	7.2	395512	397238	408103	410097	404910	403172	6503.44	1.61
Analyst 3	LQC	4.8	260517	255130	251303	252513	254041	254700.8	3562.98	1.40
	MQC	6	319784	321669	320922	330098	321002	322695	4193.49	1.30
	HQC	7.2	395231	403225	402942	415116	411531	405609	7841.43	1.93

Table 3: Robustness results of various parameters tested for 5-FU

Variables	Value	Conc. (µg/mL)	Mean peak area	SD	% RSD	Mean R_t (min)	SD	% RSD
Flow rate (mL/min)	0.8	6	407271.6	3876.19	0.95	8.94	0.11	1.22
	1	6	336113.4	1855.99	0.55	7.58	0.07	0.94
	1.2	6	243530.6	2753.34	1.13	6.02	0.05	0.93
Wavelength (nm)	264	6	337290	1709.90	0.51	7.58	0.07	0.94
	266	6	336113.4	1855.99	0.55	7.58	0.07	0.94
	268	6	334823.2	4155.27	1.24	7.58	0.07	0.94

Robustness:

Robustness of developed method was studied by varying the flow rate (0.8, 1 and 1.2mL/min) of mobile phase and the detection wavelength (264, 266 and 268nm). The observed percentage relative deviation was found less than 2% for all the samples (Table 3), indicating that the developed method was satisfactorily robust and the responses were unaffected by these changes.

System suitability:

System suitability parameters i.e., Height Equivalent to Theoretical Plate (HETP), theoretical plate, theoretical plate/meter and, tailing factor of peak and peak purity index were calculated for the analytical method. The results of the study are summarised in Table 4.

Table 4: Results of system suitability parameters

Parameters	Value
HETP	17.07
Theoretical plate	8788.27
Theoretical plate/meter	58588.48
Tailing factor	1.22
Peak purity index	1.00

CONCLUSION:

In the present study the estimation of 5-FU was carried out using RP-HPLC method. The reports of validation studies indicated that the method was accurate, precise, rugged and robust. This method can be successfully applied to estimate 5-FU and its concentration in various pharmaceutical formulations.

CONFLICT OF INTEREST:

The authors declare that there is no conflict of interest with a person or an organisation related this research work.

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RESEARCH ARTICLE

Pharmacognostical and Phytochemical Evaluation of Renowned Polyherbal Formulation, Sharbat Ahmad Shahi: A Comprehensive Approach with Modern Techniques

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ABSTRACT:

Background: An increase in the awareness about the advantages of the traditional medicines has led to the commercialization of the formulations used for the treatments. To obtain effective drug with proven efficacy, it is necessary to ascertain standards through contemporary scientific and technical procedures which would certainly enhance the reputation and acceptance of Unani medicines. Quality control of Unani polyherbal formulations is the need of the day for better acceptance of Unani medicine. Sharbat Ahmad Shahi (SAS) is a Unani polyherbal formulation containing ten ingredients, widely used in treatment of psychiatric disorders. The present study was taken up to scientifically evaluate the various physicochemical parameters to standardize the formulation. In the present research work an attempt has been made to ascertain various physicochemical and phytochemical parameters of SAS such as ash values, moisture content, extractive values, thin layer chromatography (TLC) and high-performance thin layer chromatography (HPTLC) etc, it may be useful for its quality control and standardization. **Methods:** Ingredients were identified by the experts. The method mentioned in national formulary of Unani Medicine with modification was followed for preparation of the sharbat. The formulation was also subjected to preliminary physicochemical and phytochemical evaluation. Various parameters such as organoleptic characters, extractive values for the extract and TLC and HPTLC fingerprinting were carried out for evaluation of SAS. **Results:** Preliminary phytochemical, physicochemical and chromatographic profile of the formulation was established. Qualitative chemical tests indicated the presence of alkaloid, glycoside, tannins, and phenolics. TLC and HPTLC fingerprinting studies showing the presence of various phytochemicals present in the drug. **Conclusion:** Findings of the present work can be used as a reference for preparation and standardization of SAS.

KEYWORDS: Polyherbal formulation, physicochemical parameters, phytochemical parameters, standardization.

INTRODUCTION:

Herbal medicine is still the backbone of about 70–80% of the world's population, mainly in developing countries, for primary health care because of enhanced cultural acceptability, better compatibility with the human body and lesser side effects. India has an immense heritage of traditional systems of medicine for various ailments. Due to lack of quality control measures, people are incapable to exploit the benefit of the traditional systems of medicine. Due to this scientific attentiveness a scenario has formed to undertake the research activities like standardization of traditional medicines and to develop the scientific methods for the manufacture of quality medicines. WHO has emphasized the need to certify quality control of medicinal plant products by using modern techniques and by applying appropriate parameters. In order to overcome certain predictable shortcoming of the pharmacopoeial monograph other quality control measures must be explored[1],[2],[3],[4]. Out of several oral unit dosage forms, Sharbat (syrup) is one of the most suitable/practical dosage forms due to its

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easy portability for prolong use, stability and accuracy of dose, etc. Therefore, in the present study, physiochemical parameters for Sharbat Ahmad Shahi (SAS) were investigated. Formulation selected for study is being usually used and manufactured by the Unani pharmacies. It contains ten ingredients, Aftimoon (*Cuscuta reflexa Roxb*.), Badranjboya (*Nepeta hindostana Roth.*), Berge Gaozaban (*Borago officinalis Linn.*), Bisfaij (*Polypodium vulgare Linn.*), Sana makki (*Cassia angustifolia Vahl.*), Berge Banafsha (*Viola odorata Linn.*), Gule Surkh (*Rosa damascena Mill.*), Gule Nilofer (*Nymphaea alba Linn.*), Halela siyah (*Terminalia chebula Retz.*), Tukhme Balangu (*Lallemantia royleana Benth.*). This particular formulation is mentioned in *Bayaaze kabeer* and *Qarabadeen majeedi*. It is used in the treatment of Malikhuliya (Melancholia), Junoon (Schizophrenia), Khafkan (Palpitation) and Mania[5]. Moreover, this formulation has been delineated for its pharmacological activity as anxiolytic and there is a need to develop its quality control standards.

MATERIALS AND METHODS:

All the ingredients were procured from Shamsi Dawakhana, Ballimaran, Delhi-110006, India but one of its ingredient i.e Berge banafsha (Viola odorata Linn.) was procured from Kashmir raw drug dealers with the knowledge of Unani physician. The identity and authenticity of the drugs were established by an expert from Department of Botany, School of Chemical and Life Sciences, Jamia Hamdard, New Delhi-110062. The Sharbat Ahmad Shahi was prepared as per the formulation composition given below[6].

Table 1: Ingredients of Sharbat Ahmad Shahi

S. No	Unani Name	Botanical Name	Parts Used	Quantity
1	Aftimoon	*Cuscuta reflexa* Roxb.	Dried leaves	24g
2	Badranjboya	*Nepeta hindostana* Roth.	Leaves	225g
3	Berge Gaozaban	*Borago officinalis* Linn.	Leaves	200g
4	Bisfaij	*Polypodium vulgare* Linn.	Seeds	75g
5	Sana Makki	*Cassia angustifolia* Vahl.	Leaves	9g
6	Berge Banafsha	*Viola odorata* Linn.	Leaves	50g
7	Gule Surkh	*Rosa damascena* Mill.	Flowers	40g
8	Gule Nilofer	*Nymphaea alba* Linn.	Flowers	75g
9	Halela Siyah	*Terminalia chebula* Retz.	Fruits	75g
10	Tukhme Balangu	*Lallemantia royleana* Benth.	Seeds	75g
11	Qand Safaid	Sugar		4kg

Preparation of Sharbat Ahmad Shahi:

The Sharbat Ahmad Shahi was prepared as per the formulation composition given in the Qarabadeen Majeedi (1986) and Hamdard Pharmacopoeia of Eastern Medicine. All the dry ingredients (mentioned above in the table) were soaked in 8 litres of water overnight and boiled in the next morning till only one-fourth volume of water remains. The decoction was then rubbed with the hands, cloth-filtered, and re-filtered through a cotton wool layer spread out on a sieve into another vessel. Next, the white sugar and citric acid were added to the decoction which is again heated. And any impurity that collects on the surface is removed. When the *Qiwam* (Sugary syrup) became concentrated, the sodium benzoate dissolved in a little water was added, and immediately after boiling, the *Qiwam* (Sugary syrup) was taken off the fire and sieved through a fine muslin cloth and preserved in a glass bottles.

Parameters for physicochemical standardization:

- Organoleptic properties
  - Appearance
  - Color
  - Smell
  - Taste
- Alcohol soluble matter
- Water soluble matter
- Successive extractives:
  - Pet. Ether
  - Chloroform
  - Ethyl alcohol
  - Water
- pH
  - pH of 1% aqueous solution
  - pH of 10% aqueous solution
- Ash value
  - Total ash
  - Water soluble ash
  - Acid insoluble ash
- Density
  - Bulk density

Parameters for Phytochemical Standardization:

- Total alkaloids
- Tannins
- Total phenolics
- Sugar
  - Total sugar
  - Reducing sugar
- TLC
- HPTLC

Table 2: Test for Phytochemical Analysis

Test	Reagents	Observations
Alkaloids	Mayer's Reagent	Creamy or white precipitate indicates the presence of an alkaloid.
	Dragondroff's Reagent	Orange or orange-red precipitate confirms the presence of an alkaloid.
	Hager's Reagent	Formation of an orange yellow precipitate confirms the presence of an alkaloid.
Glycosides	Killer-Killani Test	Formation of reddish brown colour at the junction of two layers and the upper layer turned bluish green in presence of glycosides.
	Borntrager's Test	pink to red colour due to the presence of glycoside
	Legal Test	pink or red colour was obtained which indicates the presence of glycosides.
Tannins	Bromine Water Test	Decolourization of bromine water occurs, shows presence of tannins
	Ferric Chloride Test	Bluish black colour is produced which disappears on addition of a few ml of dilute sulphuric acid solution followed by the formation of a yellowish-brown precipitate.
	Lead Acetate Test	yellow or red precipitate is formed
Phenolic	Ferric Chloride Test	blue or green colour is produced.
	Lead Acetate Solution Test	Formation of yellow colour precipitate indicates the presence of phenolic compounds.
	Gelatin Solution	observed for the formation of precipitate of turbidity
	Liebermann Test	A red colour is obtained on dilution, and turns blue when made alkaline with aqueous sodium hydroxide solution.
Fixed oils	Add 5 drops of each extract, few drops of 10% sodium hydroxide (NaOH) and 1 ml of 1% Copper sulphate ($CuSO_4$) solution. Appearance of clear blue colour of a solution confirmed the test.	
Volatile oils	0.05- 0.1 ml of petroleum extract of the test drug was allow to falling onto a filter paper and leave it at room temperature for 24 hours. The volatile oil evaporates completely within 24hours without leaving a translucent or greasy mark.	
Reducing Sugars	Fehling's Test	Aqueous extract of the test drug was taken and a mixture of equal volume of Fehling's solution I and Fehling's solution II were added and heated on a water bath, observed the colour of the precipitate. A brick red precipitate shows the presence of reducing sugars.
	Benedict's Test	Equal volume of Benedict's reagent and a test solution was taken in a test tube and heated on a water bath. The solution appears red, yellow or green in colour depending upon the amount of reducing sugar present in a solution (Adediran and Bark, 1991).

THIN LAYER CHROMATOGRAPHY (TLC):[7]

Thin layer chromatography is the best-known technique of plant biochemistry. It is used for preliminary separation and determination of plant constituents. It is helpful for proper identification, authentication of phytoconstituents. Different fractions of plant drug extract were subjected to TLC to find out the compounds present in them with their different Rf values. The Rf values of the spots were calculated by the following formula:

$$R_f \text{ Value} = \frac{\text{Distance travelled by spot}}{\text{Distance travelled by solvent}}$$

- **Application of the sample:**

The collected test sample was applied on TLC plate as a spot 1cm from edge of the plate with the help of a capillary tube. The plate was then placed in closed container.

- **Chromatographic chamber, conditions of saturation and development of TLC plates:**

A rectangular chromatographic glass chamber was used in the experiment, having dimensions (16.5 cm × 29.5 cm). Before using the chromatographic chamber, it was allowed to saturate. Generally, the experiments were carried out at room temperature in day light.

- **Developing Solvent System:**

So many solvent systems were tried to develope for each test sample but the suitable resolution was acquired in the solvent systems specified in respective tables.

- **Spraying Equipment:**

Spraying equipment is used to detect the different constituents present on TLC plates. It consists of air compressor which was attached to a glass sprayer. The sprayer was filled with a 50 ml of 8% H_2SO_4 and then used. The sprayer was washed with water separately, after each spray.

- **Development of spot:**

After spraying the TLC plates were kept in oven at 110ºC for 5 min till the development of spots seen visibly.

HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY (HPTLC):

HPTLC analysis of Sharbat Ahmad Shahi (SAS) extract was carried out for their qualitative analysis. Hence, simultaneous fingerprinting analysis of extracts was carried out using newly developed HPTLC method following the ICH guidelines[17].

HPTLC sample preparation, Optimization of the solvent system for HPTLC and various chromatographic condition:

The solvent system composition was adjusted by testing different solvent compositions of varying polarities. Altered tested compositions of solvent system, the preferred resolution of the compounds, together with proportioned and reproducible peaks, was achieved using Toluene: Ethyl acetatel: Formic acid (6:3:1, v/v/v) solvent system for methanolic extract and Toluene: Ethyl acetate: Formic acid (6:3:1, v/v/v) for chloroform

extract. Well separated and squeezed bands were imagined by using anisaldehyde sulphuric acid spraying reagent. HPTLC fingerprinting of extracts has been presented in below tables and figures show the qualitative analysis of extracts.

The methanolic and chloroform extract of SAS were re-formed using methanol and chloroform respectively (HPLC grade) and prepared 30mg/mL concentration of the extracts. The samples were dotted in the form of bands (4.0mm width), on pre-coated HPTLC silica gel aluminium plates ($60F_{254}$; 20 × 10cm, Merck KGaA, Germany) by a CAMAG microliter syringe using a CAMAG Linomat V (Muttenz, Switzerland) and were controlled by Win CATS software (CAMAG). A constant application rate of 10µL/s was applied and the space between two bands was 6.0mm. The slit dimension was maintained at 5.0 × 0.3mm, and 20mm/s scanning speed was employed. The solvent system of the SAS extracts consisted of toluene: ethyl acetate: formic acid, (6:3:1). Linear ascending development was carried out in a 20 × 10cm twin trough glass chamber, saturated with the solvent system. The optimized chamber saturation time for the solvent system was 15 min at room temperature. The length of chromatogram run was 80 mm. Subsequent to development, HPTLC plates were dried in an oven at 60°C for five min. Densitometric scanning was performed on a CAMAG TLC scanner IV (absorbance mode 530nm) with WinCATS software after spraying the developed plate with anisaldehyde-sulphuric acid reagent and heating it on a hot air oven at 110°C for five minutes.

OBSERVATIONS AND RESULTS:

Table 3: Organoleptic properties of SAS

S. No.	Properties	Observation
1	Appearance	Viscous liquid
2	Colour	Dark brown
3	Smell	Pleasant
4	Taste	Sweet

Table 4: Physicochemical parameters

Parameters	Batch-I	Batch-II	Batch-III	Mean±SEM
Alchohol soluble matter	19.6	20.0	19.8	19.8±0.115
Water soluble matter	30.0	29.1	30.4	29.8±0.416
Successive extractive values:				
Petroleum ether	2.40	2.38	2.30	2.36±0.030
Chloroform	1.2	1.8	1.5	1.5±0.173
Alcohol	14.24	15.30	14.20	14.58±0.360
Water	1.2	1.8	2.0	1.66±0.240
pH of 1% solution	5.07	5.12	5.10	5.09±0.014
pH of 10% solution	5.01	5.08	5.1	5.06±0.027
Loss on drying at 105 ºC	8.23	8.18	8.3	8.23±0.034
Total ash	4.5	3.9	4.3	4.23±0.176
Acid insoluble ash	2.7	2.2	2.5	2.46±0.145
Water soluble ash	1.75	1.62	1.78	4.23±0.176
Bulk density	4.36	3.98	4.20	4.18±0.110

Table 5: Phytochemical screening of individual extract of SAS

S. No	Phytochemical test	Petroleum ether	Chloroform	Methanolic	Aqueous
1	Alkaloid	+	+	+	+
2	Glycosides	+	+	+	_
3	Tannins	+	+	+	+
4	Volatile oils	+	+	+	+
5	Fixed oils	+	+	+	+
6	Flavanoids	+	+	-	+
7	Reducing sugar	+	+	+	+
8	Saponins	+	+	+	+

Table 6: Phytochemical parameters

Parameters	Batch-I	Batch-II	Batch-III	Mean±SEM
Total Alkaloid	0.04	0.03	0.04	0.036±0.003
Reducing sugar	20.20	21.51	19.80	20.50±0.516
Non-reducing sugar	40.16	43.20	43.40	42.25±1.048
Phenolic	5.0	4.8	4.4	4.46±0.290
Tannin	0.52	0.58	0.66	0.58±0.040

Table 7: R_f value of SAS

Fraction	Methanol	Chloroform
Solvent system	Toluene: Ethyl acetatel: Formic acid (6:3:1, v/v/v)	Toluene: Ethyl acetatel: Formic acid (6:3:1, v/v/v)
No of spot	9	8
R_f values	0.16, 0.22, 0.25, 0.29, 0.35, 0.41, 0.58, 0.7, 0.83	0.15, 0.2, 0.26, 0.4, 0.5, 0.59, 0.7, 0.73

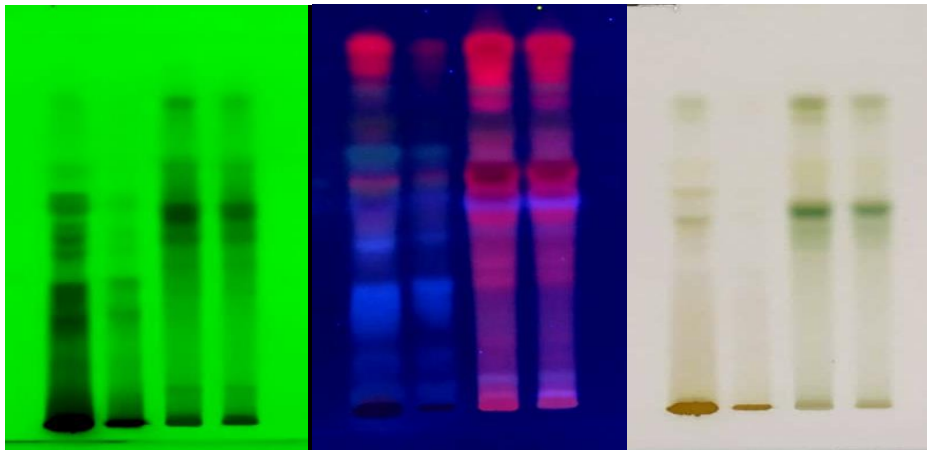

Fig 1, 2 and 3 **Under UV light of 254 nm** **Under UV light of 366 nm** **Under Day light**
Track 1 and 2 Methanolic extract ; Track 3 and 4 Chloroform extract

Table 8: R_f values for Methanolic extract at 366 nm (Track 1)

Peak	R_f value	Maximum Height (AU)	Maximum percentage	Area percentage
1	0.13	824.4	21.67	38.60
2	0.29	482.0	12.67	10.35
3	0.43	530.9	13.96	21.10
4	0.53	287.3	7.55	5.05
5	0.67	440.9	11.59	9.61
6	0.71	388.2	10.20	3.84
7	0.75	379.7	9.98	4.62
8	0.80	388.0	10.20	5.43
9	0.88	83.2	2.19	1.40

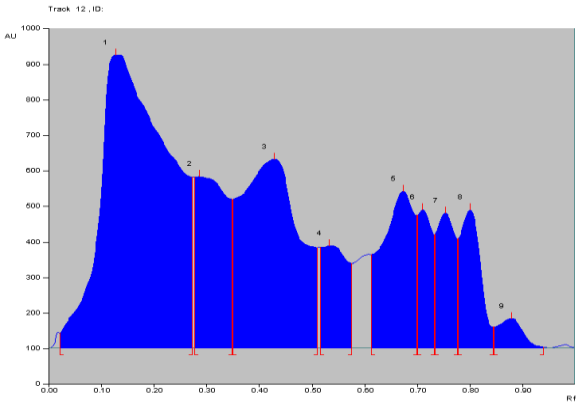

Fig 4: HPTLC chromatogram of methanolic extract of SAS at 366 nm

Table 9: R_f values for Methanolic extract at 254 nm (Track 2)

Peak	R_f value	Maximum Height (AU)	Maximum percentage	Area percentage
1	0.03	29.2	1.94	0.64
2	0.13	687.2	45.71	52.04
3	0.31	161.4	10.74	7.76
4	0.45	195.3	12.99	17.83
5	0.54	155.5	10.35	8.20
6	0.65	75.3	5.01	3.64
7	0.70	82.4	5.48	2.87
8	0.78	85.4	5.68	5.28
9	0.89	31.6	2.10	1.74

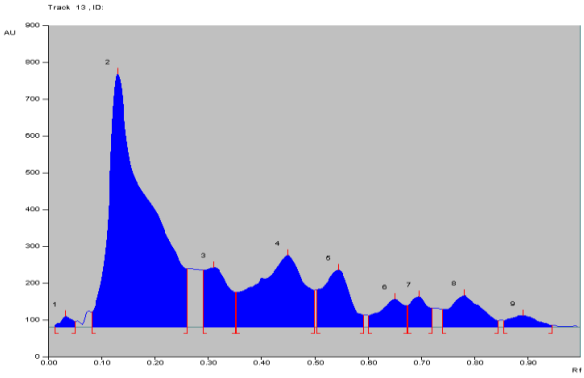

Fig 5: HPTLC chromatogram of methanolic extract of SAS at 254 nm

Table 10: R_f values for Chloroform extract at 366 nm (Track 3)

Peak	R_f value	Maximum Height (AU)	Maximum percentage	Area percentage
1	0.04	32.6	1.70	0.87
2	0.07	41.6	2.17	0.48
3	0.13	460.8	24.03	17.65
4	0.23	216.3	11.28	11.04
5	0.58	263.5	13.74	19.01
6	0.68	437.3	22.81	21.33
7	0.75	465.4	24.27	29.62

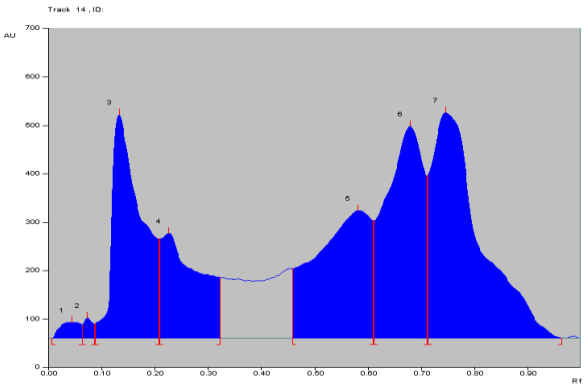

Fig 6: HPTLC chromatogram of chloroform extract of SAS at 366 nm

Table 11: R_f values for Chloroform extract at 254 nm (Track 4)

Peak	R_f value	Maximum Height (AU)	Maximum percentage	Area percentage
1	0.04	11.3	0.62	0.24
2	0.07	43.0	2.38	0.44
3	0.13	481.1	26.58	22.78
4	0.23	199.2	11.01	10.13
5	0.46	106.8	5.90	6.38
6	0.53	147.8	8.16	6.89
7	0.60	175.3	9.68	8.72
8	0.68	242.6	13.41	14.10
9	0.76	272.1	15.04	24.92
10	0.88	130.6	7.22	5.40

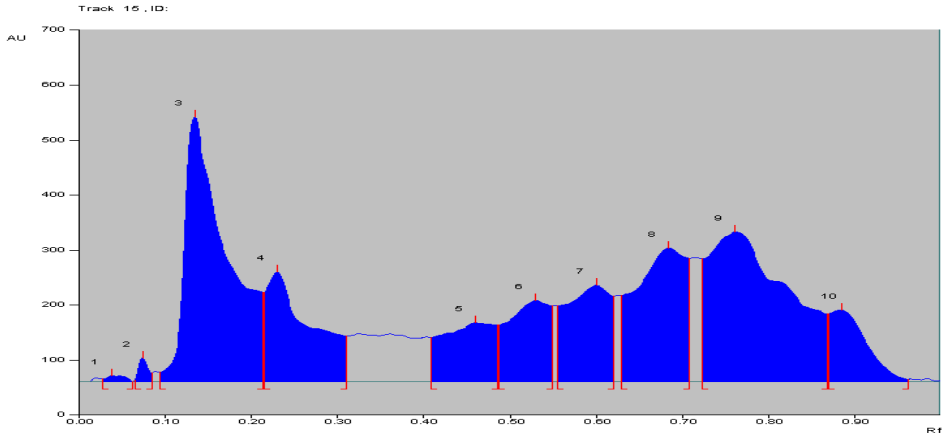

Fig 7: HPTLC chromatogram of chloroform extract of SAS at 254 nm

CONCLUSION:

In this investigation various standardizations parameters such as physicochemical parameters, preliminary phytochemical investigation and TLC profiles of sequential extraction of sample in petroleum ether, chloroform and methanol extracts were studied, which are being reported for the first time in this formulation. The above findings of the present work can be used as a reference for preparation and standardization of SAS.

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CONFLICT OF INTEREST:

There are no conflicts of interest.

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RESEARCH ARTICLE

Enhancement of Solubility and effect of Granulation methods on drug release in sustained release matrix tablets of a poorly soluble drug

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ABSTRACT:

Domperidone, a BCS Class II drug chosen as a model drug which is highly permeable and poorly soluble, mainly used in the treatment of Emesis. It has a strong affinity for D_2 receptors, chemically related to Haloperidol, but pharmacologically related to metaclopramide. Sustained release tablet of Domperidone are preferred because of prolonged drug release in order to reduce the frequency of dosing. In the present study, it was decided to design controlled release formulation of Domperidone with pH dependent release profile so as to minimize/prevent initial drug release in the stomach in order to reduce the possible gastro-irritant and ulcerogenic effects of the drug The study was carried out using release retarding polymers like HPMC (hydrophilic matrix polymer), Eudragit (polyacrylate polymers) and natural polymers like guar gum and xanthan gum were used. An ideal matrix formulation prepared using different polymer and diluent concentrations. The formulation were prepared using various compression techniques like wet granulation technique and direct compression techniques in order to release their contents in a sustained manner over a certain period of time. As Domperidone is class II drug having low solubility and more permeability and, the solubility of Domperidone was initially enhanced by preparing solid dispersions using solvent evaporation method by using drug and polymer (β-cyclodextrin) in three different ratios i.e. 1:0.75, 1:1, 1:1.5 and the solid dispersion mixture containing drug and polymer in the ratio 1:1.5 showed 97% drug release in one hour was optimized as the best mixture. In the present work drug and polymer mixture in the ratio 1:1.5 was further formulated into tablets by incorporating natural and synthetic gums by using different granulating techniques like direct compression and wet granulation in three different concentrations. Formulation (F3) containing drug and Xanthan gum in the ratio 1:1 prepared by wet granulation technique could sustain the drug release over a period of 12h and hence considering all the post compression parameters it was optimised as the better formulation. FTIR, DSC, X-Ray Diffraction, SEM studies were performed for optimised solid dispersion mixture and also the optimised formulation.

KEYWORDS: Solubility, Solid Dispersions, Matrix tablets, Direct Compression, Wet granulation.

INTRODUCTION:

Among all the routes that have been explored for the systemic delivery of drugs, Oral drug delivery has been known for decades as the most widely utilized route of administration. Nowadays most of the pharmaceutical scientists are involved in developing an ideal drug delivery system. This ideal system should have an advantage of single dose for long duration of the treatment and it should deliver the drug directly at specific site. The goal in designing sustained delivery systems is to reduce the frequency of the dosing or to increase effectiveness of the drug by localization at the

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site of action, reducing the dose required or providing uniform drug delivery.[1]

During the past two decades, there has been a profound increase in the development of sustained release drug delivery system due to various factors like expiration of various international patents, prohibitive cost of developing new drug entities, discovery of new polymeric materials that are suitable for retarding the release of the drug, improvement in therapeutic efficiency and safety[2]. Of all sustained release dosage forms, matrix tablets are considered to be the commercially feasible dosage forms that involve the least processing variables, utilize the conventional facilities and accommodate large doses of drug. Matrix devices had gained steady popularity in pharmaceutical research due to their chemical inertness, drug embedding ability and drug release character[3].

Vomiting is regulated centrally by the vomiting centre and the chemoreceptor trigger zone (CTZ), which are present in the medulla. The CTZ is sensitive to chemical srimuli and is the main site of action of many emetic and antiemetic drugs. Dopamine (acting through D2 receptors) is an inhibitory transmitter in the GIT normally acts to delay gastric emptying when food is present in stomach. It also appears to cause gastric dilation and LES relaxation attending nausea and vomiting[4]. Domperidone blocks D2 receptors and has an opposite effect-hastening gastric emptying and enhancing LES tone by augmenting Ach release, clinically this action is exerted through 5HT receptors. The central anti dopamergic action on CTZ responsible for antiemetic property[5].

The main aim of the present work is to formulate and evaluate sustained release matrix tablets of Domperidone by using natural polymers like Xanthan gum and Guar gum using various compression techniques like wet granulation technique and direct compression techniques.

MATERIALS AND METHODS:

MATERIALS:

Domperidone, Xanthan gum, Guar gum, Eudragit RS 100, and Hydroxyl propyl ß cyclodextriene were obtained from Yarrow chem products, Mumbai. Lactose was purchased from and Talc from Otto Chemika Biochemika reagents.

METHOD:

Domperidone a BCS class II drug is a poorly soluble in water. Therefore, solubility of Domperidone was enhancing by preparing solid dispersions using solvent evaporation technique. Solvent evaporated mixtures were by using drug and carrier in different ratios such as 1:0.75, 1:1 1:1.5. Solid dispersions were characterised by FTIR spectroscopy, DSC studies**,** X-ray diffraction, and dissolution studies. Dissolution studies were performed for the solvent evaporated mixtures using USP dissolution type II apparatus (paddle method) with 50 rpm 6.8pH phosphate buffer as dissolution mediums at 37±0.5ºC. Based on physicochemical characterization, solvent evaporated mixture containing drug and carrier in the ratio of 1:1.5 (equivalent to 30mg of drug) was selected to formulate into sustain release tablets.

Table 1: Formulation table for Solid Dispersions

Formulation code	Carriers	Method	Drug: Polymer ratio
SD 1	HP-β-CD	Solvent evaporation	1: 0.75
SD 2	HP-β-CD	Solvent evaporation	1: 1
SD 3	HP-β-CD	Solvent evaporation	1:1.5

Sustained release matrix tablets of Domperidone were prepared by using direct compression technique and wet granulation techniques.

DIRECT COMPRESSION TECHNIQUE:

All the ingredients were weighed separately dried for 30 mins at 50˚C and cooled to room temperature. They were then passed through #40 sieve separately to attain fine powder. The sieved powders were weighed and added geometrically (except glidant and lubricant), mixed properly and then finally lubricant and talc added just before compression. This mixture was directly compressed using rotary tablet punching machine equipped with 8mm flat faced circular punches at a constant compression force[6].

WET GRANULATION TECHNIQUE:

In this technique all the ingredients except lubricants and anti- adherent were mixed properly and then the mixture is formed into dough using 2% starch paste as a binding agent. Then the dough is then passed through the sieve #22 to form the granules.

Table 2: Formulation table for Preparation of tablets:

Ingredients	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12
Method	**Wet Granulation Method**						**Direct Compression Method**					
Domperidone	30	30	30	30	30	30	30	30	30	30	30	30
Xanthan gum	7.5	15	30	-	-	-	7.5	15	30	-	-	-
Guar gum	-	-	-	7.5	15	30	-	-	-	7.5	15	30
Lactose	158	150	--	158	150	135.5	158	150	135.5	158	150	135.5
Mg Stearate	3	3	3	3	3	3	3	3	3	3	3	3
Talc	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5

The so formed granules were dried at 50°C and then cooled at room temperature. Lubricant and glidant were added. Then the mixture was compressed using rotary tablet punching machine equipped with 8mm flat faced circular punches at a constant compression force.[7]

Evaluation Parameters:

Pre Compression Parameters:

Prior to the compression of tablets, the blend of drug and excipients of all the batches were evaluated for various micromeritic properties like Angle of repose, bulk density, tapped density, Carr's Compressibility index and Hausner's ratio[8].

Post Compression Parameters:

Thickness:

Thickness of the sustained release tablets were tested using calibrated Vernier-calipers. The tablet thickness was controlled within a ±5% variation.

Hardness test or crushing strength:

Hardness is the force required to break a tablet across the diameter. It is measured in kilograms and a crushing strength of usually 4kg is considered to be the minimum satisfactory for tablets. The hardness was tested using Monsanto hardness tester.

Friability test:

This is an in process quality control test performed to ensure the ability of tablets to withstand shocks during processing, handling, transportation, and shipment. It is usually measured by using Roche Friabilator[9].

$$\% \text{ friability} = \frac{\text{Weight}_{initial} - \text{Weight}_{final}}{\text{Weight}_{initial}} \times 100$$

Uniformity of weight or Weight variation test:

This test is performed to check the weight of the tablet frequently (every half an hour) so that in case of any corrections will be made during the compression of tablets. Any variation in the weight of the tablet may lead to over dose or under dose of medication. Therefore every tablet in each batch should have a uniform weight[10].

$$\% \text{ deviation} = \frac{\text{individual weight-average weight}}{\text{average weight}} \times 100$$

Estimation of drug content:

Drug content was determined accurately by weighing 5 tablets and crushing them in motor with the help of a pestle. Content uniformity was calculated using the following formula.

% purity =10 C (Au/As)

Where, C= Concentration, Au and As= absorbance obtained from the standard preparation and assay preparation respectively**.**

***In–vitro* drug release studies:**

The dissolution behaviour of Domperidone was recorded using a dissolution apparatus (Disso 2000 LAB INDIA, Mumbai). USP dissolution apparatus with rotating paddle assembly (type II) was used at 50 rpm, in 900 ml of deionised water (phosphate buffer pH 6.8). The mean of the three determinations was used to calculate the drug release from the tablets. The samples were withdrawn at predetermined time intervals, and equal amount of fresh buffer was replaced. The obtained samples were filtered and assayed spectrophotometrically at 285nm[11].

FT-IR Study:

The FT-IR spectra of pure drug, polymers and optimised formulation were scanned over a frequency range 4000-400 cm-1 by placing sample on diamond ATR and analyzing for the presence of characteristic peaks[12].

Thermal Analysis:

DSC was performed using DSC calorimeter to study the thermal behaviour of pure drug, polymers and mixture of optimised formulation. The required amounts of samples were heated in sealed aluminium pans under nitrogen flow (30ml/min) at a scanning rate 5°C per min from 40°C to 250°C. The heat flow as a function of temperature and enthalpy change was measured for the drug, polymers and mixture of optimised formulation[13].

X-ray diffraction analysis:

X-ray diffraction is an important tool to investigate the crystal structure as well as the average structural spacing between the layers or rows of atoms in an unknown material. Formulations were subjected to X-ray diffraction analysis, using Cu target slit 10 mm to investigate the physical state of Domperidone, HP-β-CD and Domperidone solvent evaporated mixtures.[14]

Scanning Electron Microscopy:

Scanning electron microscope (SEM) allows viewing and surface analysis of solid dispersions as well as spatial pattern formation for the scanned object. This is very important for the qualitative assessment of their properties such as particle size, their shape, morphology, porosity, presence of crystalline forms, as well as tested powders texture monitoring[15].

Characterization of pure drug Domperidone:

Identification of Domperidone by UV- visible spectrophotometer:

Standard plot of Domperidone was constructed by taking series of concentration in pH 6.8 phosphate buffer and pH 1.2 N HCl buffer. The absorption spectra were recorded in the wavelength region of 200-400 nm in the UV visible spectrophotometer. The absorption maximum of Domperidone was found to be 285 nm.

Melting point:
Melting point of Domperidone was determined using melting point apparatus and DSC. The temperature at which the drug starts to melts is recorded and compared to its standard value. Melting point of Domperidone was found to be 245.98°C by DSC and 242-246°C by melting method, which complies with the standard values in the official monograph as per USP- NF. The above determined results show the drug is pure and free from impurities[16].

Solubility:
Solubility determination of Domperidone was carried out in water and organic solvents. As it is class II drug, Domperidone shows poor solubility. It was found that Domperidone is practically insoluble in water, but soluble in organic solvents like ethanol, dimethylsulfoxide. The above estimation done was beneficial for further studies[17].

Compatibility studies of solid dispersions:
Visual inspection:
Visual inspections were conducted to check the compatibility between drug and excipients in its physical mixture when stored in glass container at room temperature for a certain period of time. The main objective of this inspection was to observe any colour change in the physical mixture. Throughout the examination, there was no change in the colour of the physical mixture throughout the examination period, indicating that drug and excipients are quite compatible each other.

FTIR (Fourier transformer infrared spectroscopy):
The physical mixture of drug and excipients was characterized by FTIR spectrum by yris Diamond TG/ DTA. The FTIR of the pure drug and solid dispersion did not show any peak indicating the absence of any chemical reaction between Domperidone and β-cyclodextrin

DSC (Differential scanning Calorimetry):
DSC thermogram of Domperidone showed a sharp peak at due to the melting point of the drug and indicating its crystalline nature. This shows that the drug considered for the research is in pure form. The measured melting endotherm of the β- cyclodextrin is 91.27°C.

XRD (X-ray diffraction):
The X-ray diffractograms of Domperidone showed characteristics sharp intensity diffraction peaks at 27.353°C

Determination of drug content and percent yield in solid dispersions:
All the solid dispersions prepared by solvent evaporation method were in the form of free flowing powders. The values of % drug content and % yield values were determined.

***IN- VITRO* DISSOLUTION STUDIES OF SD'S:**
The *in vitro* dissolution studies were performed for the solid dispersions. The drug release from solid dispersions was 74.57, 89.51 and 96.53% respectively in 1 hr.

Table 3: *In vitro* dissolution profile for solid dispersions.

Time (mins)	SD 1	SD 2	SD 3
5	32.69±1.534	35.46±3.98	51.68±3.92
10	48.31±1.630	49.29±2.41	54.35±2.31
15	51.49±1.75	53.42±3.06	64.21±5.04
30	54.36±2.26	65.49±1.91	81.58±3.92
45	66.39±2.06	74.51±2.41	89.58±3.40
60	74.51±2.08	89.51±2.57	**96.53±5.16**

From the above data Solid dispersion SD3 was optimised as the best formulation and was used further for compression into tablets.

PRECOMPRESSION PARAMETERS:
Precompression studies are conducted in order to determine the flow-ability, compressibility studies of the powder blend and granules.

Different tablet batch formulations F1-F6 were prepared by wet granulation and F7–F12 by direct compression methods. Solid dispersion mixture (75mg) and other all excipients were passed individually through #40 sieve and mixed well for 10 min in a mortar and pestle to form a powder blend. This blend was compressed into tablets by wet granulation and direct compression techniques using single punch rotary tablet punching machine using 8mm flat punches.

Table 4: Post compression parameters of various formulations prepared by Wet granulation and Direct Compression Techniques

Formulation code	Thickness (mm)	Weight variation(mg)	Hardness (kg/cm$^{2)}$	Friability (%)	Drug content (%)
F1	5.3±2.14	201.5±2.65	7.5±2.25	0.86±2.21	100.65±2.15
F2	5.5±2.16	198.5±2.45	8.5±2.29	0.75±2.02	99.96±2.13
F3	5.6±2.09	196.2±2.26	6.5±2.03	0.95±2.02	98.25±2.15
F4	5.8±2.25	197.5±2.24	7.5±2.23	0.86±2.04	99.56±2.12
F5	5.2±2.39	195.5±2.35	8.5±2.15	0.72±2.49	98.52±2.18
F6	5.4±2.24	201.2±2.39	7.9±2.18	0.73±2.42	100.56±2.12
F7	5.5±2.15	201.5±2.65	6.5±2.21	0.75±2.21	98.20±2012
F8	5.3±2.12	198.5±2.45	7.5±2.20	0.88±2.02	100.96±2.12
F9	5.6±2.03	197.2±2.21	6.5±2.04	0.85±2.02	98.37±2.03
F10	5.8±2.23	198.5±2.24	6±2.03	0.76±2.05	98.92±2.12
F11	5.2±2.35	195.5±2.35	6.5±2.15	0.72±2.11	101.55±2.12
F12	5.4±2.26	199.1±2.31	7±2.15	0.73±2.12	99.54±2.13

***In-vitro* drug release studies:**

Prepared tablets were evaluated by conventional *In-vitro* dissolution testing USP type II apparatus at 50 rpm. Initially the dissolution studies were out in 900 ml of 0.2 N HCl for 2 hours. After the completion of the 2 hours the remaining time period i.e., 10 hours the dissolution studies were performed in 6.8 pH phosphate buffer. Both the media was maintained at the temperature 37±5°C. The volume in order to maintain the sink condition; 5 ml of the dissolution media was withdrawn at the predetermined intervals and fresh preheated dissolution media was replaced.

Table 5: *In vitro* dissolution profile of Domperidone Sustained release tablets by wet granulation and Direct Compression Techniques.

TIME	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12
	Wet granulation Technique						Direct Compression Technique					
1	5.96±1.4	7.42±1.2	8.01±1.9	10.52±1.3	9.62±1.5	10.56±1.2	7.56±1.2	14.68±1.5	15.58±1.2	14.52±1.2	19.52±1.2	16.46±1.2
2	14.64±1.2	19.44±1.5	16.03±1.2	18.26±2.3	21.45±1.6	25.44±1.3	12.56±1.3	15.51±1.6	19.21±1.3	20.21±1.3	22.52±1.6	22.51±1.3
3	25.4±2.1	30.48±1.3	28.8±1.3	27.56±1.4	31.62±1.4	35.65±1.4	28.45±1.3	31.56±1.2	31.52±1.4	32.25±1.4	29.56±1.4	32.52±1.3
4	34.21±2.5	36.04±1.2	39.62±1.5	38.52±1.5	38.52±1.2	49.26±1.4	43.21±1.5	45.25±1.3	43.52±1.5	44.23±1.5	33.56±1.2	33.64±1.2
5	49.25±1.7	40.06±1.6	48.58±1.7	48.29±1.6	41.25±1.3	55.26±1.3	71.56±1.2	69.72±1.5	52.56±1.5	55.56±1.4	40.56±1.5	44.42±1.4
6	59.52±1.3	47.96±1.3	59.04±1.6	54.65±1.5	43.21±1.6	58.36±1.3	88.86±1.5	81.54±1.3	68.52±1.4	69.72±1.6	56.21±1.3	49.51±1.3
7	65.21±1.6	59.41±1.4	65.82±1.5	68.25±1.7	48.54±1.6	63.24±1.6	98.32±1.8	95.56±1.5	85.86±1.5	73.59±1.8	67.56±1.5	56.52±1.2
8	88.92±1.5	69.54±1.4	73.21±1.4	85.86±1.5	54.52±1.2	69.71±1.6	-	-	96.26±1.4	85.46±1.7	73.21±1.5	61.52±1.7
9	96.42±1.4	72.51±1.7	80.21±1.5	93.34±1.4	61.65±1.2	74.21±1.8	-	-	-	97.26±1.4	86.39±1.9	66.21±1.4
10	-	81.54±1.3	87.51±1.1	-	65.66±1.3	83.52±1.6	-	-	-	-	94.22±1.5	71.52v1.5
11	-	92.22±2.3	91.56±1.5	-	69.24±1.5	89.51±1.3	-	-	-	-	-	75.21±1.5
12	-	-	98.26±1.4	-	73.41±1.5	93.56±1.4	-	-	-	-	-	75.21±1.8

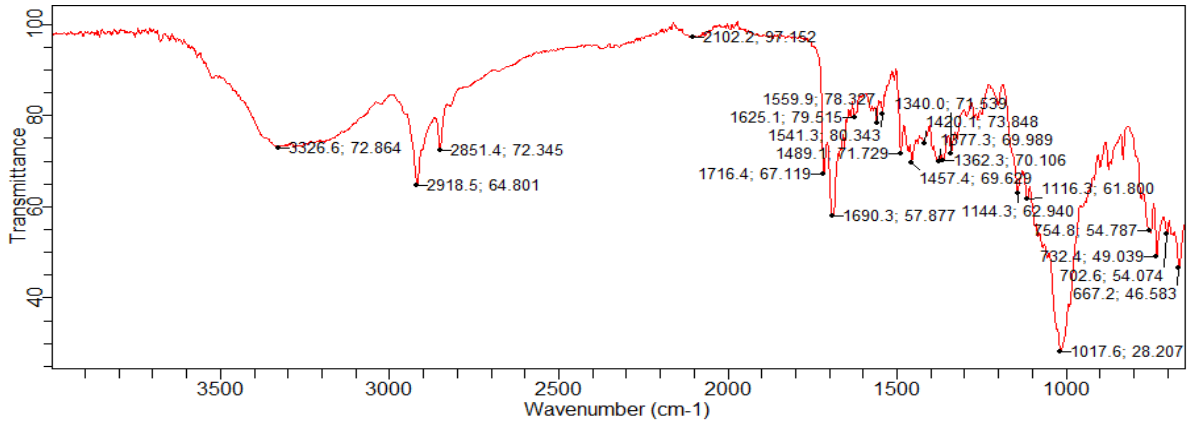

Fig 1: FTIR spectrum of Optimised Formulation

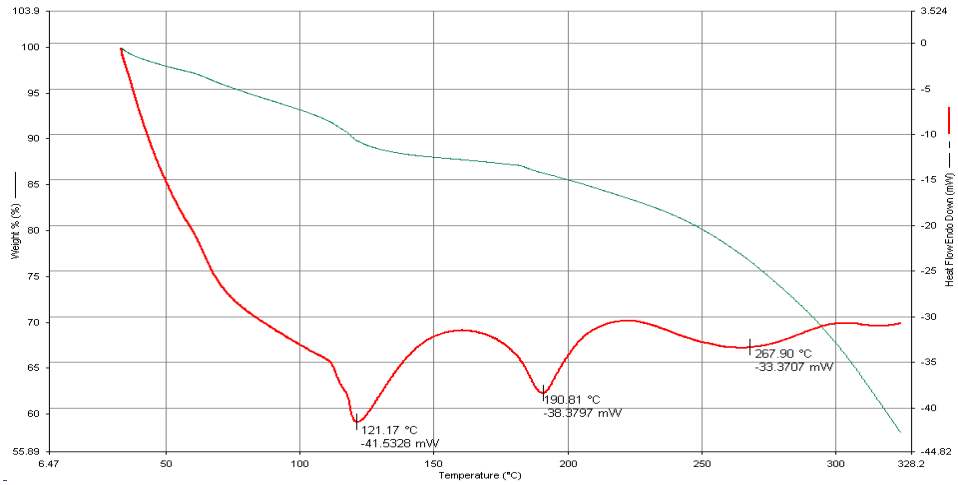

Fig 2: DSC thermo gram of Optimized formulation

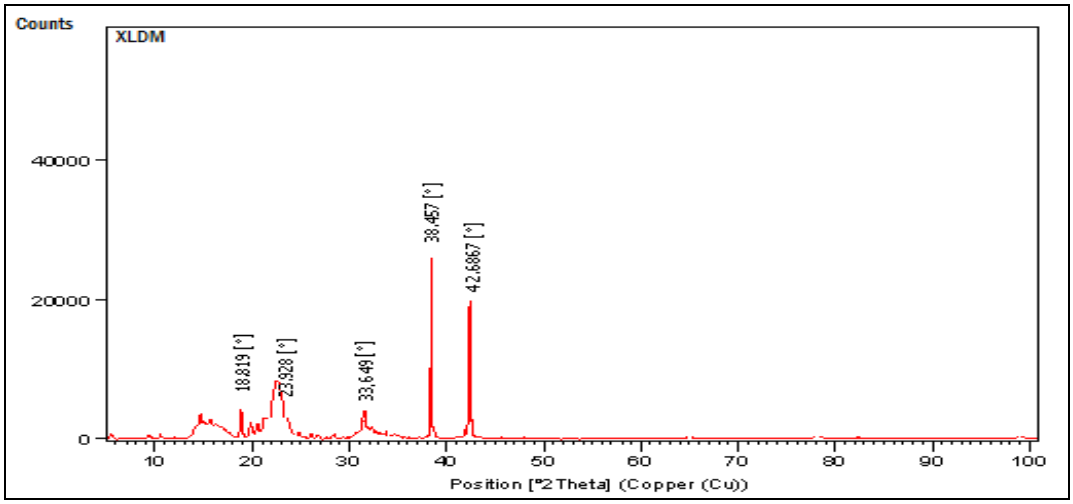

Fig 3: XRD of Optimised formulation

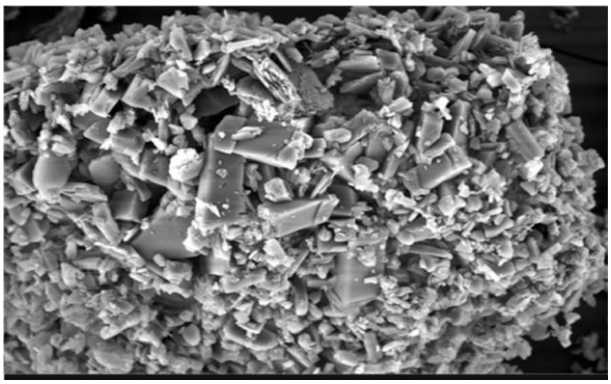

Fig 4: SEM image of Solid Dispersion mixture

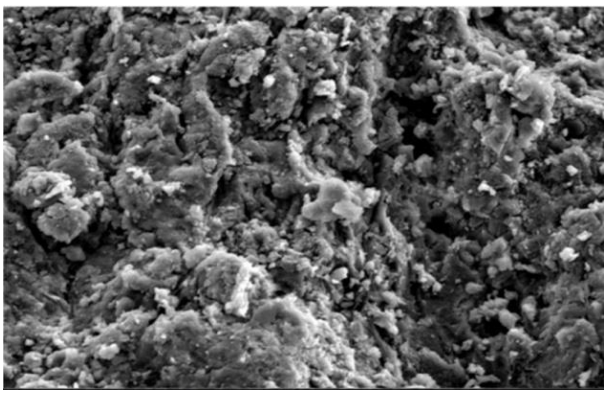

Fig 5: SEM image of optimized drug mixture

DISCUSSION:

As Domperidone is BCS Class II drug, having less solubility and more permeability. Therefore, the solubility of Domperidone was enhanced by preparing solid dispersions using solvent evaporation technique. β-cyclodextrin inclusion complexes were prepared in three different ratios like 1:0.75, 1:1, 1:1.5. In the present work drug:polymer in the ration 1:1.5which showed 97% of drug release in 1 hour was optimised as the best mixture and further formulated into tablets by incorporating natural gums in three different ratios using wet granulation and direct compression techniques.

The active pharmaceutical ingredient Domperidone was evaluated for its physical characteristics and drug polymer compatibility studies. The precompression powder blends of different batches were evaluated for various parameters like angle of repose, bulk density, tapped density, compressibility index Hausner's ratio. The results obtained were found to be satisfactory and within the specified limits.

The matrix tablets were prepared by wet granulation (F1-F6) and direct compression (F7-F12) containing different ratios of drug and polymer were subjected to various evaluation tests such as thickness, uniformity of weight, drug content, hardness and friability. The results have complied with the official pharmacopeial limits. It has been observed in this investigation that the formulation (F3) containing drug and Xanthan gum in the ratio 1:1 prepared by wet granulation technique could sustain the drug release over a period of 12h and hence considering all the post compression parameters it was optimised as the better formulation.

CONCLUSION:

Domperidone a BCS class II drug having low solubility and highly permeability was chosen as a model drug. It is mainly used in the treatment of Emesis. It has a strong affinity for D_2 receptors, chemically related to Haloperide, but pharmacologically related to Metaclopramide. Sustained release tablet of Domperidone is preferred because of its prolonged drug release manner, and therefore frequency of the dosing can be reduced.

In the present study, it was decided to design controlled release formulation of Domperidone with pH dependent release profile so as to minimize/ prevent initial drug

release in the stomach that will reduce the possible gastro-irritant and ulcerogenic effects of the drug. At the same time, there was no compromise on the biopharmaceutical profile of the drug as Domperidone is reported to be well absorbed through\out the GI tract by increasing the half-life eventually producing the sustained action. The study was carried out using release retarding polymers like Xanthan gum, Guar gum and different granulation techniques. This gives advantage in reducing the frequency of the dosage to increase effectiveness of the drug by localizing at the site of action.

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RESEARCH ARTICLE

In vitro Antioxidant and Phytochemical analysis of crude extracts of endophytic fungi (*Cladosporium sp*) from *Boerhaavia diffusa* Linn

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ABSTRACT:
In recent years, fungal endophytes from diverse host species have been widely studied for valuable compounds (enzymes, antimicrobial compounds). Fungal endophyte can act as the source of several secondary metabolites that can be used in medicine. In this study, we explored fungal endophytes associated with the medicinal plant *Boerhaavia diffusa* to determine the Phytochemicals and antioxidant properties. Solvent extraction was performed to obtain the crude from the fungus isolated from *B.diffusa*. DPPH assay was performed to determine the antioxidant property. Phytochemical characterization determined the phytochemical groups in the crude extract. GC-MS and FTIR were also performed for the extracts. FT IR analysis of the crude extract revealed the presence of chemical groups.

KEYWORDS: Endophytic fungi, *Boerhaavia diffusa*, Nyctaginaceae, Medicinal plant, Phytochemicals, Antioxidant, FT-IR, Gas chromatography-Mass spectrometry.

INTRODUCTION:
Endophytes form a synergetic relationship with their host plant, usually causing no apparent symptoms of the disease[1,2]. Endophytes are universal, extremely common and found in almost all the species of plants in various amounts[3,4]. Like the host plant endophytes can also produce similar secondary metabolites and they are the potential producers of novel biologically active compounds[1]. It has been described that over 4000 bioactive metabolites are from the fungal origin[5]. In a natural ecosystem, endophytic fungi play a significant role in enhancing the ability to resist disease and promoting the growth of the plant[6]. Endophytes have large biological diversity, in which each species of plant may be a host to a number of endophytes[7]. By discharging active metabolites, the fungal endophytes protect their hosts from contagious agents and also to withstand adverse conditions[8].

Medicinal plants are recognized to harbor several endophytic fungi which were thought to be linked with the production of a wide variety of pharmaceutical products[8]. *Boerhaavia diffusa* is an herbaceous plant belongs to the family Nyctaginaceae, in Sanskrit, it is commonly referred to as punarnava[9]. They are widely distributed in the tropics and subtropics. For centuries it was used as a medicinal plant by indigenous and tribal people in India[10,11]. Different therapeutic value is seen in each part of the plant and it should be prepared on its own for the maximum benefits[12]. *Boerhaavia diffusa* plant as several medicinal properties, in which they are used to treat inflammations, wounds and also in hypertension[11]. Mainly, the roots and the whole plant is used for the medicinal purpose. The plant roots are used to treat anasarca, jaundice and ascites and also used in the treatment of several hepatic disorders[13,14]. In Arab countries as an Unani medicine, they are used in the treatment of dyspepsia, enlargement of spleen, stress, abdominal pain, diabetes and congestive heart failure[10]. In Brazil, the plant extract is used for treating gallstones, urinary disorders, liver support, cystitis, edema, nephritis and bile insufficiency. Several reports have been reported that in Ayurvedic and Unani *B.diffusa* plants were used to treat 22 alinments[9].

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Scientific classification:

Botanical Name	: *Boerhaavia diffusa*
Kingdom	: Plantae
Class	: Magnoliopsida
Order	: Caryophyllales
Family	: Nyctaginaceae
Genus	: Boerhaavia
Species	: *B. diffusa*

MATERIALS AND METHOD:

Sample collection:

Healthy and mature plants of *Boerhaavia diffusa* were collected in and around Brahmapuram, in Vellore, Tamilnadu, India. After the selection of a plant, a sterile scalpel was used to excise the disease-free stem part of the plant. These samples were collected in a sterile bag and then transported to the laboratory for processing. To reduce the chance of contamination, plant materials were freshly collected and are utilized for the isolation of endophytic fungi[15].

Isolation and extraction of endophytes:

The collected plant materials were washed thoroughly in sterile water, the stem is cut into small pieces under sterile conditions and surface sterilized by soaking in 1% sodium hypochlorite for 2 mins and 70% ethanol for 30 sec and eventually the plant material was washed in sterile demineralized water. After drying, the small pieces of the stem were placed on potato dextrose agar medium and the plates were incubated at 37˚C for 3 days. After incubation, the actively rising pure cultures agar blocks were placed in Erlenmeyer flasks containing 300ml of potato dextrose broth for mass cultivation of endophytic fungi. With periodical shaking the flasks were incubated at 37˚C for 21 days. The cultures were filtered after the incubation period under the sterile condition to remove the mycelia mat and the filtered fungal broth was taken in separating funnel along with the equal volume of the respective solvents like DCM and methanol, ethyl acetate solvents were added to the fungal mat respectively. Then the content was shaken vigorously and left undisturbed, the cell mass gets separated and the solvent obtained was collected in separate beakers and evaporated to harvest crude extract and this extract was dissolved in different solvents to perform further activities[16].

Identification of fungi:

Endophytic fungi are recognized by studying their cultural characteristics, spore formation and mycelium. Slides were prepared by tease mount method using Lactophenol cotton blue reagent and observed at 40 x, 100 x[17].

Phytochemical analysis:

Phytochemical analysis of different fungal extracts such as dichloromethane, methanol mat and ethyl acetate mat extract of the fungal mat were checked for the presence of the following secondary metabolites such as Alkaloids, Terpenoids, Phenols, Carbohydrates, Saponins by standard procedure[18].

Phenols:

To the 1ml of fungal extract a few drops of neutral 5% ferric chloride solution were added. A dark green color indicated the presence of phenolic compounds.

Flavonoids:

A few drops of 20% NaOH solution was added to 1ml of fungal crude extract. A change to yellow color was observed on the addition of acid it changed to colorless solution indicates the presence of flavonoids.

Saponins:

The fungal extract was vigorously shaken with distilled water and was allowed to stand for 10 min. The formation of froth indicated the presence of saponins.

Tannins:

To the fungal crude extract alcoholic $FeCl_3$ reagent was added. A bluish-black color, which disappears on the addition of dilute H_2SO_4, was followed by the formation of yellowish-brown precipitate indicated the presence of tannins.

Terpenoids:

1ml of fungal crude extract was mixed in 2ml of chloroform. 3ml of concentrated H_2SO_4 was then added to form a layer. At the interface formation of a reddish-brown precipitate indicates the presence of terpenoids.

Total phenolic content:

Total phenol content of different endophytic fungal extracts such as dichloromethane, methanol mat and ethyl acetate mat extract of the fungal mat was estimated using Folin – Ciocalteau based assay using Gallic acid as standard. Each extract was dissolved in methanol (1mg/ml) and 500µl of 40% Folin – Ciocalteau reagent was added. Followed by 1.5ml of 20% of Na_2Co_3 was added to the mixture. Then the final volume was made to 5ml by adding distilled water. The mixture was incubated for 30 minutes at room temperature and the absorbance of the developed color was noted at 765nm using UV-Vis spectrophotometer. The same procedure was done with 1mL aliquots of 5, 10 to 50µg/ml of methanolic gallic acid solution used as a standard for the calibration[19].

Antioxidant:

DPPH (2,2 Diphenyl-L-Picrylhydrazyl) assay:

For the DPPH assay, different concentrations of aliquots of different extracts were added to 2ml of (DPPH) methanolic solution (0.1mM). The reaction mixture was

shaken well and the contents were incubated at room temperature for 10 minutes. The absorbance of the resulting solution was read at 517nm against a blank. Gallic acid was used as a standard. This radical scavenging activity was measured as a decrease in the absorbance of DPPH and was calculated using the following equation[20].

Scavenging ability (%) =
[Abs of control-Abs of sample/Abs of control] × 100

Fourier-transform infrared spectroscopy (FTIR):
Based on the peak value in the region of infrared radiation, the FTIR spectrum was used to identify the functional group of the active components. The crude fungal extracts from *Cladosporium sp*. were subjected to FTIR analysis to determine the different functional groups present in sample[21].

Gas chromatography-mass spectrometry (GC-MS) analysis:
The fungal extracts were subjected to GC MS analysis to identify the bioactive compound. The sample were analyzed in GC Clarus 500 Perkin Elmer by using software Turbo mass 5.2 equipped with mass detector Turbo mass gold Perkin Elmer. 2µl sample was introduced via an all-glass injector working in the split mode, with Helium as the carrier gas with a linear velocity of 32cm/s. The HP-5 fused silica capillary column (Length – 30 m; Film thickness- 25µm I.D - 0.2 mm) were used. The identification of components were accomplished using computer searches in NIST version 2005[22].

RESULT AND DISCUSSION:

Isolation and identification of fungi:
The healthy stem of the plant *B.diffusa* was subjected to isolate the endophytic fungi and the identification was based on colony morphology and microscopic studies. The colony morphology of the endophytic fungus was noticed. The result was presented in Table.1, Figure.1.

Table.1: Morphology of isolated fungi

Endophytic fungi	Colony morphology	Part of the plant used
Cladosporium sp.	Non-spore forming, grey fungus	Stem

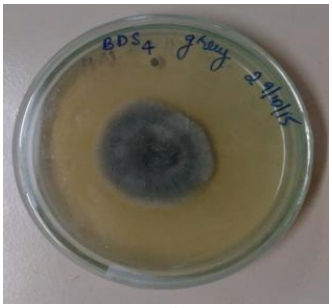

Figure.1: Colony morphology of the endophytic fungal strain *cladosporium* sp., isolated from *B.diffusa*.

Phytochemical screening:
The different extracts from endophytic fungi such as dichloromethane, ethyl acetate fungal mat and methanol fungal mat demonstrate the presence of Phenolic compounds, Saponins, Flavonoids, Tannins and Terpenoids. These chemical constituents are responsible for different medicinal properties of the extracts. Table.2 shows the results of a qualitative phytochemical analysis of endophytic fungi.

Table.2: Qualitative phytochemical composition of extracts of endophytic fungus.

Phytochemical	DCM	EA	MM
Phenols	+	++	+
Flavonoids	+	+	+
Saponins	-	+++	+
Terpenoids	++	+++	+
Tanninis	-	++	-

(+) - Positive; (+++) - Highly positive; (-) – Negative.
DCM-dichloromethane; EA-Ethyl acetate mat; MM-methanol mat.

Determination of total phenolic content:
The total phenolic concentrations in different extracts of endophytic fungi, calculated from the calibration curve were shown in Table.3. The ethyl acetate fungal mat extract of *B.diffusa* showed the highest concentration of phenol (15.15 mg/g GAE) Whereas, DCM extract contained a significantly least concentration of phenol.

Table.3: Total phenolic content (TPC) in the different extracts of endophytic fungus.

S. No	Extracts	Total phenolic content (mg of GAE/g of extract)	Total phenolic content (mg of GAE/g of extract)
1	DCM	12.61	12.63±0.0015
2	Ethyl acetate mat	15.15	15.15±0.001
3	Methanol mat	14.68	14.68±0.0020

Antioxidant: DPPH radical scavenging activity:
In the present study, the antioxidant activity of four different fungal extracts was investigated using the DPPH method. Different extracts showed antioxidant activity up to a varying extent. DCM Fungal extracts showed significant antioxidant activity of 71.46 % at the concentration of 150 µg/ml. Ascorbic acid was taken as a standard showing 97.77 % antioxidant activity. The percentage of DPPH radical scavenging activity of endophytic fungi and ascorbic acid is shown in Table.4.

Table.4: DPPH radical scavenging activity of extracts from endophytic fungus

S.No	Solvent extraction	Concentrations (µg/ml)		
		50	100	150
1	Standard	96.18±1.241	97.18±0.631	97.77±0.311
2	DCM	56.66±0.841	68.58±1.619	71.46±0.622
3	Ethyl acetate mat	22.37±0.777	45.51±0.650	50±0.834
4	Methanol mat	21.81±0.424	37.94±0.162	40.88±0.650

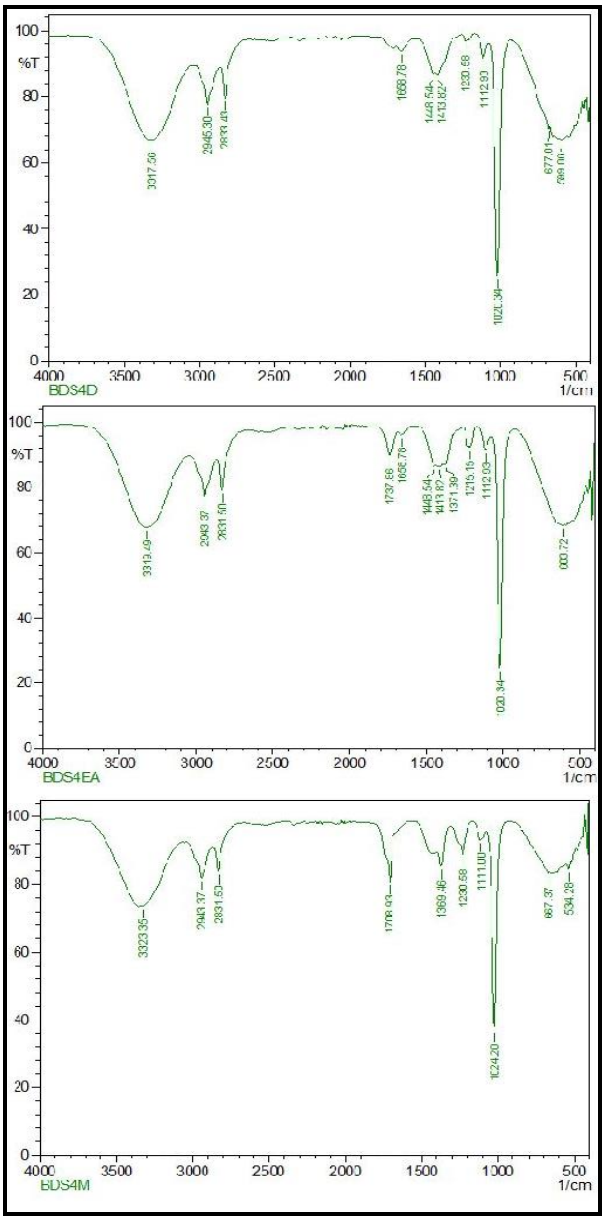

Figure.2: FTIR spectra of BDS4 fungal extracts. (D) FTIR spectrum analysis of DCM extracts-stem, (EA) FTIR spectrum analysis of Ethyl acetate mat extracts-stem, (M) FTIR spectrum analysis of Methanol mat extracts-stem

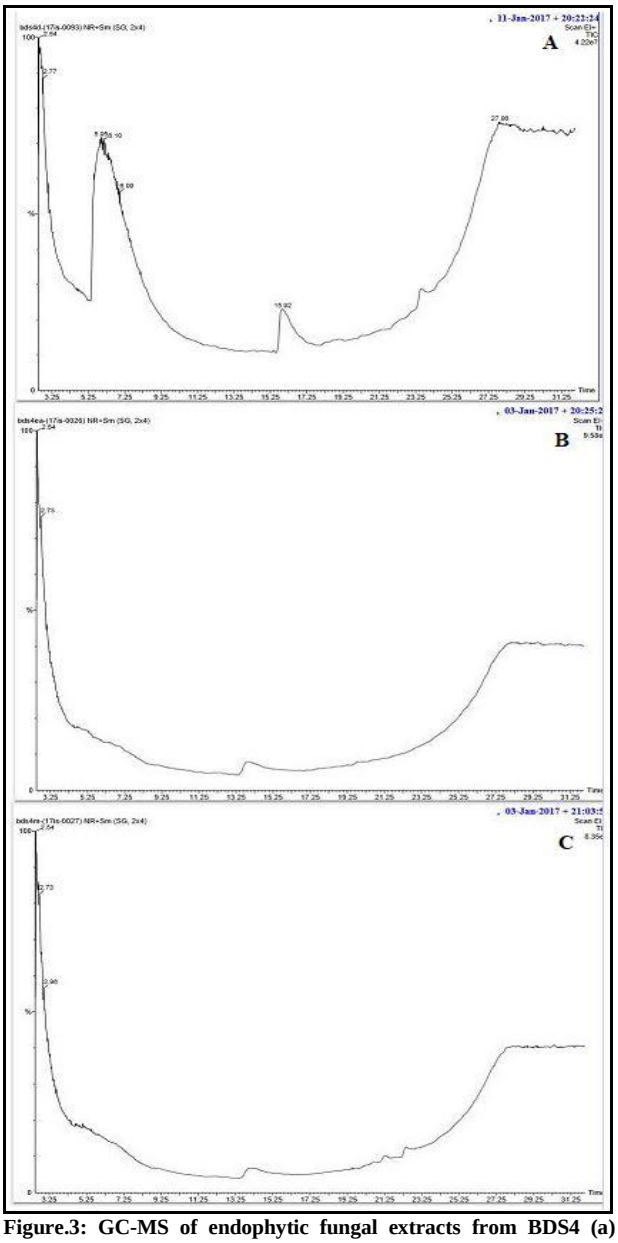

Figure.3: GC-MS of endophytic fungal extracts from BDS4 (a) DCM extract, (b) ethyl acetate mat extract, (c) methanol mat extract.

Fourier-transform infrared spectroscopy (FTIR):

FTIR analysis of different endophytic fungal extracts such as Dichloromethane, methanol mat and ethyl acetate mat extract of the fungal mat of *B.diffusa* plant was passed into the FTIR and the functional group of the components was separated based on its peak ratio. The FTIR results of the plant extracts are shown in Figure.2.

Gas chromatography-mass spectrometry (GC-MS) analysis:

The chromatogram obtained from the GC-MS analysis is shown in Figure for the different fungal extracts. The list of compounds matched with the NIST library search in the GC-MS analysis were shown in chromatogram in Figure.3.

CONCLUSION:

Fungal endophytes are poorly explored group of microorganisms and they serve as an exuberant source of bioactive and chemically novel secondary metabolites which launches a diverse area for research and in several pharmaceutical and industrial fields. As a plant harbors at least one fungal endophyte, it can be used as a plentiful source of drugs. Investigation of these fungal endophytes provides the possibility for the discovery of

novel drugs. The results of this study indicate that endophytic fungi may serve as the potential source of natural antioxidants. This study will offer an introduction to more comprehensive work on bioactive compounds produced by these endophytes.

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CONFLICTS OF INTEREST:

Declared none.

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RESEARCH ARTICLE

Formulation and *in vitro* Evaluation of Lisinopril floating Gastroretentive Tablets

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ABSTRACT:
The present study was aimed to develop sustained release floating tablet containing lisinopril by wet granulation technique. HPMC K4 M and Carbopol 934 P were used to sustain the drug release. The results of micromeretic properties of granules showed good flow behavior. Tablet weight variation, friability and drug content were within the specified limits. Tablet hardness and thickness were satisfactory. The tablets were hydrated gradually reaching a plateau after 8 h. The floating lag time varied from 22.9 - 68.6 seconds depending on polymer type and concentration. The *in vitro* drug release profile of tablets containing crosprovidone at its higher level and HPMC alone showed more than 50% of cumulative percentage drug release at the end of first 4 h of the dissolution study. However, from these tablets, at the end of 8 h the cumulative percentage drug release was found to be 71.03±4.975%. Formulations F11 containing HPMC K4M and Carbopol 934 and higher level of Croscarmellose sodium showed a higher percentage of drug release (79.15±2.688%) when compared to the other formulations. The developed floating system enhanced the retention time and prolonged the drug release in 0.1 N HCl (pH 1.2), which could improve the local availability of the drug.

KEYWORDS: Floating tablets, gastroretentive drug delivery, controlled release, sustained release.

INTRODUCTION:
The primary goal behind development of drug delivery carrier is to target a drug directly to its site of action to obtain a better therapeutic effect[1]. Oral route persist the route of choice due to the ease of dose administration and patient compliance. Oral route of drug administration offers various advantages for administration of drugs used to deliver drugs to act locally to the gastrointestinal tract. Development of oral drug delivery systems has various benefits like low cost, flexible formulation approach, drug delivery for a long time period and improved bioavailability[2].

Oral pharmaceutical formulations are classified into two classes namely conventional and modified release systems. Conventional release systems are designated to disintegrate quickly, and allow drug release in a short time. The main limitation of such conventional system is fluctuations in drug concentration in blood plasma, which cause reduced or loss in intended therapeutic effects or increased frequency of side effects. In case when the drug concentration decreased due to its metabolism and excretion, administration of multiple dosages in a day is needed.

Tablets are the most conventional and economic pharmaceutical formulations prepared to release the medicament after oral administration. Time and cost effectiveness make tablets still the favoured dosage forms. The performance of tablet depends on its matrix and surface properties, which govern the mechanical and chemical properties of tablet. Conventional release tablets result in relatively increased number of dosages. These conventional tablets may show more fluctuations in plasma drug concentration. To avoid the fast sub-therapeutics level of the drug another dose is usually given for treating chronic diseased conditions[3].

To overcome the limitation of conventional tablets, development of various modified release drug products is gaining more attention to control the drug release. Modified release products use polymers to alter the rate of drug release under controlled pH conditions of

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gastrointestinal tract (g.i.t). The term controlled-release was originally used to depict various extended release formulations such as prolonged action, sustained-release, slow-release, long-action and programmed delivery. The basic rational of controlled or sustained release formulation is to control drug at target site, avoiding the frequent dosing and improve efficacy effect of a drugs by altering its pharmacokinetics and pharmacodynamic profile[4,5,6].

However, such controlled delivery systems extend limited advantages for bioactives having narrow therapeutic window. Various drugs such as gliclazide and pioglitazone are absorbed from duodenum and jejunum[7]. However, limited absorption may be possible at these sites due to the quick passage of dosage form (about 1-2 h)[8-11]. To meliorate the oral availability of these therapeutics, the retention time of the delivery system need to be extended in the stomach, so that the drug will be available in the solution form when it reached to the area from where its maximum absorption is possible[12]. This can be successfully accomplished by developing gastroretentive controlled release carrier that can resist the grinding, crushing, contractions, and peristaltic movements and allow prolonged drug release[13-15]. Retention for prolonged period of time leads to improved oral bioavailability, and clinical efficacy, also reduces the number of dosage administration and improves patient compliance[16]. Hence, extended release drug delivery systems with gastric retention are recommended as potential delivery systems for effective drug delivery[17].

Absorption of lisinopril is slow, variably, and incomplete (~30%) after oral administration. To overcome this limitation of lisinopril, the present study was designed to develop floating gastroretentive tablets by wet granulation technique.

MATERIAL AND METHODS:

Materials:

Lisinopril was received as gift sample from Wockhardt Ltd., Mumbai, India. Crosprovidone and Croscarmellose sodium were received as gift sample from Signet Chemicals, Mumbai, India. HPMC K4 M and Carbopol 934 P were purchased from Sigma Aldrich Chemicals, Bangalore. Lactose, polyvinylpyrrolidone (PVP), sodium bicarbonate, citric acid and magnesium stearate were purchased from Fines, Mumbai, India.

Methods:

Preparation of granules for tablet formulation:

Accurately weighed amount of lisinopril was mixed in a clean and dry mortar with all ingredients except magnesium stearate (Table 1 and 2). The powder was blended well for 10 min. PVP-K30 solution in 5% w/v isopropyl alcohol was used for wet granulation. It was passed through sieve # 20 and dried at 40°C in a hot air oven. Lubricated of the dried granules was carried using magnesium stearate.

Micromeretic characterization of granules:

Determination of bulk density and tapped density:

Pre-weighed granules were placed in a 10 mL measuring cylinder. The initial volume was recorded. The cylinder containing granules was tapped 100 times. The density determinations were calculated using below formula[18]:

$$\text{Bulk Density} = \frac{\text{Weight of Granules}}{\text{Initial Volume}}$$

Determination of Hausner's ratio:

The density findings of the granules were utilized to calculate Hausner's ratio using below formula[18]:

$$\text{Hausner's ratio} = \frac{\text{Tapped Density}}{\text{Bulk Density}}$$

Determination of Carr's index:

The density (bulk and tapped) results were utilized to calculate Carr's index by below formula[18]:

$$\text{Carr's index (\%)} = \frac{\text{Tapped Density - Bulk Density}}{\text{Tapped Density}}$$

Table 1: Composition of sustained release gastroretentive floating tablets of Lisinopril.

Ingredients (mg/tablet)	LFT_1	LFT_2	LFT_3	LFT_4	LFT_5	LFT_6	LFT_7	LFT_8	LFT_9	LFT_{10}	LFT_{11}
Lisinopril	20	20	20	20	20	20	20	20	20	20	20
HPMC K4 M	100	90	100	50	40	100	50	55	90	100	50
Carbopol 934 P	-	20	30	30	50	80	30	50	50	60	40
Crosprovidone	4	-	-	-	3	2	-	2	-	-	-
Croscarmellose sodium	-	-	3	2	-	-	2	-	-	-	4
Lactose	91	78	48	99	95	13	113	81	41	35	94
PVP (% w/v)	5	5	5	5	5	5	5	5	5	5	5
Sodium bicarbonate	45	50	55	55	50	45	45	50	55	45	50
Citric acid	8	10	12	12	10	8	8	10	12	8	10
Magnesium stearate	7	7	7	7	7	7	7	7	7	7	7

Determination of angle of repose:
The granules were allowed to pass freely through a fixed funnel (lower tip at 2cm above the surface). The granules were poured until the tip of pile touched funnel. The tan-1 was calculated using pile height and radius of its base ratio as per the below formula[18]:

$\tan^{-1} = h/ r$

Where, h is the height of the pile and r is the radius of pile base

Formulation of floating tablets containing lisinopril:
The granules were compressed into tablets by wet granulation method using 9mm diameter die cavity and flat faced punches in a rotary tablet press (Rolax manual tablet compression machine, Ambala, India). The composition of controlled release gastroretentive floating tablets of lisinopril is given in Table 1 and 2. The weight of tablets was adjusted to 270mg[19].

Evaluation of tablets:
Determination of weight variation:
For the determination of tablet weight variation, USP procedure for uniformity of weight was followed. Briefly, 20 tablets were taken randomly from each batch and the weight was recorded individually and collectively using a calibrated weighing balance. The average tablet weight was used to estimate tablet weight variation.

Determination of tablet hardness:
Ten tablets were taken randomly from each batch and tested for hardness by Monsanto tablet hardness tester.

Determination of tablet thickness:
Ten tablets were randomly taken from each batch and tested for thickness using vernier caliper.

Determination of friability:
From each batch, twenty tablets were initially weighed and placed in the chamber of a USP friability tester (Electrolab EF-2 friabilator). The friabilator was operated at 25rpm for 100 revolutions. After 100 revolutions, the tablets were collected, de-dusted and re-weighed. The percentage friability was calculated using below formula:

$$\text{Friability (\%)} = \frac{\text{Initial weight - Final weight}}{\text{Initial weight}} \times 100$$

Determination of drug content:
It was estimated by crushing 6 tablets from each batch. The tablet powder (≡ average tablet weight) was taken in 100mL of 0.1N HCl and stirred for 24 h. After 24 h, the mixture was filtered through Whatman filter papers. The filtrate was diluted suitably and analyzed spectrophotometrically at 206nm.

Swelling studies:
The swelling of tablet was examined in petri plate containing 0.1 N HCl, pH 1.2. At predetermined intervals, the tablet was taken out from the petri plate and the percentage swelling was determined using below formula:

$$\text{Swelling (\%)} = \frac{\text{Weight of the swollen tablet - Initial weight of tablet}}{\text{Initial weight of tablet}} \times 100$$

Determination of in vitro buoyancy:
The floating lag time and floating duration were determined by placing 3 tablets from each batch in 100 mL 0.1N HCl (pH 1.2) and allowed to float. The floating lag time was determined on the basis of time taken by the tablet to float at the top of the media constantly for a long time. The floating duration was determined on the basis to recording the total time period for which the tablet floated constantly.

In vitro release study:
The release of lisinopril was investigated using USP tablet dissolution apparatus type - II (DS 8000, Lab India, Mumbai, India). The dissolution experiments were done in 900mL 0.1N HCl (pH 1.2) at 100 rpm and 37±0.5°C. Five milliliter sample was taken at of every 15 min for first hour followed by 1 hour for first 4 hour and by every 2 hour interval till 8th hour. Sink condition was maintain by adding same amount of buffer after each sampling added to. The samples were diluted sufficiently and analyzed using a UV spectrophotometer (1700, Shimadzu, Japan) at 206nm. Each sampling time was done in triplicate.

RESULTS AND DISCUSSION:

Micromeritic characterization of granules:
Bulk density:
The bulk density was observed in the range of 0.459 to 0.564g/mL (Table 2). The results confirmed that the obtained bulk densities were almost similar for all batches indicating almost identical flow behavior and packing arrangement of particles.

Tapped density:
The results of tapped density are shown in Table 2. The tapped density was recorded in the range of 0.567 to 0.623g/mL. The results indicating very small or no difference in granule volume even after 100 tapping which shows almost similar flow properties.

Hausner's ratio:
It also indicates flow properties of the granular mass. In the present study, the value of Hausner's ratio was from 1.083 to 1.910 (Table 2), indicating good flow of granules. However, formulation LFT4, LFT8, LFT4, LFT11 had Hausner's ratio value > 1.25 indicating poor flow.

Carr's index:
It is suggested that the value of Carr's index in the range of 5 to 15 indicates excellent and from 15 to 20 indicates good flow of material. On the other side, the value < 30 indicates cohesiveness of particles with poor flow. The value of Carr's index was observed from 7.692 – 40.444 indicating excellent to very poor flow properties (Table 2).

Angle of repose:
It was determined to estimate flow behavior of granules. The angle of repose for all the formulations was ranged from 24.17 – 27.61 (Table 2), which indicates good flow of granular and indicating it was non-aggregating.

Evaluation of floating tablets:

Weight variation:
The average weight of the tablet was found in the range of 281.384mg to 278.616mg (Table 3). The maximum percentage deviation was found to be 1.384. None of the batch shows a deviation of more than ± 7.5% (Indian Pharmacopoeial limit). The results conclude that the tablets complied with weight variation test.

Tablet hardness:
The hardness for different batches was found between 3.6±0.120 to 4.2±0.521kg/cm2 showing satisfactory mechanical strength (Table 3).

Tablet thickness:
The maximum average thickness from all the formulations was 1.32 mm. The minimum average thickness from all the formulations was 1.09 mm. The average thickness from all the formulations was 1.14 mm. The percentage deviation in thickness was found to be 0.02 to 0.18.

Friability test:
The friability loss was less than 1% in all the formulations (0.19±0.082% to 0.82±0.170) (Table 3).

Table 2. Micrometric properties of granules (Formulation LFT_1-LFT_{11}).

Micrometric property	Formulation code										
	LFT_1	LFT_2	LFT_3	LFT_4	LFT_5	LFT_6	LFT_7	LFT_8	LFT_9	LFT_{10}	LFT_{11}
Bulk density (g/mL) ± SD	0.505 ± 0.15	0.511 ± 0.24	0.464 ± 0.61	0.513 ± 0.09	0.534 ± 0.11	0.508± 0.43	0.564 ± 0.37	0.487 ± 0.09	0.514 ± 0.61	0.512 ± 0.31	0.459 ± 0.22
Tapped density (g/mL) ± SD	0.587 ± 0.34	0.567 ± 0.27	0.615 ± 0.51	0.611 ± 0.54	0.598 ± 0.21	0.608 ± 0.22	0.611 ± 0.17	0.623 ± 0.81	0.584 ± 0.52	0.612 ± 0.27	0.609 ± 0.22
Angle of repose (θ) ± SD	24.17 ± 0.51	26.11 ± 0.41	27.61 ± 0.34	26.27 ± 0.31	24.97 ± 0.72	26.31 ± 0.41	24.43 ± 0.42	26.00 ± 0.34	27.81 ± 0.21	25.90 ± 0.17	25.47 ± 0.48
Hausner's ratio	1.162 ± 0.10	1.109 ± 0.12	1.325 ± 0.34	1.910 ± 0.22	1.119 ± 0.31	1.196 ± 0.22	1.083 ± 0.21	1.270 ± 0.34	1.136 ± 0.31	1.195 ± 0.22	1.326 ± 0.35
Carr's Index (%) ± SD	13.969 ± 2.19	9.876 ± 1.05	24.552 ± 2.31	16.039 ± 0.94	10.702 ± 0.28	16.447 ± 0.64	7.692 ± 0.52	21.829 ± 1.02	11.986 ± 1.23	16.339 ± 2.17	40.444 ± 1.34
Drug content (%) ± SD	96.15 ± 1.37	92.02 ± 0.82	96.11 ± 2.34	98.14 ± 2.37	99.04 ± 1.81	99.11 ± 1.34	95.27 ± 1.38	97.13 ± 1.62	96.34 ± 2.72	98.37 ± 1.92	99.04 ± 1.63

Table 3. Various evaluation parameters of developed floating tablets of lisinopril.

Formulation code	Hardness (kg/cm^2)	Friability (%)	Weight uniformity (mg)	Drug content (mg)
LFT_1	3.6 ± 0.120	0.61 ± 0.131	280 ± 1.028	99.11 ± 1.34
LFT_2	3.9 ± 0.620	0.55 ± 0.108	280 ± 1.067	99.04 ± 1.81
LFT_3	3.6 ± 0.130	0.37 ± 0.100	280 ± 0.987	98.14 ± 2.37
LFT_4	4.1 ± 0.110	0.82 ± 0.170	280 ± 1.027	96.11 ± 2.34
LFT_5	3.4 ± 0.550	0.19 ± 0.082	280 ± 0.982	92.02 ± 0.82
LFT_6	3.9 ± 0.076	0.27 ± 0.055	280 ± 1.384	96.15 ± 1.37
LFT_7	4.1 ± 0.225	0.62 ± 0.071	280 ± 1.327	99.04 ± 1.63
LFT_8	4.2 ± 0.521	0.62 ± 0.082	280 ± 0.972	98.37 ± 1.92
LFT_9	4.1 ± 0.132	0.37 ± 0.061	280 ± 1.368	96.34 ± 2.72
LFT_{10}	3.9 ± 0.521	0.52 ± 0.052	280 ± 1.357	97.13 ± 1.62
LFT_{11}	3.7 ± 0.214	0.36 ± 0.105	280 ± 1.270	95.27 ± 1.38

Drug content:

The maximum and minimum percentage drug content was found to be 100.18% and 96.64%, respectively (Table 3). The limit is within the specified pharmacopoeial specification (IP i.e. ±10). The results of drug content study were complying pharmacopoeial specifications.

Swelling studies:

Water uptake approach was used to determine swelling behavior of prepared tablets. Due to the simultaneous swelling of polymer matrix and continuous dissolution of drug into bulk the strength of matrix gets reduced. However, swelling may also lead to sustain the rate of drug release because of the increase in diffusional path. In the present study, maximum swelling was recorded after 8 hour (Table 4). Highest percent swelling was ascertained at the end of 8 hour (Figure 1). Percentage swelling was increased with an increase in polymer concentration.

All the tablets had similar hydration behaviour, as they reached a plateau after 8 hour and remained unchanged until 12 hour. Almost all the formulations reached > 45% hydration. High water uptake capacity of the tablets may be due to the quick hydration of polymers and swelling rate of the tablets increased with an increase in polymers concentration.

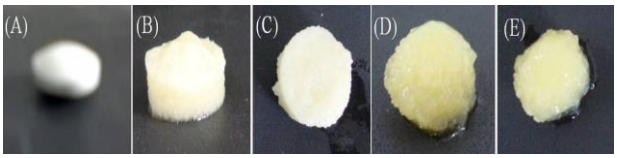

Figure 1. Swelling behavior of lisinopril floating tablet in 0.1 N HCl, 1 h (A), 2 h (B), 4 h (C), 8 h (D), and after 12 h (E).

Table 4. Results of percentage hydration.

Formulation code	Hydration (%)				
	1 h	2 h	4 h	8 h	12 h
LFT_1	48.92	51.86	68.32	71.12	72.05
LFT_2	46.41	50.76	56.31	64.91	65.37
LFT_3	47.82	53.33	68.34	83.51	84.32
LFT_4	50.26	62.79	75.61	90.21	91.10
LFT_5	48.69	58.21	74.58	88.96	89.08
LFT_6	53.51	59.36	73.11	90.61	91.05
LFT_7	49.14	67.87	71.51	84.12	94.31
LFT_8	44.55	65.23	72.87	86.37	86.92
LFT_9	49.34	62.54	73.95	81.06	81.67
LFT_{10}	52.22	52.35	67.36	80.01	81.12
LFT_{11}	54.94	54.35	68.04	81.17	81.52

Determination of buoyancy:

The optimum floatation (less lag time and more total floatation) was observed at 55 mg/ tablet of sodium bi-carbonate so this was considered as optimized level of gas forming agent. The tablets with HPMC exhibited short buoyancy lag time and remained buoyant for longer duration than the tablets containing carbopol. The percentage buoyancy of all the formulations was found to be good (Figure 2). The floating lag time varied from 22.9 sec to 68.6 sec depending on polymer type and concentration (Table 5).

Table 5. Various evaluation parameters of developed floating tablets of lisinopril.

Formulation code	Floating lag time (s)	Total floating time (h)
LFT_1	34.3 ± 3.0	12.0 ± 2.5
LFT_2	38.0 ± 5.0	16.0 ± 1.5
LFT_3	22.2 ± 4.0	20.0 ± 1.5
LFT_4	25.1 ± 6.0	18.0 ± 1.4
LFT_5	22.9 ± 4.0	20.0 ± 1.0
LFT_6	68.6 ± 3.0	12.0 ± 0.5
LFT_7	67.0 ± 2.0	16.0 ± 1.0
LFT_8	51.7 ± 3.0	12.0 ± 2.0
LFT_9	23.0 ± 2.0	20.0 ± 0.5
LFT_{10}	67.5 ± 4.0	13.0 ± 0.5
LFT_{11}	49.3 ± 2.0	16.4 ± 1.0

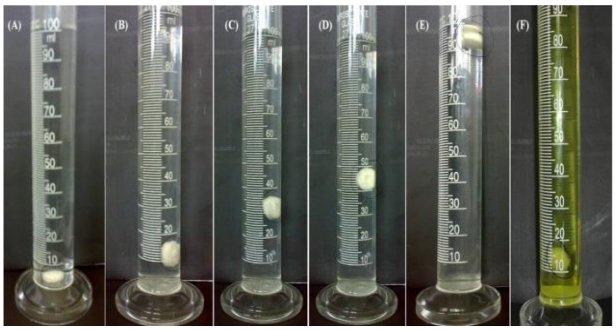

Figure 5.7 Buoyancy sequence of lisinopril floating tablet in 0.1 N HCl, at (A) 0 sec, (B) 5 sec, (C) 10 sec, (D) 12 sec, (E) 4 h, and (F) after 18 h.

In-vitro dissolution studies:

Release of drug from the tablets changed depending on the type and ratio of matrix-forming polymer. HPMC and Carbopol had excellent gelling properties, and also helped in sustaining effect. The *in-vitro* drug release profile of tablets containing Carbopol 934P with HPMC K4M showed sustained drug release profile (Figure 3 and Figure 4). On physical examination of tablets during dissolution study, it was observed that the tablets were swollen over the period of time.

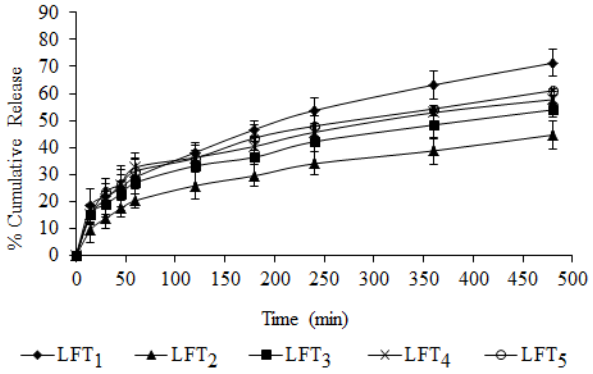

Figure 3. Dissolution profile of Lisinopril from floating matrix tablets (formulation LFT_1 to LFT_5). All values are expressed as mean± SD, n=3.

The *in-vitro* drug release profile of tablets containing crosprovidone at its higher level and HPMC alone showed more than 50% of cumulative percentage drug release at the end of first 4 h of the dissolution study. However, from these tablets, at the end of 8 h the cumulative percentage drug release was found to be 71.03 ± 4.975%. Formulations F11 containing HPMC K4M and Carbopol 934 (50:40 ratio) and higher level of Croscarmellose sodium (4 mg) showed a higher percentage of drug release (79.15 ± 2.688%) when compared to the other formulations. HPMC K4M alone could not have prolonged sustaining effect. However, formulations containing HPMC K4M at its higher level in combination with Carbopol has shown more sustaining effect.

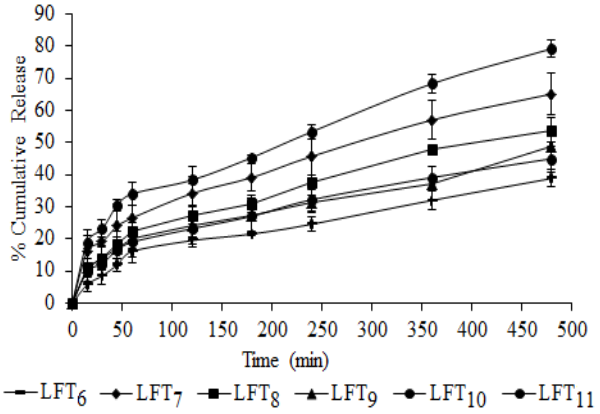

Figure 4. Dissolution profile of Lisinopril from floating matrix tablets (formulation LFT_6 to LFT_{11}). All values are expressed as mean± SD, n=3.

The drug release profile form the prepared floating tablets could be attributed to the increased hydration and increased swelling behaviour of polymer. The higher swelling of polymer may increase the diffusional path-length to allow drug to diffuse out.

Kinetic treatment to dissolution data:

Various mathematical models such as zero order, first order kinetics, Higuchi's equation, and Peppas' equation have been established that could explain exactly which type of drug release kinetics and mechanism followed. In case of first order plot, the in vitro release data was plotted as log cumulative percentage remaining to release against time. For Higuchi's plot, a plot of percentage cumulative drug released against square root of time was plotted.

For Peppas' plot, a plot of log percentage cumulative drug released against log time was plotted. The type of drug release kinetics and mechanism was confirmed based on the values of r^2 values. The results suggested that the drug release from the floating tablets followed first order kinetics and Higuchi's equation (highest r^2 coefficients) (Table 6). Higuchi's plots were found to be linear in all the formulations indicating diffusion controlled release from all the floating tablets. The release exponent "n" was in the range more than 0.5 and less than 1 for Peppas' plots, confirming drug release via diffusion with swelling. Similar kind of drug release kinetic and mechanism has been reported earlie[20-22].

Table 6. Results of drug release kinetic studies.

Formulation code	Zero order (r^2)	First order (r^2)	Higuchi's model (r^2)	Peppas' model (r^2)	
				r^2	n
LFT_1	0.9005	0.9709	0.9760	0.9718	0.5117
LFT_2	0.9093	0.9620	0.9962	0.9844	0.5613
LFT_3	0.9119	0.9618	0.9855	0.9869	0.5346
LFT_4	0.9051	0.9588	0. 9838	0.9839	0.5133
LFT_5	0.9162	0.9736	0.9891	0.9897	0.5234
LFT_6	0.9179	0.9499	0.9956	0.9752	0.5157
LFT_7	0.9034	0.9709	0.9959	0.9915	0.5057
LFT_8	0.9143	0.9943	0.9920	0.9902	0.5492
LFT_9	0.9035	0.9429	0.9732	0.9831	0.5192
LFT_{10}	0.9011	0.9472	0.9946	0.9917	0.5330
LFT_{11}	0.9094	0.9739	0.9827	0.9787	0.5976

CONCLUSION:

Sustained release floating gastroretentive tablets of lisinopril were successfully prepared by wet granulation technique. The study was conducted to assess the effect of polymer type and concentration on matrix characteristics such as buoyancy behavior and release profiles. The type and quantity of polymer played main role for providing buoyancy and sustained release profile. As the concentration of polymer increases, lag time also increase. It was observed that the prepared tablets had ideal properties to be used as gastroretentive system. The developed floating system enhanced the retention time and prolonged the drug release in the stomach, which, in turn, improved the local availability of the drug. Overall, a floating system for lisinopril was formulated successfully with less buoyancy lag time, long buoyancy time and sustained drug release rate from the hydrophilic matrices. The tablets are anticipated to provide a new choice of safe product with better

bioavailability for the treatment of high blood pressure (hypertension).

CONFLICT OF INTEREST:

The authors declare no conflict of interest.

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RESEARCH ARTICLE

Development of Validated Analytical Area under Curve UV Spectrophotometric Method for Assay of Cetrizine Dihydrochloride

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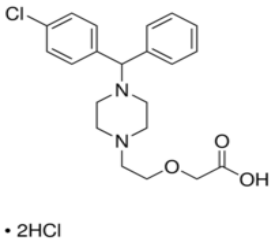
ABSTRACT:
A simple and efficient method for the determination and estimation of area under curve of Cetirizine dihydrochloride has been developed using distilled water as solvent. Cetirizine dihydrochloride obeys Beer's Law in the concentration range of 10-30µg/ml at Area of 225nm to 235nm using UV-spectrophotometer SHIMADZU model UV 1800. The method was applied to the tablet formulations and found to be accurate, precise and economic for routine determination of Cetrizine in pharmaceutical dosage forms.

KEYWORDS: Cetrizine, analytical method, area under curve, UV spectrophotometric, determination.

INTRODUCTION:
Cetrizine dihydrochloride:
Cetirizine hydrochloride is an antihistamine that treats symptoms, such as itching, runny nose, watery eyes, and sneezing from hay fever (allergic rhinitis) and other allergies, such as allergies to molds and dust mites.

Structure:



Synonyms:
[2-[4-[(4-Chlorophenyl)-phenylmethyl]-1-piperazinyl] ethoxy] acetic acid
Levo Cetrizine Hydroxyzine
CTZ
Formula: $C_{21}H_{25}ClN_2O_4$

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REVIEW OF LITERATURE:
Khalid Abdel-Salam M. Attia, reported Four simple, accurate, selective and sensitive spectrophotometric methods for the determination of cetirizine (CTZ) in presence of its oxidative degradation products without previous separation. Method A, Bivariate method using optimum wavelengths (211 and 231nm) which formed by Kaiser's method, with mean percentage recovery of 100.81±0.560. Method B, Area under curve method using two wavelength regions (220−230nm) and (240−250nm) with mean percentage recovery of 100.43±1.068. Method C, Dual wavelength method using two wavelengths 230nm and 244nm with mean percentage recovery of 100.07±0.549. Method D, Simultaneous equation method, using two wavelengths one for maximum absorbance of drug and one for degradate at 230nm and 258nm respectively. Results were statistically compared to a reported method and no significant difference was noticed regarding accuracy and precision.[1]

A M Y Jaber H., developed a HPLC method for the determination of cetirizine dihydrochloride (CZ) as well as its related impurities in commercial oral solution and tablet formulations. Furthermore, two preservatives associated with the drug formulations, namely, propyl (PP) and butylparabens (BP) were successfully determined by this method.[2]

A.F.M EI Walilya reported Derivative spectrophotometric, colorimetric and high-performance liquid chromatographic methods, for the determination of the antihistaminic cetirizine dihydrochloride in tablet form were described. Spectrophotometrically, cetirizine was determined by the measurement of its first (1D) and second (2D) derivative amplitudes at 239 (peak) and 243–233nm (peak-to-trough), respectively. The aqueous solutions obeyed Beer's law in the concentration ranges of 1.2–10.0 and 0.8–10.0µg ml−1 for 1D and 2D measurements, respectively. The colorimetric procedure was based on measuring the absorbency of the coloured chromogen resulted from the reaction between cetirizine sodium salt in polar solvent (DMF) and chloranil at 556 nm. The relation with concentrations was linear over 120–250µg ml−1. Optimization of the reaction conditions was studied. At the same time, investigation of the complex formed was made with respect to its composition and the associated constant.[3]

M.F Zaater carried out the development and evaluation of HPLC method for quantifying cetirizine in human serum is described. The method involves liquid phase extraction of cetirizine in methylene chloride, adding diazepam as an internal standard, followed by separation on a reversed phase C18 Novapak column (150×3.9nm; 4µm), and employing a UV-detection set at 230nm at ambient temperature. The mobile phase consists of a 13 mM phosphoric acid solution and acetonitrile (61:39 v/v) adjusted to pH 2.8 with 5 M NaOH. The assay is linear from 10 to 500ng ml−1 with a detection limit of 5 ng ml−1 and a mean recovery of 96.5%. The applicability of this method in pharmacokinetic studies is evaluated.[4]

V. Krishna Reddy developed and validated a UPLC method, simple, precise, accurate and specific chromatographic method for the determination of cetirizine dihydrochloride in tablets. An ultra performance liquid chromatography instrument and silica, 33 x 4.6 mm, 3 µ were used for determination of cetirizine dihydrochloride. The flow rate of 1.0 mL/min was set with isocratic, the temperature of column compartment maintained at 25°C and Ultra violet detection done at 230nm wavelength. The injection volume was 5 µL. The cetirizine dihydrochloride peaks eluted at 1.202 minute and run time was set for about two minutes.[5]

T Veeraiah reported an Extractive spectrophotometric methods for the determination of Cetirizine using dyes viz., bromocresol green, bromothymol blue and Tropaeolin OO as analytical reagents have been developed and described. The developed methods involved the formation of coloured ion-pair complexes of Cetirizine with dyes in acidic medium. The ion-pair complexes of Cetirizine, with bromocresol green, bromothymol blue and Tropaeolin OO, quantitatively extracted into chloroform, absorbed maximally at 410, 414, and 410nm. The stoichiometry of the complex in three cases is found to be 1:1. The Beer's law is obeyed in the concentration ranges 2.5-30µg/ml. The effect of concentration of dye, pH and interference of excipients have been studied and optimized.[6]

OBJECTIVES OF STUDY:

Following the literature review, the objectives of the current work was

1) To develop new alternative analytical method for uv-spectrophotometric method for determination of Cetirizine hydrochloride.
2) To establish a validated simple and standard, reproducible method for quality control and assurance of Cetirizine hydrochloride
3) Development of area under curve UV spectrometric method for the quantitative estimation of Cetirizine hydrochloride.

MATERIALS AND METHODS:

Source of data:

Standard bulk drug obtained from bulk manufacturers as gift sample.

All the reagents and solvents of analytical grade are used.

Data were collected using instrument such as Shimadzu 1800 Spectronic model UV spectrophotometer.

Method of collecting data:

The analytical method development and validation study was carried out in the department of Quality Assurance, BLDE A's SSM College of Pharmacy and Research Centre, Vijayapur.

Strategy:

Statistical analysis:

The statistical analysis will be carried out for the calculation by applying statistical software.

Data collection:

Preparation of standard stock solution:

Standard stock solution of Cetirizine hydrochloride was prepared by dissolving accurately weighted quantity of Cetirizine hydrochloride 100mg in 1000ml of distilled water and transferred it to 100ml volumetric flask .volume made up to the mark with distilled water for obtaining standard stock solution of 100µg/ml concentrations.

Method:

Linearity curve for AUC:

From the standard stock solution of Cetirizine

hydrochloride appropriate aliquots were 1000ml of volumetric flask and dilutions were made with distilled water to produced working standard solution of Cetirizine hydrochloride 10,15, 20, 25, 30µg/ml. The difference in the area under curve of Cetirizine hydrochloride was measured in the area from 225-235nm. The calibration curve of the drug of Cetirizine hydrochloride was plotted. The concentration range over which the drug followed linearity was chosen as analytical concentration range I.e.10 - 30µg/ml for Cetirizine dihydrochloride. The data is given under the table 1.

Table 1: Area under Curve (AUC) in distilled water ranging from 10-30µg/ml

Sr. No.	Concentrations	A U C
1	10g/ml	2.2349
2	15g/ml	3.3369
3	20g/ml	4.3539
4	25g/ml	5.3284
5	30g/ml	6.3172

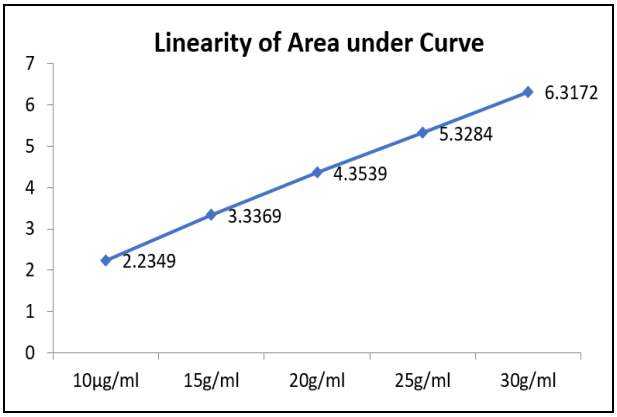

Fig 1: UV Spectrum showing Area under curve of Cetirizine hydrochloride 10µg/ml

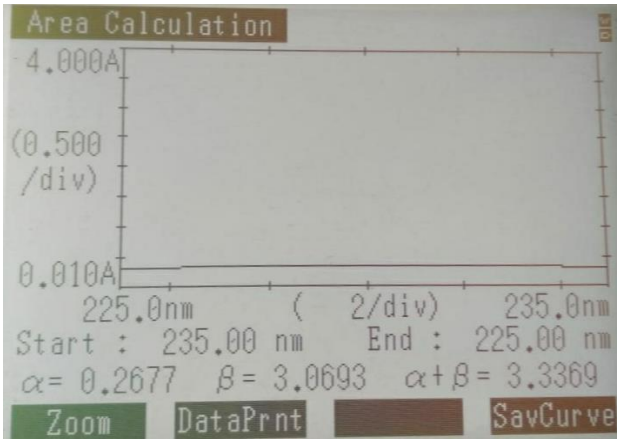

Fig 2: UV Spectrum showing Area under curve of Cetirizine hydrochloride 15µg/ml

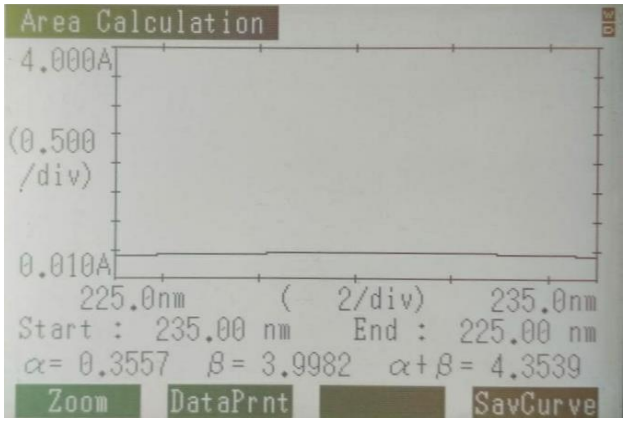

Fig 3: UV Spectrum showing Area under curve of Cetirizine hydrochloride 20µg/ml

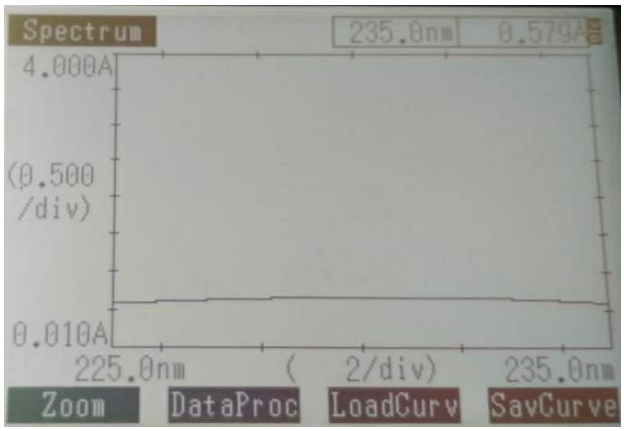

Fig 4: UV Spectrum showing Area under curve of Cetirizine hydrochloride 25µg/ml

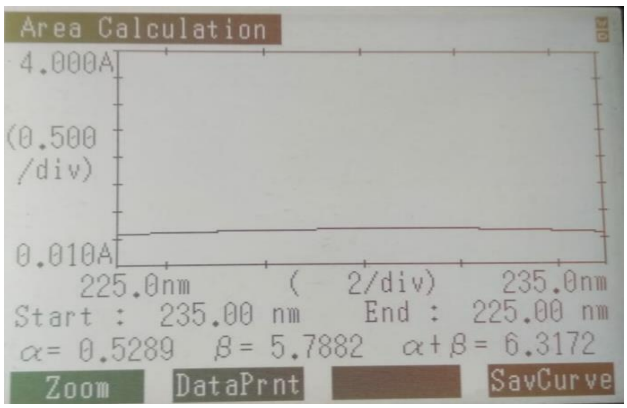

Fig 5: UV Spectrum showing Area under curve of Cetirizine hydrochloride 30µg/ml

Method Validation:

Estimation of Drug from Dosage Form: (Tablet Assay Study) Brand name- Zyncet ® 10

Standard:

From the standard stock solution of Cetrizine, appropriate aliquots were pipette out into 25ml volumetric flask and dilutions were made with methanol to obtain working standard solution of Cetrizine

24µg/ml. This concentration was scanned at area of 225nm to 235nm.

Sample:

Ten tablets of brand Zyncet ® 10 containing 10 mg of Cetrizine weighed, and finally powered with the help of mortar. Each uncoated tablet contains 25mg of Cetrizine. A quantity of powder sample of equivalent to 25mg of Cetrizine was taken into volumetric flask. And dilution was made to get concentration of 10µg/ml respectively. These concentrations were scanned at area between 225nm to 235nm. (Table 2)

Table 2: Assay for Cetrizine in Tablet Formulations

Brand Name	Label Claim (mg/tablet)	Amount Found (mg/tablet)	% of Label Claim	Mean	SD	CV
GLUCOBAY ® 25	10	9.99	99.46	99.87	0.2356	0.0023
	10	10.12	100.02			
	10	9.98	99.96			
	10	10.14	100.02			
	10	9.97	99.90			

Table 3: Result for accuracy parameters of Cetrizine (Brand Name: Zyncet ® 10):

Label % recovery	Amount present (mg/tablet)	Amount of Standard added (mg/tablet)	Amount Recovered (mg/tablet)	Total % recovery	%mean recovery	SD	CV
80	10	8	9.99	99.95	100.08	0.1258	0.0012
80	10	8	10.02	100.1			
80	10	8	10.04	100.2			
100	10	10	9.98	99.92	100.03	0.2351	0.0023
100	10	10	10.01	100.3			
100	10	10	10.02	99.87			
120	10	12	9.98	99.96	99.5633	0.6612	0.0066
120	10	12	9.98	98.8			
120	10	12	9.99	99.93			

Accuracy (Recovery study):

Recovery experiments are used for the study of accuracy method. This study was carried out by adding known amount bulk sample to the tablet and recovery was performed at three levels, 80, 100 and 120% of Cetrizine standard concentration. Samples for recovery studies were prepared according to before mentioned procedure. Three samples were prepared for each recovery level. The solutions of sample were analyzed and % recoveries were calculated by using following formula.

$$\% \text{ Recovery} = \frac{\text{Observed amount of compound in sample}}{\text{Amount of all compound present in sample}} \times 100$$

The recovery values are summarized in following table 3.

Precision:

The precision (inter–day) was carried out by using four independent sample of Cetrizine. The intermediate precision (inter-day precision) of the method was evaluated using four different analysts in the same laboratory. (Table 4)

Table 4: Data for Precision of Area under Curve method

Sample Number	Assay of Cetrizine as % of Labelled amount (inter – day precision)			
	Analyst 1	Analyst 2	Analyst 3	Analyst 4
1	99.45	99.65	99.84	99.44
2	100.01	99.33	99.26	99.71
3	99.36	99.28	100.71	100.79
4	100.98	100.20	99.74	99.97
Mean	99.82	99.61	99.88	99.97
SD	0.7294	0.3311	0.57685	0.6246
CV	0.0072	0.0033	0.0057	0.0062

RESULTS AND DISCUSSION:

The standard solution of Cetirizine hydrochloride in distilled water was found to be linear at concentration range of 10-30µg/ml in area between 225 to 235nm using Shimadzu 1800 Spectronic UV-visible spectrophotometer. The calibration curve of Cetirizine hydrochloride was found to be linear at conc. Range 10-30µg/ml at area between 235nm-225nm. The regression equation for the straight line is y= 4.92021-1.22708.

Therefore, it was clear that Cetirizine hydrochloride can be determined in the presence of distilled water as the only reagent with no intervention of any irrelevant substance in pharmaceutical products. The novel method for the quantitative investigation of Cetirizine hydrochloride subjected to study the linearity and different levels of concentrations and calibration standards where the determination range was optimized, accuracy was proved.

CONCLUSION:

The newly developed method is simple, rapid, cost effective and suitable for the determination Cetirizine hydrochloride. From the above experimental studies, it can be concluded that Area under curve method by UV-Spectrophotometry was developed for estimation of Cetirizine hydrochloride. The proposed method for the selected drug was found precise and accurate. The most important features of area under curve processing are their rapidity and simplicity. The method is an excellent

alternative to HPLC methods for routine analysis and accurate and better than the zero order UV-spectrophotometric method.

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RESEARCH ARTICLE

Comparative Evaluation of Protease Production from Okara, Broken wheat, Chickpea and black gram by the soil isolate of *Aspergillus terreus*

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ABSTRACT:

Proteases, the enzymes that digest proteins, are present in all organism and play an essential role in proliferation and differentiation. They are crucial for different applications in industries like detergent, food, laundry and pharmaceutical. Proteases from bacteria and fungi have emerged as a salvation to the industrial sectors. In the presented work, protease producing *Aspergillus terreus* was grown from the field soil of Birring village, Jalandhar. Further, *Aspergillus terreus* was screened for growth on different substrates like Okara, Broken Wheat, Chick Pea and Black Gram. After, substrate selection, the solid-state fermentation was found to be an efficient method for enzyme production. Upon characterisation, the crude enzyme was found to be extracellular. The optimum enzyme activity was at pH of 10.0 and a temperature of 55ºC. Also, Mn^{2+}enhanced the enzyme activity. Thus, the low-cost substrate and effective protease activity, can make this enzyme as ideal candidate for various industrial applications.

KEYWORDS: *Aspergillus terreus*, Birring village, Fungal protease, Jalandhar, Proteases.

INTRODUCTION:

In the era of industrialisation, there is a massive demand for some novel or enhanced enzymes for various industrial processes[1]. These enzymes are commonly sourced from microorganisms, and quite often from fungi[2,3,4]. Proteaseshydrolyse the peptide bonds and produce the amino acids along with ammonia. The name protease does not correspond to one enzyme, but it comprises amidases, proteinases, and peptidases[5]. Plant and animals are also protease-producers but are unable to meet the current demand. Thus, microbial enzymes were exploited as they grow at a high rate and easily amendable by genetic engineering. So extensive research is being done in this direction to meet the demand for detergent, food, leather, and pharmaceutical industries[6,7]. Proteases also find applications as anti-inflammatory enzymes. Such proteases have been extracted from plants previously[8].

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Different bacteria, fungi and actinomycetes have been documented as producers of proteases[9]. Out of these, *Aspergillus, Penicillium, Rhizopus* and *Trichoderma* are the potential ones[10]. The fungal strains favour the moist environment, and thus, solid-state fermentation favours the growth and development of filamentous hyphae and mycelia which adhere to the solid surface and remains attached to it[11]. These strains have not only found applications in enzyme production, but also decolorization and degradation of dyes[12]), degradation of natural rubber[13] and Biosorption of copper[14]. There are several reports which have used the different types of substrates such as corn stover, pre-filtered palm oil effluent, mustard seed oil et cetera[15,16,17]. *Aspergillus terreus*, is one predominant fungal species which secretes protease enzyme at a highconcentration through the fermentation process[18]. Aspergillus terreus has been known to show anti-microbial activity against the pathogenic Streptococcus mutans. So, having one fungus with multiple biotechnological applications is considered an advantage[19].

The current study focusses on the isolation of *Aspergillus terreus* and the production of protease enzyme by utilising Okara, Broken wheat, Chickpea and

Black gram as a substrate along with the optimisation of solid-state fermentation for enzyme production.

MATERIAL AND METHODS:

Soil Collection:

The soil samples were collected from the fields of village Birring, Jalandhar City [Longitude: 75.5761679; Latitude: 31.3260302] from the top soil and the soil was collected in the sterile zipped polybags.

Isolation and Screening of the sample:

For experimenting, the 10^{-4}-fold diluted culture was spread-plated on Skimmed-Milk Agar and Czapek Dox Agar, supplemented with or without Amphotericin B and Tetracycline. The cultures were grown at 30ºC for 4-5 days[20]. On observing the fungal growth, *Aspergillus terreus* was identified based on morphological characteristics[21]. Further, the culture was maintained by streaking it on the Czapek Dox agar+ 0.75%

Preliminary Screening of Proteolytic Fungi by Plate Assay

The culture was screened for protease production by plate-assay with modifications in Kamath et al. (2010) protocol[22]. The fungal isolate was grown on Czapek Dox Agar supplemented with 0.75% casein for three days at 30ºC. The zone of hydrolysis was observed for protease production.

Selection of Substrate for Solid-State Fermentation

Substrates like broken wheat (BW), Okara (OK), Chickpea (CP) and Black gram (BG) were cleaned with 0.2% H_2SO_4 as well as distilled water and dried in a hot air oven at 60ºC for 24 hours. The substrates were then tested individually for optimal production of the protease[23].

Inoculum Preparation:

The 7-day old culture of isolated *Aspergillus terreus*, preserved on the Czapek Dox Agar supplemented with 0.75% casein was suspended in a 10% Tween-80 solution. The fungal spores were inoculated with a needle into the solution for preparing the pre-fermentation culture and was kept for incubation of 5 days at 30ºC[24].

Solid-State Fermentation Condition and Enzyme Extraction:

Twenty grams of each substrate, i.e. BW, OK, CP and BG were added to 50mL of culture medium and incubated overnight before inoculation. Excess water was drained using the muslin cloth, and the substrate was transferred to 250ml flasks. Next, the flasks were sterilised by autoclaving and 4mL of spore-suspension was added for conducting the solid-state fermentation and flasks were incubated at 30ºC.

Additionally, vegetative cells were also inoculated to enhance fungal growth. The substrate showing no growth of *Aspergillus terreus* was discarded. Further, the assessment of protease production by solid-state fermentation was done after 5 and 19 days[25]. The protease was extracted by adding the 60ml of NaCl (1%), in each of the flasks. The flasks were then incubated at 27ºC at 110rpm with shaking for two hours. Next, residues were removed with the help of muslin cloth and the filtrate was sedimented at 8000rpm/10minutes. The crude enzyme extract (supernatant) was collected, whereas the pellet was dissolved in 200µL of lysis buffer and then both were stored at 4ºC for further analysis[11].

Determination of localisation of protease by Gel Diffusion Method:

For determination of the enzyme localization, Czapek Dox Agar supplemented with 0.75% casein was poured in petri plates, 10mm holes were bored and 100µL of crude enzyme extract (supernatant and lysate) was added into the wells. The plates were incubated in the upright position at 30ºC for 24 hours. After incubation, the zone of clearance was observed for the presence of protease enzyme[26].

Assessment of crude protease activity:

For estimating the protease activity, the modified protocol of Anson was followed where in casein protein was used as substrate. The enzyme dilutions were prepared by dissolving the crude enzyme in a mixture of 10mM sodium acetate plus 5mM calcium acetate (pH 7.5) and 5ml of 0.65% buffered casein solution was added and incubated at 37ºC for 30 minutes. After the incubation, 5ml of TCA (Trichloroacetic acid) was added to cease the reaction, followed by 30 minutes incubation at 37ºC. The solution was filtered with 0.45µm polyethersulfone filters. Next, 5mL sodium carbonate and 1mL of Folin's reagent was mixed with 2mL of the filtrate. Enzyme activity was calculated through absorbance measurement at 660nm and comparison with the tyrosine standard. The unit definition of protease implies the concentration of enzyme needed for the liberation of 1µg of tyrosine per ml per minute[27].

Determination of Protein Concentration:

Total protein in the enzyme preparation was estimated using the Bradford method[28]. The concentration of protein was estimated by measuring the absorbance at 595nm and comparing it with the BSA standard curve.

Effect of pH on Protease Activity:

Effect of pH on protease activity was determined by measuring its activity with varying pH. The buffers used for analysis were sodium acetate (pH 3, 4, 5), potassium

phosphate (pH 6, 7, 8) and sodium carbonate (9, 10). The reaction mixtures containing 300µL of the buffer, 500µL of casein and 200µL of the enzyme were incubated at 37°C for 30 minutes. The protease activity was determined using the standard Lowry's method[22,29].

Variation of Enzyme Activity with Temperature:
The dependence of enzyme activity on temperature was determined by measuring its activity upon varing temperature ranging from 6ºC, 25ºC, 35ºC, 45ºC, 55ºC, 65ºC, 75ºC and 90ºC. The reaction mixtures containing 300µL of sodium carbonate buffer, 500µL of casein and 200µL of enzyme were incubated at 37ºC for 30 minutes. The protease activity was determined using the standard Lowry's method[22,29,30].

Determination of Metal Ion Cofactor:
Effect of metal on protease activity was determined by measuring the enzyme activity with or without metal ions like Mg^{2+}, Mn^{2+}, Cu^{2+} and Ca^{2+}. The reaction mixtures were then incubated at 55°C for 60 minutes and the protease activity was determined by standard Folin's protocol[22,29,30].

RESULTS:

Isolation, Identification and Screening of Protease-producing *Aspergillus* strain:
The culture plate of both, Skim milked agar and Czapek Dox agar (with and without Tetracycline and Amphotericin B) were observed for the growth of fungal hyphae. Based on the size and morphology, one distinct colony of cinnamon brown colour with white-creamish vegetative part was selected as shown in Figure 1a and b. On visualizing the fungal colony, at 40X the biseriate, compact, dense columnar and smooth conidiophores were observed. The characteristics were similar with the *Aspergillus terreus*[21]. The fungal strain showed the zone of clearance on the Czapek Dox agar supplemented with 0.75% casein as shown in Figure 1c, which confirmed the production of protease enzyme.

Selection of Substrate and inoculum preparation for Solid-state fermentation:
The substrate flasks inoculated with the spores of *Aspergillus terreus* were observed for fungal growth. It was observed that OK, CP and BG showed the growth and no growth was observed for BW. Thus, fresh inoculum of *Aspergillus terreus* was prepared for inoculation of OK, CP and BG for fermentation.

Solid-State Fermentation for Protease Extraction:
In SSF, the substrate not only served the purpose of anchoring material but also supplements as the nutrient media. Hence, the selected substrates OK, CP and BG were inoculated with prepared inoculum for 5 and 19-days long fermentation process. After the 5th and 19th day, the supernatant and cell-free extract were collected for each substrate for further analysis.

Determination of localization of Protease Enzyme by Gel Diffusion Method:
The protease localization was studied using the standard agar diffusion assay and it was found that the culture supernatant showed the enzyme activity after 5th as well as 19th day of fermentation. The zone of clearance as shown in Figure 1d proved the enzyme to be extracellular.

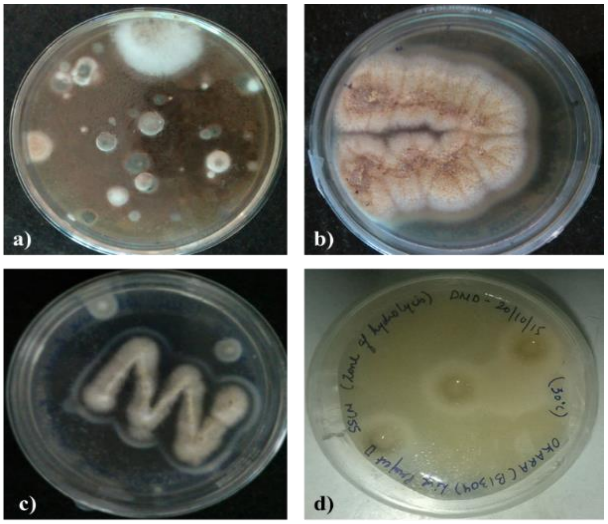

Fig 1: a) Czapek-Dox Agar Plate showing the fungal isolate of '*Aspergillus terreus*',b) Pure culture of *Aspergillus terreus*, c) Isolated *Aspergillus terreus* showing the zone of hydrolysis on Czapek Dox Agar supplemented with 0.75% casein, d) Czapek-Dox-Casein Agar plate showing the localization of protease enzyme

Assessment of Crude Protease Activity and Protein Estimation:
The crude protease activity was estimated by using casein as the substrate and measuring the absorbance at 660nm. The enzyme activity was determined by comparison with the tyrosine standard and using the following formula:

Enzyme Activity = {(551.9µM/0.903) x Absorbance of Unknown Sample}/30

The enzyme activity of sample is illustrated in Table 1 and Figure 2a and 2bfor both 5th and 19th day extract. In both cases, the maximum protease activity was observed for the substrate OK in comparison to CP and BG as illustrated in Table 1. Protein estimation was done using the BSA standard curve and calculated as per the following formula:

Protein content = (500 µg/ml/0.044) x Absorbance of Unknown Sample

The Protein content of all the substrate is illustrated in Table 2 for both 5th and 19th day extract. In both cases, the maximum value for protein content was found for substrate OK as illustrated in Table 2.

Effect of pH on Protease Activity:

The effect of pH on enzyme activity was studied by carrying out protease assay in buffers of different pH. The protease showed the highest activity of 62.40µM/ml/min at the alkaline pH of 10. This confirmed the protease was an alkaline protease and the results were plotted in bar graph as in Figure 2c.

Variation of Enzyme Activity with Temperature:

The effect of temperature was studied by performing the standard protease assay at varying temperatures, followed by quantification of tyrosine released. It was recorded that protease showed the maximum activity of 34.22µM/ml/min at 55ºC as illustrated in Figure 2d.

Determination of Metal ion Cofactor:

The optimum cofactor metal ion for the protease was identified through protease assay in presence or absence of (2mM and 5mM) different metal ions. Whereas Mg^{2+}, Ca^{2+} and Cu^{2+} also showed the increased activity, it was found that the most favourable ion for our protease was Mn^{2+} at a concentration of 5mM. The activity was found to be 94.73µM/ml/minas illustrated in Figure 2e.

Table 1: Crude Enzyme Activity on different substrate with varied dilution at different time interval of 5 and 19 days.

Substrate		BG		CP		OK	
Enzyme Activity (µM/ml/min)	Dilution	Day 5	Day 19	Day 5	Day 19	Day 5	Day 19
	0.4	0.91	1.95	5.19	4.91	9.41	16.0
	0.6	3.5	2.56	5.43	7.51	10.5	19.67

Table 2: Protein Content Estimation of different substrate at different time interval of 5 and 19 days.

Substrate	Protein Content (µg/ml)	
	Day 5	Day 19
BG	613.66	1602.27
CP	886.36	1193.18
OK	2613.63	5352.27

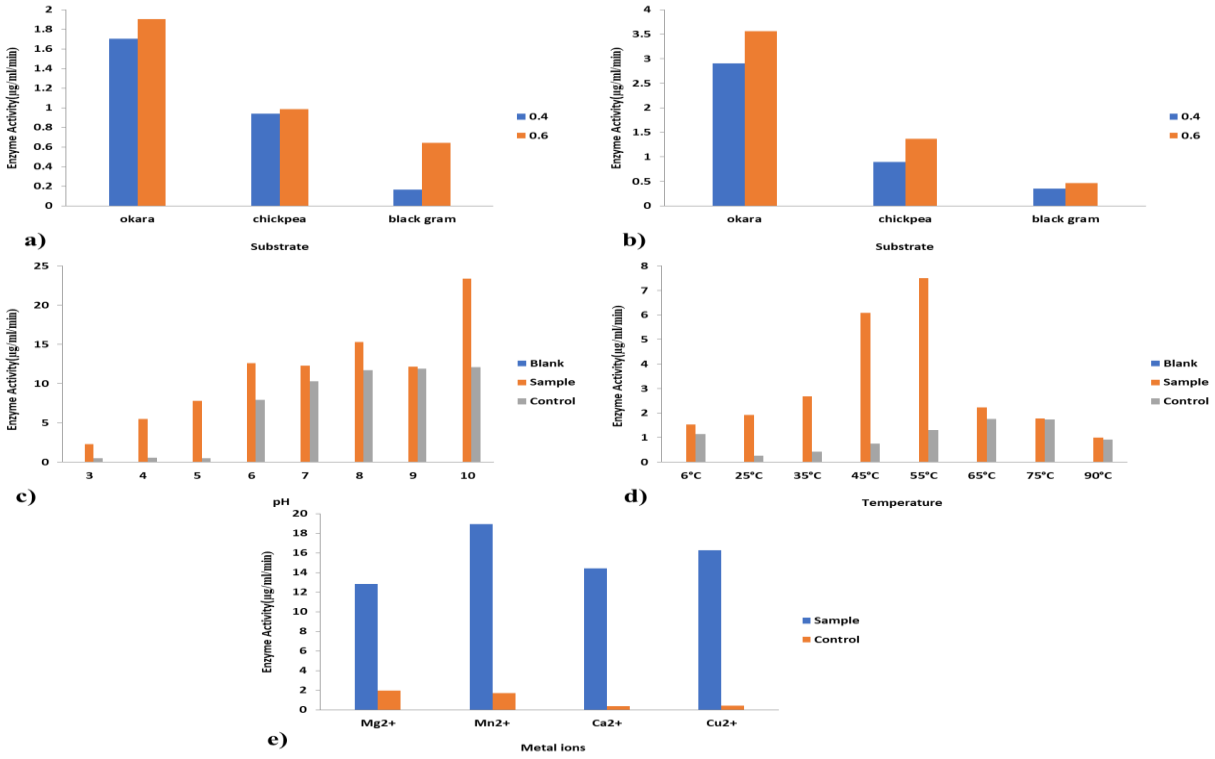

Fig 2: Bar graphs showing enzyme production from different substrates. a) Protease activity from different substrates (after 5 days); b) Protease activity from different substrates (after 19 days); c) pH optima; d) Temperature optima; e) Effect of Metal ions on Protease activity.

DISCUSSION:

Many fungal genera like *Aspergillus, Penicillium, Rhizopus* and *Trichoderma* have reported to be the potent producers of protease. Fungal proteases have also been isolated from fungal strains previously[31]. Amongst these, very less information is available on *Aspergillus terreus* for producing protease enzyme which is supported by the previous reports. The standard protocols were followed for the isolation of *Aspergillus terreus* by referring the method followed by (Gaddeyya et al., 2012)[32]. The morphological characteristics of the fungal strain were in accordance with the report by Balajee et al (2007)[21]. The plate assay showed positive results for protease production on the Czapek Dox Agar supplemented with 0.75% casein similar to results obtained by Pilli and Siddalingeshwara (2016)[33]. The zone of hydrolysis was the confirmation for protease production.

Various substrates like corn-stover, pre-filtered palm oil and mustard oil have been used for the production of protease. But, low-cost substrate for protease production are required. Thus, Broken wheat (BW), Okara (OK), Chickpea (CP) and Black gram (BG) were used as the substrate for the growth of *Aspergillus terreus*. The OK, CP and BG favoured the substrate for their growth and proliferation. Various studies have proposed that the solid-state fermentation is an effective method for the enzyme production[34,35,36]. Therefore, the selected substrates were used for the solid-state fermentation. The supernatant and cell-free extract were collected for each substrate for the determination of localization of the protease enzyme by agar-diffusion assay[37]. Generally, enzymes like lipase, cellulase, xylanase, protease are extracellular in nature[38,39,40].

Several reports claims that pH 7.0-7.5 is optimum for the protease production. But, the *Aspergillus terreus* isolate showed the highest activity at pH 10.0 which is in concordance with activity reported for *Bacillus pumillus*[41]. Literature review has revealed that most of fungi like *Aspergillus oryzae, Beauveria feline, Penicillium* sp. produce protease under SSF condition within the range of 25-30°C[42,43,44,45]. *Aspergillus oryzae* has been reported to exhibit protease activity at 57°C which is quite close with our result[46]. According to reults published previously, Ca^{2+}, Mn^{2+}, Mg^{2+} ions aid in the protease production. Similar result has been recorded for *Pseudomonas thermaerum* GW1[47]. Thus, our study reports about the protease producing strain of *Aspergillus terreus*, which could play essential role in the enzyme industry. Alkalophilic protease have also been isolated from different soils and show enhanced activity in presence of calcium and manganous ions[48].

CONCLUSION:

In the era of biotechnology, the usage of biocatalysts has gained considerable attention. Thus, there is need to explore the microbes which have ability to serve the industrial demand. The major challenges which need attention are high production cost, specific growing condition in SSF and loss of enzyme activity with respect to time. Therefore, to confer these challenges we need to isolate and characterize the new strains of microbes which can grow on the low-cost substrate in a continuous process. There are various factors that influence the protease activity like pH, ionic strength, temperature and handling. Isolation of newer enzymes can increase the rate of industrial process in comparison to the current enzyme. The protease from the *Aspergillus* terreus isolate worked best at 55ºC and at a pH of 10.0, which reflects the advantage of production of a highly effective enzyme using low-cost substrates. Further, genetic manipulations in enzyme-coding genes can aid in enhancing the production of enzyme as well as make them ready to work under harsh condition in the industrial process.

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RESEARCH ARTICLE

Formulation and Evaluation of Fast Dissolving Buccal Patches of Tenofovir Disoproxil Fumarate

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ABSTRACT:

Background and objectives: Tenofovir disoproxil fumarate is a anti-retroviral agent. It is used in the treatment of HIV-1 infection in adults and pediatric patients of 2 years of age and older. It is also indicated for the treatment of chronic hepatitis-B in adults and pediatric patients 12 years of age and older. The present work is designed to prepare and evaluate mucoadhesive buccal film of Tenofovir disoproxil fumarate as a novel form of fast releasing dosage form. The objective of this study was to develop oral drug delivery system in the form of fast dissolving film which overcomes first pass metabolism and the drug achieve to specific site, for greater therapeutic action. **Methods:** Buccal films of Tenofovir disoproxil fumarate were prepared by solvent casting method. The prepared films were evaluated for the various evaluation parameters like thickness, surface pH, weight uniformity, content uniformity, folding endurance, swelling index, in vitro drug release study. **Results:** All the formulations exhibited good results for physicochemical characterizations. In in vitro drug release study, the films exhibited fast release within 5 hours. The formulation F1 (containing HPMC3cps and croscarmellose) showed no irritant effect on buccal mucosa. It was revealed that Superdisintegrants composition had significant influence on drug release. Thus, conclusion can be made that stable dosage form can be developed for Tenofovir disoproxil fumarate for fast release by buccal patches.

KEYWORDS: Tenofovir disproxil fumarate, Buccal patches, Superdisintegrants, Hydroxy propyl methyl cellulose.

INTRODUCTION:

Oral route has been the commonly adopted and most convenient route for drug delivery. Oral route of administration has been received more attention in the pharmaceutical field because of the more flexibility in the designing of dosage form than drug delivery design for other routes, ease of administration as well as traditional belief that by oral administration the drug is well absorbed as the food stuffs that are ingested daily.[1]

The mucosa of the mouth is very different from the rest of the gastro intestinal tract and morphologically is more similar to skin.

Although the permeability of skin is widely regarded as poor, it is not generally appreciated that the oral mucosa lacks the good permeability demonstrated by the intestine.[2]

Tenofovir disoproxil fumarate, marketed by Gilead Sciences, belongs to a class of anti-retroviral drugs known as nucleoside analogue reverse transcriptase inhibitors (NRTI's), an enzyme crucial to viral production in HIV infected people. Tenofovir is indicated in combination with other anti-retroviral agents for the treatment of HIV-1 infection in adults and pediatric patients of 2 years of age and older. It is also indicated for the treatment of chronic hepatitis-B in adults and pediatric patients 12 years of age and older.[3,4,5]

MATERIALS:

Tenofovir disoproxil fumarate was obtained as gift sample from the Hetero drugs, Hyderabad. HPMC3cps, Superdisintegrants were obtained from Otto reagents,

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SSG, Citric acid and Sodium lauryl sulfate were obtained from SD fine chemicals, Mumbai. All other reagents and solvent used were of analytical grade.

Method of preparation of patches:[6]

The mucoadhesive buccal patches were formulated using solvent casting technique.

The fast dissolving buccal patch was prepared by dissolving the film forming polymers (HPMC3cps) in the water followed by addition of glycerol as plasticizer to the resulted polymer solution. The above solution was allowed to stir on the magnetic stirrer for 4 h to homogenize the solution. Then the solution kept in the vacuum desiccators to remove the air bubbles. Meanwhile, in the separate beaker the solution of all ingredients with TDF is prepared and allowed standing for 45 minutes. Finally, both the solutions were mixed and homogenized on the magnetic stirrer for another 1 h. Then the solution was casted into the Petriplates for drying into the oven at 50°C for 24 h. After drying the film was cut into suitable size and stored in aluminum foil in well closed container.

Table 1: Composition of buccal patches of Tenofovir disoproxil fumarate

Ingredients	Category	F1	F2	F3	F4	F5	F6
TDF (mg)	Drug	300	300	300	300	300	300
Glycerol (mg)	Plasticizer	200	194	200	194	200	194
HPMC 3cps (mg)	Film forming agent	500	500	500	500	500	500
Croscarmellose (mg)	Superdisintegrants	18	24	-	-	-	-
Crospovidone (mg)		-	-	18	24	-	-
SSG (mg)		-	-	-	-	18	24
Citric acid (mg)	Acidifier	6	6	6	6	6	6
SLS (mg)	Surfactant	1	1	1	1	1	1
Orange spirit (mg)	Flavoring agent	5	5	5	5	5	5
Methanol: Ethanol: Chloroform	solvents	6:9:12	6:9:12	6:9:12	6:9:12	6:9:12	6:9:12

Calibration curve of Tenofovir Disoproxil Fumarate:[6]

A. Preparation of 6.8 p^H phosphate buffer:

Dissolve 28.8g of disodium hydrogen phosphate and 11.45g of potassium dihydrogen phosphate in 1000ml of distilled water to produce 6.8 p^H phosphate buffer.

B. Calibration curve TDF:

100mg of pure drug was taken in a 500ml standard flask and to this add 6.8 p^H phosphate buffer and shake vigorously. From this stock solution with draw 1ml of solution and dilute to 100ml with phosphate buffer. Finally, different concentrations like 10µg/ml, 20µg/ml, 30µg/ml, 40µgml, and 50µg/ml were prepared. These are further diluted and samples were subjected to spectrophotometric analysis (UV-Visible) to determine absorbance at λ_{max} of 260nm. From the curve obtained, a line equation and regression analysis were determined.

Evaluation tests for patches:[7]

1. Weight Variation:

The patches were cut in the required sizes and weights were calculated individually.

2. Thickness:

The thickness of each patches was measured using thickness tester (screw gauge) at different positions of the film and the average was calculated.

3. Moisture Absorption:

The buccal patches were weighed (w1) and placed in desiccators having anhydrous Aluminium chloride. After 3 days, the patches were reweighed (w2). The % moisture absorption was calculated using

$$\% \text{ Moisture absorption} = \frac{\text{Final weight-Initial weight}}{\text{Initial weight}} \times 100$$

4. Moisture Loss:

The buccal patches were weighed (w1) and placed in desiccators having anhydrous. Calcium chloride. After 3 days, the patches were reweighed (w2). The % moisture absorption was calculated using

$$\% \text{ Moisture loss} = \frac{\text{Initial weight - Final weight}}{\text{Initial weight}} \times 100$$

5. Folding Endurance:

Folding endurance of the buccal patches was determined by taking 20mm diameter of patch was repeatedly folding at the same place till it broke. The no of times of patch could be folded at the same place without breaking gave the value of the folding endurance.

6. Drug content uniformity:

The films were tested for the content uniformity. A film of size 1 cm^2 was cut and placed in the beaker containing 100 ml of 6.8 p^H phosphate buffer. The films were allowed to dissolve in the solution. The contents were transferred to a volumetric flask. The absorbance of the solution was subjected to spectrophotometric analysis (UV-Visible) to determine absorbance at λ_{max} of 260nm.

7. Surface p^H:

For surface p^H determination, the patches were left to swell for 2 hours in 6.8 p^H phosphate buffer. The surface

p^H was measured by means of p^H paper placed on the surface of the patches. The mean of three readings was recorded.

8. Swelling Index:

A drug loaded film of 1 cm^2 was weighed and then placed in the 50 ml of 6.8 p^H phosphate buffer. After 2 hours the patch was removed and again reweighed. The difference in the final and initial weights gave the results of weight increase due to the absorption of water and swelling of film.

$$\text{Swelling index} = \frac{\text{Final weight - Initial weight}}{\text{Initial weight}} \text{ X } 100$$

9. Disintegration time:

10. It is an important tool in designing the dosage form. It is the time required for the dosage form to break up into granules of specified size under carefully specified conditions.

11. Diffusion Studies:

The prepared patches were cut according the required size and the attached to the open-end glass test tube; it is placed in the beaker containing 6.8 p^H phosphate buffer. The beaker is kept on the magnetic stirrer and temperature of 37 ± 0.5^0 C was maintained. Aliquot amount of samples are withdrawn at an interval of 1 minute and same amount of fresh medium was replaced. The absorbance of the solution was subjected to spectrophotometric analysis (UV-Visible) to determine absorbance at λ_{max} of 260 nm.

12. Kinetic study:

The matrix systems were reported to follow the zero order release rate and the Diffusion mechanism for the release of the drug. To analyze the mechanism for the release and release rate kinetics of the dosage form, the data obtained was fitted into, Zero order, First order, Higuchi matrix and peppa's model. In this by comparing the r Values obtained, the best fit model was selected.

a. Zero order kinetics:

Drug dissolution from pharmaceutical dosage forms that do not disaggregate and release the drug slowly, assuming that the area does not change and no equilibrium conditions are obtained can be represented by the following equation

$$Qt = Q_0 + K_0 t$$

Where Qt is the amount of drug dissolved in time t, Q_0 is the initial amount of drug in the solution and Ko is the zero order release constant.

b. First order kinetics:

To study the first order release kinetics the release rate data were fitted to the following equation.

$$\log Qt = \log Q_0 + k1t/2.303.$$

Where Qt is the amount of the drug released in time t, Qo is the initial amount of the drug in the solution and K1 is the first order release constant.

c. Higuchi model:

Higuchi developed several theoretical models to study the release of water Soluble and low soluble drugs incorporated in semisolids and or solid matrices. Mathematical expressions were obtained for drug particles dispersed in a uniform matrix behaving as the diffusion media. And the equation is

$$Qt = KH.t_{1/2}$$

Where Qt is the amount of drug released in time t, KH is the Higuchi Dissolution constant.

d. Korsmeyer and Peppa's model:

To study this model the release rate data are fitted to the following equation.

$$Mt/M = K.tn$$

Where Mt/M is the fraction of drug release, K is the release constant, t is the release time and n is the Diffusion exponent for the drug release that is dependent on the shape of the matrix dosage form.

RESULTS AND DISCUSSION:

1. Preformulation studies:

1. Preparation of Standard curve of Tenofovir disoproxil fumarate:

The absorbance of the solution was measured between 200 – 400 nm, using UV spectrophotometer with distilled water as blank. The values are shown in table no. 5. A graph of absorbance Vs Concentration was plotted which indicated in compliance to Beer's law in the concentration range.

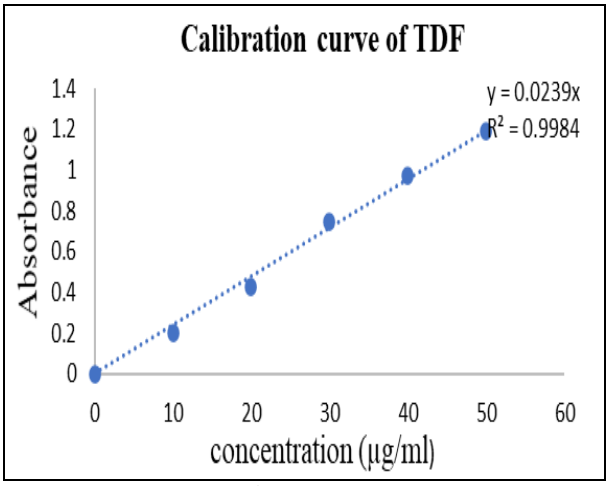

Fig No.1. Standard graph of TDF

Identification of pure drug:

FTIR Spectroscopy:

The FTIR spectrum of pure drug of Tenofovir Disoproxil Fumarate shows the following functional groups at their frequencies. The IR spectra of Tenofovir Disoproxil Fumarate shown in spectrum no.1

Solubility:

Tenofovir Disoproxil Fumarate is soluble in methanol, ethanol. Slightly soluble in DMSO.

Melting point:

It has melting point of 113-115^{0}C.

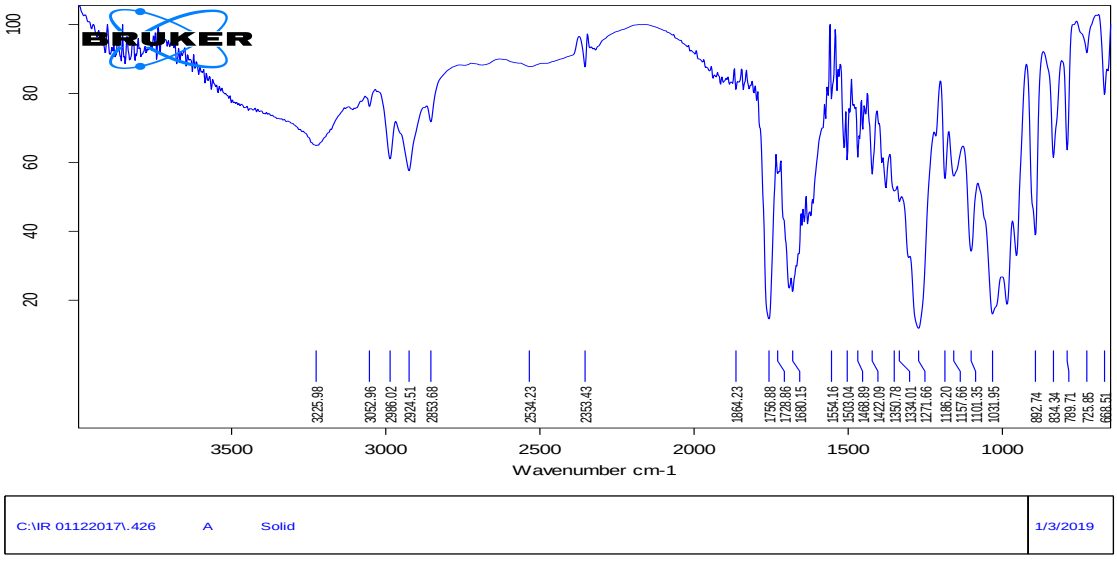

Fig No. 2.: FT-IR Spectrum of pure drug (Tenofovir Disoproxil Fumarate)

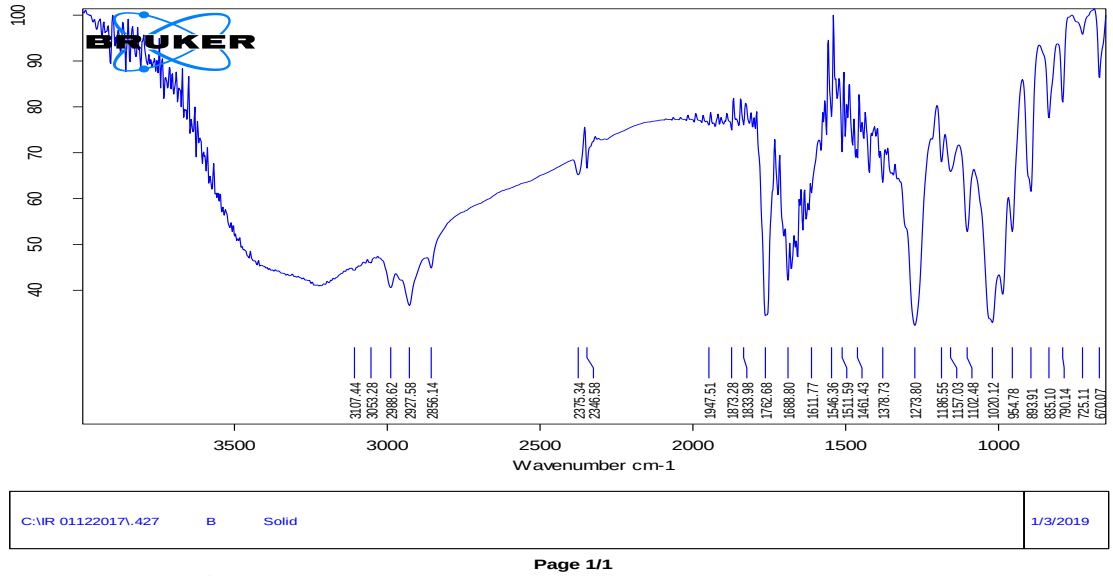

Fig No. 3.: FT-IR Spectrum of TDF+HPMC K4M

Evaluation Results of Prepared TDF Buccal Patches:

1. Weight variation:

Weight variation test was performed. The weights of the patches were between 0.30 gm to 0.35 gm. Hence all patches formulations passed weight variation test. Results are shown in table no.2.

2. Thickness:

Thickness of all the formulations was between 0.21 mm to 0.28 mm. Results are shown in Table No.2.

3. % Moisture absorption:

% Moisture absorption of all formulations was between 6.45 % to 14.70 %. Results are shown in Table No. 2.

4. % Moisture loss:
% Moisture loss of all the formulations was between 9.09 % to 16.12 %. Results are shown in Table No.2.

5. Folding endurance:
The number of times the strip could be folded at the same place without breaking gives the exact value of folding endurance (a measure of fragility). The folding endurance was measured manually for the prepared films. A strip of 2×2cm was cut evenly and repeatedly folded at the same place till it broke.

6. Drug content:
Percentage drug content of all formulations was between 55.34 to 86.53%. The results are shown in Table no.2.

7. Surface pH:
The surface pH of prepared inserts was found be in range of 6.2 to 7. This indicated that the prepared inserts would not alter the pH of the tear fluid in the eye. Results are shown in Table no.2

8. Swelling index:
Swelling index of all the formulations was between 0.52% to 0.86%. Results are shown in Table No.2.

9. Disintegration time:
Disintegration time of all the formulations was between 32 to 59. Results are shown in Table No.2.

10. In vitro release studies:
The in vitro drug release studies were performed by using a modified US Pharmacopoeia paddle-type dissolution apparatus (using 900ml of PBS 7.4 as dissolution medium). The dissolution studies are crucial because one needs to maintain the drug concentration on the surface of the Stratum corneum consistently and keep it substantially higher than the drug concentration in the body, to achieve a constant rate of drug permeation. A circular film with an internal diameter of 1 cm was used for the study and a stainless-steel ring was employed to hold the patch at bottom. All dissolution studies were performed at 32±0.5◦C, at 50rpm. Samples were withdrawn at different time intervals and analysed spectrophotometrically. % drug released were plotted against time for different formulations.[7]

Table No. 2: Evaluation studies of formulated buccal patches

Formula No.	Weight variation (mg)	Thickness (mm)	% of Moisture absorption	% of Moisture loss	Folding endurance	Drug content (%)	Surface pH	Swelling Index (%)	Disintegration Time (sec)
F1	0.33	0.25	9.09	9.09	292	92.34	6.2	0.52	38
F2	0.34	0.25	14.70	14.7	256	86.53	6.4	0.63	32
F3	0.30	0.21	13.30	10.01	285	61.96	6.7	0.57	50
F4	0.32	0.23	9.37	15.62	187	58.76	6.8	0.67	46
F5	0.35	0.28	9.09	11.42	198	67.09	6.9	0.76	59
F6	0.31	0.22	6.45	16.12	198	69.44	7.0	0.86	55

Table No. 3. Cumulative % drug release of various formulation

TIME (min)	F 1	F 2	F 3	F 4	F 5	F 6
0	0	0	0	0	0	0
0.5	22.95	31.85	26.15	28.29	29	22.95
1	31.67	39.14	34.69	35.23	32.56	31.67
1.5	41.81	47.86	49.11	41.99	41.81	41.81
2	61.38	62.27	57.82	66.54	56.04	61.38
3	70.64	74.91	69.92	73.3	69.21	70.64
4	71.7	84.51	81.49	79.18	80.07	71.7
5	99.45	93.26	93.59	86.14	97.86	98.57

Table No. 4. Higuchi model: √T v/s % CDR

TIME (min)	F1	F2	F3	F4	F5	F6
0	0	0	0	0	0	0
0.70	22.95	31.85	26.15	28.29	29	22.95
1	31.67	39.14	34.69	35.23	32.56	31.67
1.22	41.81	47.86	49.11	41.99	41.81	41.81
1.41	61.38	62.27	57.82	66.54	56.04	61.38
1.73	70.64	74.91	69.92	73.3	69.21	70.64
2	71.7	84.51	81.49	79.18	80.07	71.7
2.23	98.57	93.26	93.59	86.14	97.86	98.57

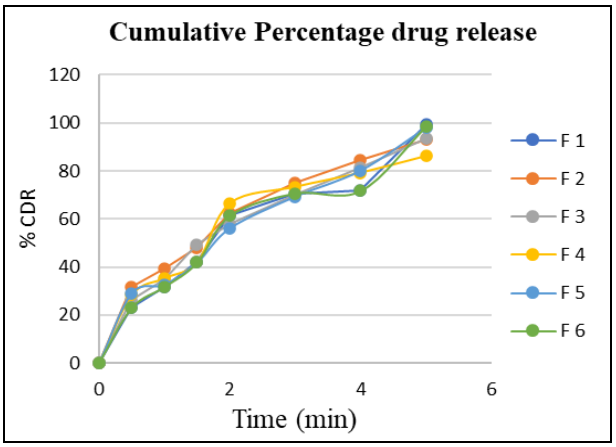

Fig No. 4. Cumulative Percentage drug release of Formulation – 1

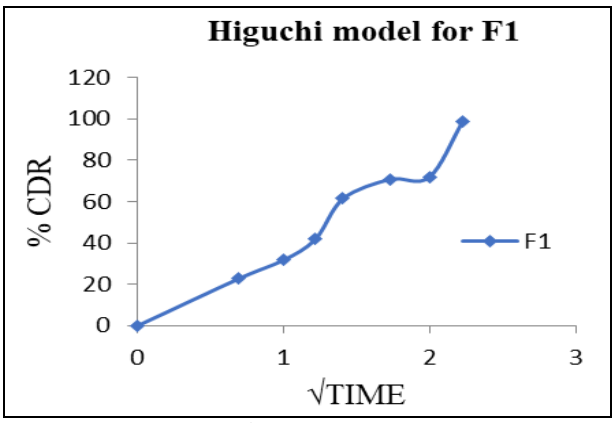

Fig No. 5. Higuchi model of Formulation – 1

CONCLUSION:

These results indicated that F1 (drug 300 mg) with croscarmellose of tenofovir buccal patches has achieved the objective of considerable influence on the physio chemical characteristics and releasing property. The concentration of Croscarmellose in the formulation determines the drug release from the patches. As the concentration of Croscarmellose increases, drug release also increases. So, finally the best concentration of Croscarmellose was found to be 24 mg and formulation 1 gave the best results.

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RESEARCH ARTICLE

Hinguvachadi Choornam, An *Insilico* approach to confirm the Therapeutic Efficacy towards PCOS

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ABSTRACT:
Polycystic Ovary Syndrome (PCOS) in other way known as hyperandrogenic anovulation. Itis the common endocrinological and metabolic disorder in women of reproductive age which may leads to infertility. The condition estimate by the development of more than 10 small ovarian cysts of diameter ranging from 2-9mm.Disordered gonadotropin secretion in PCOS women leads to increased LH level which disturbs the LH: FSH ratio in body. Stimulation of FSH receptors with plant phytoconstituents elevate the production of FSH which reduce the over activity of LH and reduce the PCOS activity. In addition, inhibition of androgen receptors reduce the excess androgenic activity in body. Hinguvachadi choornam; an Ayurvedic formulation used for treating Poly Cystic Ovarian Syndrome. In the present study, *insilico* evaluation of FSH receptor (4AY9) and androgen receptor (2PIV) by phytoconstituents present in the choornam were studied using PyRx, PyMOL, Open babel software. ADME parameters are evaluated with SWISS ADME software. The results revealed that all the phytoconstituents possess binding energy ranges from -6.0kcal/mol to -10.0kcal/mol with the two receptors. In addition, the drug likness of the constituents present in the choornam; asarone, ferulic acid, alantolactone, quercetin, naringenin and gingerol predicts through analyzing the ADME properties.

KEYWORDS: Poly Cystic Ovarian Syndrome, Hinguvachadi choornam, Swiss ADME, FSH receptor, *insilico* study.

INTRODUCTION:
Poly Cystic Ovarian syndrome (PCOS) previously known as Stein-Leventhal syndrome was first described in 1935 by American gynecologists Irvin F Stein, and Michael L Leventhal[1]. The cardinal features includes; hyperandrogenism, anovulation and cystic ovaries. The exact etiology of PCOS was still not clear. Altered gonadotropin secretion results in the over production of Luteinizing Hormone (LH) which further leads to the androgen secretion from thecal cells of ovary and results in hyperandrogenism[2]. The factors that may affect the risk of PCOS includes; genetic aspects, stress, over weight, insulin resistance and sleep apnea. Environmental risk factors like eating plastic packed foods, contacting pesticides, eating fruit with pericarp, alcohol consumption may leads to cystic ovaries[3].

Women with PCOS possess reduced glucose availability in the oocyte and follicular cells which may impair oocyte quality and further leads to infertility[4]. Altered endothelial function should be observed in PCOS women with increased carotid intima media thickness (measure of thickness of inner two layers of carotid artery) and lower degree of adiponectin[5].

In ayurveda mainly four etiological factors like unhealthy lifestyle (*Mithyachara)*, menstrual disorders (*Artavadushti*), genetic defects (*Beejadosha*) and certain unknown factors (*Daivata*) are responsible for PCOS[6,7]. Hinguvachadi choornam is the combination of effective potent herbs for the management of reproduction related problems. Hingu means Asafoetida which is the first ingredient of this product highly effective in PCOS[8].

Granulosa cells in ovary releases FSH, binds to its receptor leading to the production of aromatase which aromatize the excess androgen. Targeting these receptors with chemical drugs can be effective but possess side effects. The plant-based compounds with FSH receptor stimulatory and androgen receptor inhibitory action can

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be important in the treatment of PCOS. *Insilico* studies mainly aims to find out the binding potential of these ligands with FSH receptor and androgen receptor, the important biomarkers of PCOS. Docking was performed by Auto dock Vina program in PyRx software and visualized using PyMol software[9].

MATERIALS AND METHODS:

Hinguvachadi choornam:

The major ingredients present in Hinguvachadi Choornam includes *Ferula asafetida*, *Acorus clamus*, *Punica granatum*, *Inula recemosa*, *Zingiber officinale*, *Tamarindus indica* etc.

ADME Properties:

Absorption, Distribution, Metabolism and Elimination are the pharmacokinetic parameters of the ligand important for determining their therapeutic action. ADME properties analysed using Swiss ADME, an online ADME prediction tool which is quick, accurate and easy to use[9].

Macromolecule structure retrieval:

From RCSB protein data bank (http://www.rcsb.org/pdb/), 3D structure of FSH receptor (PDB ID- 4AY9) and androgen receptor (PDB ID-2PIV) are retrieved. Protein processing done through PyMOL viewer by removing co-crystallised ligands and water molecules. Hydrogen atoms are added to produce the processed 3D structure of macromolecules in pdb format[9].

Ligand structure retrieval:

Compounds were reprocessed from pubchem data bases ie; ferulic acid (CID-445858), alantolactone (CID-72724), asarone (CID-636822), gingerol (CID-442793), naringenin (CID-932) and quercetin (5280343). The compounds in SDF format should converted into PDB format using online smile translators[10].

Docking and visualization:

AutoDockVina was used for docking of ligands to the receptors. Receptor grids are prepared for proteins to such an extent that ligands should be inside the active pockets while docking. Docked 3D structures were visualized using PyMOL open software tool[11].

RESULT AND DISCUSSION:

ADME and Drug likeness prediction:

Analysis of physicochemical and drug like properties of phytoconstituents should be carried out through SWISS ADME software. The properties like molecular weight, log P value, number of hydrogen bond acceptors and donors are listed in **table 1**. The drug potential of all the ligands are tested using Lipinski rule of five, Ghose, Veber and Egan rule, shown in **table 2.**

Table 1: Physicochemical properties, ferulic acid (CID-445858), alantolactone (CID-72724), asarone (CID-636822), gingerol (CID-442793), naringenin (CID-932) and quercetin (5280343) by Swiss ADME

SL No	Plant name	Constituent	Formula	Molecular weight (gm/mol)	Log *P*	No. of hydrogen bond acceptor	No. of hydrogen bond donor
1	*Ferula asafoetida*	Ferulic acid	$C_{10}H_{10}O_4$	194.18	1.62	4	2
2	*Acorus calamus*	Asarone	$C_{12}H_{16}O_3$	208.25	3.02	3	0
3	*Punica granatum*	Quercetin	$C_{15}H_{10}O_7$	302.24	1.63	7	5
4	*Inula recemosa*	Alantolactone	$C_{15}H_{20}O_2$	232.32	2.75	2	0
5	*Zingiber officinale*	Gingerol	$C_{17}H_{26}O_4$	294.39	3.48	4	2
6	*Tamarindus indica*	Naringenin	$C_{15}H_{12}O_5$	272.25	1.75	5	3

Table 2: Drug likeness properties offerulic acid (CID-445858), alantolactone (CID-72724), asarone (CID-636822), gingerol (CID-442793), naringenin (CID-932) and quercetin (5280343) by Swiss ADME.

SL No	Plant name	Constituent	Lipinski rule	Ghoserule	Veber rule	Eganrule	Bioavailability Score
1	*Ferula asafoetida*	Ferulic acid	Yes	Yes	Yes	Yes	0.56
2	*Acorus calamus*	Asarone	Yes	Yes	Yes	Yes	0.55
3	*Punica granatum*	Quercetin	Yes	Yes	Yes	Yes	0.55
4	*Inula recemosa*	Alantolactone	Yes	Yes	Yes	Yes	0.55
5	*Zingiber officinale*	Gingerol	Yes	Yes	Yes	Yes	0.55
6	*Tamarindus indica*	Naringenin	Yes	Yes	Yes	Yes	0.55

SwissADME enables the estimation of the pharmacokinetic properties of various constituents was shown in the **table.1**. All the constituents possess molecular weight less than 500gm/mol. The lipophilicity, number of hydrogen bond donors and acceptors are within the normal range. Increased number of hydrogen bonds shows increased binding affinity of ligand with receptor. Lead like properties of the compounds are evaluated on the basis of four different rule-based filters. Lipinski rule is the pioneer rule. A drug should follow Lipinski rule of five only if its molecular weight is less than 500gm/mol, number of hydrogen bond acceptors should ≤10, number of hydrogen bond donors should ≤5, log P value should be less than 5 and the number of rotatable bonds should be <8. Ghose rule suggest under the criteria that Log P lies in the range -0.4 - 5.6, Molecular refractivity within 40 - 130, Molecular weight should be in a range of 160 - 480,

Number of atoms (20-70) and Polar surface area should <140. Adrug should follow Veber rule if its rotatable bonds should be less than or equal to 10 in number and the polar surface area should less than or equal to 140.Egan rule defined as Topological polar surface area (TPSA) should be > 131.6Å. All the six constituents mentioned above were cleared the rule filters and possess drug like properties shown in **table 2**.

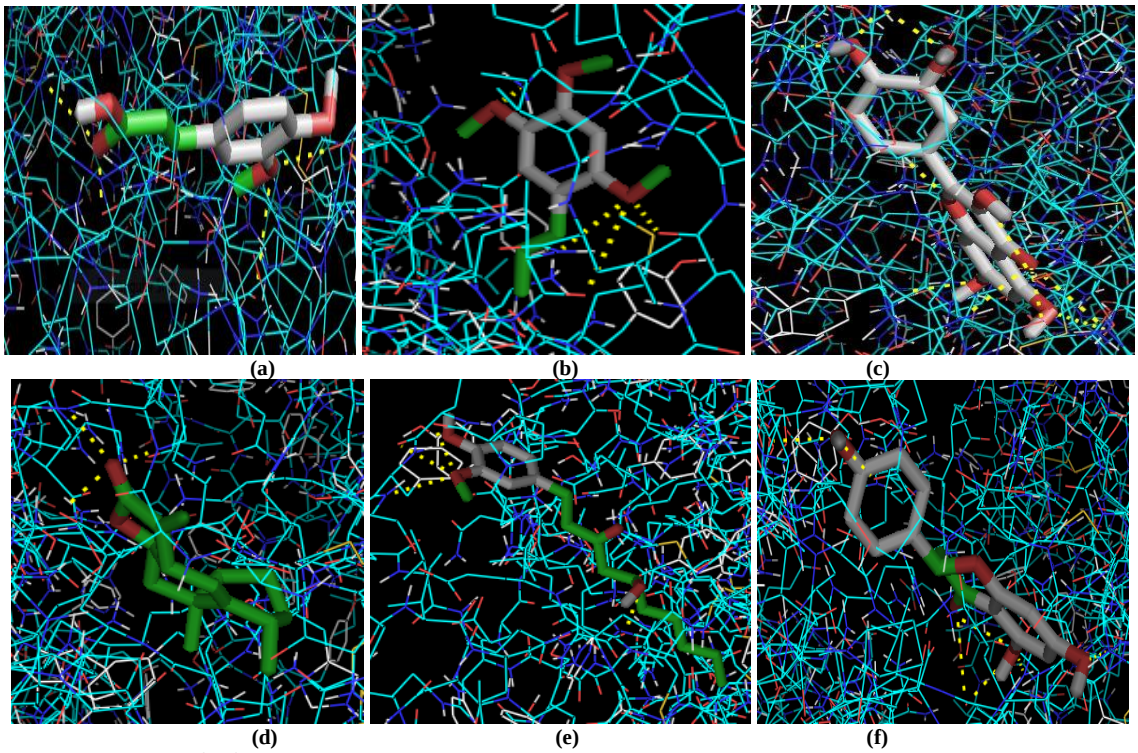

Figure 1: Docked images of(a)ferulic acid (CID-445858), (b) asarone (CID-72724), (c) quercetin (CID-636822), (d) alantolactone(CID-442793), (e) gingerol(CID-932) and (f) naringenin(5280343) with FSH receptor (4AY9) by PyMol.

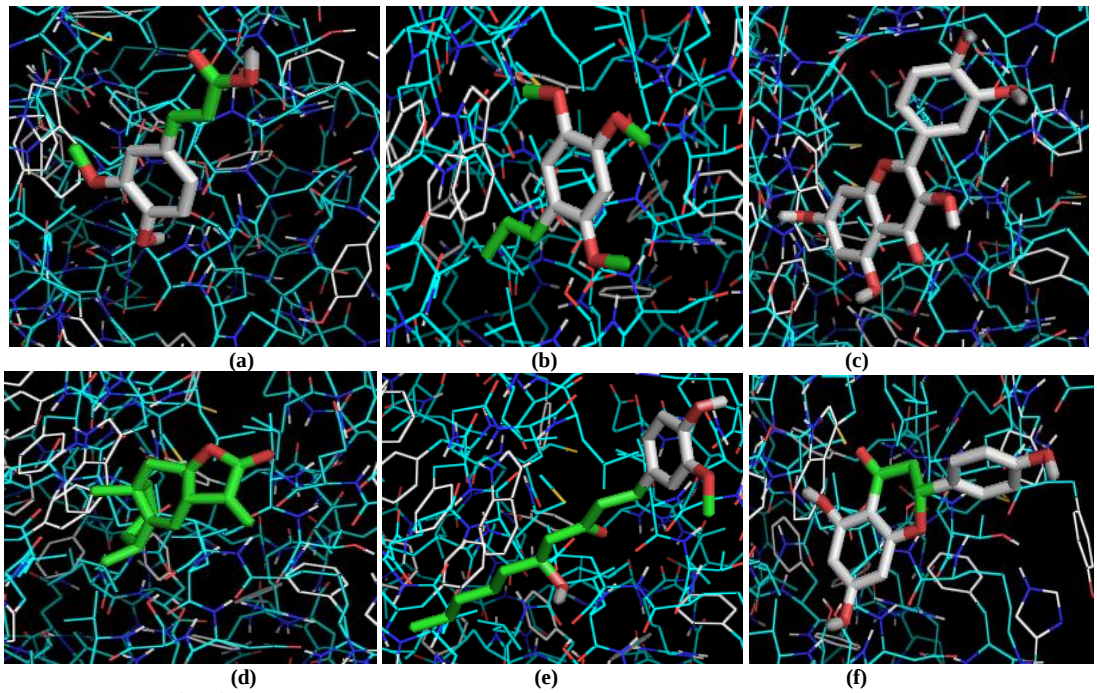

Figure 2: Docking image of (a)ferulicacid (CID-445858), (b) asarone (CID-72724), (c) quercetin (CID-636822), (d) alantolactone(CID-442793), (e) gingerol(CID-932) and (f) naringennin (5280343) with androgen receptor (2PIV) by PyMol.

Table 3: Binding affinity and hydrogen bond interactions of ferulic acid (CID-445858), alantolactone (CID-72724), asarone (CID-636822), gingerol (CID-442793), naringenin (CID-932) and quercetin (5280343) by PyRex.

SL No.	Constituents	Binding affinity(kcal/mol) with		Hydrogen bond interaction with 4AY9
		4AY9	2PIV	
1	Ferulic acid	-7.4	-6.2	4
2	Asarone	-6.2	-6.0	4
3	Quercetin	-10.0	-8.6	10
4	Alantolactone	-6.2	-8.3	3
5	Gingerol	-6.3	-7.3	4
6	Naringenin	-9.7	-9.1	7

Molecular docking studies:

Docking is the process of binding of small molecule compounds into the binding site of a receptor and estimating the binding affinity of the complex. In the present study, molecular docking and virtual screening are carried out through Auto Dock Vina program. **Figure 1** shows the docked images of phytoconstituents with FSH receptor (4AY9). Docked images of phytoconstituents with androgen receptor (2PIV) are shown in **Figure 2**. Even though there is no hydrogen bond interactions, all the constituents possess favourable binding affinity with the androgen receptor. Binding energy and hydrogen bond interaction of constituents with the FSH and androgen receptors are listed in **table 3**.

CONCLUSION:

Currently, the drug available are effective against PCOS, but possess serious side effects. Hence, the identification of new ligands to the target is important. In the present study, we performed the docking studies in order to analyze and evaluate the binding affinity of phytoconstituents present in the choornam with FSH and androgen receptor using in silico tools. All the six constituents show better docking score. Among them, quercetin possess highest binding affinity with FSH receptor and in case of androgen receptor, naringenin shows highest binding affinity. Along with this all the constituents shows drug like properties. It reveals that the compounds possess potential towards PCOS. Hence Hinguvachadi choornam can be analyzed by further *invivo* and invitro studies and can be leads to a potential therapy for PCOS.

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RESEARCH ARTICLE

Inhibitory effect of Phenolic and Flavonoidal content of *H. indicum* Root Extract on 1,1- diphenyl-2-picrylhydrazyl radicals

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ABSTRACT:
The present work was targeted to determine the total phenolic and flavonoidal content along with the determination of anti-oxidant behavior of methanolic root extract of *Heliotropium indicum* linn. The result reveals that 400 ± 7.31 mg Gal1ic acid equiva1ent of phenolic content and 540 ±59.34 mg quercetin equiva1ents of flavonoidal content was present in per gram dry weight of plant extract. The antioxidant data obtained from the work reveal that as the concentration increases the radical scavenging activity also increased. At 1000 µg/ml it has shown 91% inhibition. From this study, It was understood that anti-oxidant behavior of root extract is associated with total phenolic and flavonoidal content of extract.

KEYWORDS: *Heliotropium indicum*, total phenolic content, total flavonoidal content, anti-oxidant behavior, 1,1- diphenyl-2-picrylhydrazyl (DPPH) radicals.

INTRODUCTION:
Reactive oxygen species (ROS) are the responsible cause of oxidative stress. These species are formed as by-products in the ce11s of aerobic living body. This leads to conversion of target molecules into free radicals and damage resulted[1]. ROS include family of oxygen-containing extremely reactive and free radicals. Examples are superoxide (O_2 –), hydroxyl radicals (OH –), singlet oxygen, hydrogen peroxide (H_20_2), and 1ipid peroxides[2]. ROS are having very short ½ lives. They cause damage to main components of cell like proteins, lipid and DNA. This damage resulted in a chronic disorder like atherosclerosis, neurodegenerative disease, cancer, inflammation, aging etc[3,4,5]. ROS resulted in decrement of proliferation of cell and modulation of apoptosis and cell differentiation[6]. Epidemio1ogical evidence pointed that aging and rise in ROS are possible cause of bone loss in humans as well as in animals[7].

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Under standard physiological circumstances, cells of our body can wrestle free radical or oxidative tension by its antioxidant defense mechanism. Many endogenous guards are present inside our body, like chelating proteins, glutathione peroxidase, antioxidant enzymes cata1ase and superoxide dismutase[8]. Exogenous antioxidants derive from food, available in fruits and vegetab1es. Phytoconstituents like carotinoids, lycopene, polyphenols and vitamin C and E are effective antioxidant[9]. When endogenous or exogenous antioxidants are not able to resist oxidative damage, DNA repair enzymes, transferase, protease and lipase came into play[10]. When antioxidants defeated in fight against oxidative stress, disorder related to oxidative stress grow, which may include cancer, cardiovascular disease, diabetes, osteoporosis and neurological diseases[11].

Heliotropium indicum linn. Belongs to family Boraginaceae. Hatishur is local name of H. indicum. It is an herb distributed throughout in India. In folklore systems and traditional medicine systems *Heliotropium indicum* was employed for treating a variety of diseases. *Heliotropium indicum* was used by traditional healers of Kancheepuram in Tamil Nadu for nervous disorders,

skin diseases, stomachache and poison bites. In African countries, it is employed for treating malaria. Jamaican people use flower infusion for treating menorrhagia in female. In Thailand, its inflorescence is taken along with water or milk in the first three days menes for proper flow. In Eastern Nicaragua whooping cough of child is treated with leaf and root decoction[12]. In Cachar district of Assam juice of root is employed as opthalmia and extract of leaf is employed for treatment of wound and cuts[13]. People of Rayal seema of Andhra use paste of leaf for rheumatism. In Coimbatore district juice of its leaf along with coconut oil was employed to treat dandruff[14].

Flavonoids are coming under poly-phenolic compound. In nature, flavonoids are ubiquitous. Presence of minimum 1 aromatic ring and 1 or more –OH group are characteristic of phenolic compound[15]. These day's phenols and flavonoids have received great concern due to their valuable outcomes on human. There are a small number of studies on effect of *Heliotropium Indicum* on free radicals by DPPH method and its total flavonoidal and phenolic contents. Thus, in present work an effort has been given to study the TFC and TPC and to estimate the antioxidant actions of methanolic *Heliotropium Indicum* root extract by adopting broadly accepted model of free radical scavenging system (DPPH).

MATERIAL AND METHODS:

The roots of H. Indicum were gather from a village of Tirunelveli district of Tamil Nadu. It was confirmed by research officer of CCRAS Mr. V. Chelladurai. It was cleansed with water and dried at normal temperature. It was then converted into fine powder by mechanical grinder. By applying Soxhalet apparatus and methanol the extract was prepared. The extract was dried by rota evaporator. The dried extract was kept at 4oC for study.

Total phenolic content:-

It was determined in the extract by using Folin Ciocalteau technique16. Stock so1ution of Standard Gallic acid: l0mg of ga11ic acid was disso1ved in l0 ml of distilled water to attain the concentration 1 mg/m1. Working so1ution: 50, 100, 150, 200 and 250 µ1 of standard gal1ic acid were obtained from stock so1ution and the vo1ume prepared up to 1 ml by addition of disti1led water to attained the fina1 concentration of 50 - 250µg/m1. 1 mg/m1 of test plants extracts was arranged. The vo1ume was made to 45 m1 with disti11ed water in volumetric f1ask. l ml of Fo1in-Ciocalteu reagent (di1uted l:2 with disti1led water) was added and the content was mixed proper1y. Three minutes later, 3 m1 of 2O % sodium carbonate added to the combination and it was permitted to position for 2 hours with infrequent shaking. The absorbance of obtained blue colour that deve1oped was studied at 760 nm by using spectrophotometer.

Values of total phenol can be represented by Gallic acid equivalent (mg/ g of dry weight). Calculation of total phenolic content by following formula:

$$T = \frac{CV}{M}$$

T=Total Phenolic amount
C= Concentration of gal1ic acid from ca1iberation curve (µg/m1)
V= Volume of extract (m1)
M= Wt. of plant extract

Total flavonoids content:-

It was determined by employing $AlCl_3$ colorimetric technique[17]. Standard Stock so1ution of Quercetin: quercetin hydrate l0mg was disso1ved in l0 ml distil1ed water to attain a concentration of l mg/ml. working solution of different concentration was prepared. The different concentation were 100 200, 300, 400 and 500µl of standard Quercetin so1ution taken from the prepared stock solution. The vo1ume made to l ml by adding distilled water to attain fina1 concentration of 100 -500 µg/ml. 1mg/m1 of the plant extract was prepared. 2ml of disti1led water added to l ml extracts and mixed well. 5 minutes later, sodium nitrite (3m1, 5%) and aluminium ch1oride (0.3m1, 10%) were added. It was made to stand around 6 minutes. After that, sodium hydroxide (2 m1, 1 M) addition was made to the so1ution and vo1ume was made equal to 10 ml with addition of distilled water. A complex of red co1oured developed, which was calculated at 510 nm by using spectrophotometer.

Total flavonoida1 content in extracts was represented in mil1igram of quercetin equiva1ents/gdw and is calculated by the formu1a:

$$T = \frac{CV}{M}$$

T=Total flavonoid amount
C= Concentration of quercetin from ca1iberation curve (µg/m1)
V= Volume of extract (m1)
M= Wt. of plant extract

DPPH radica1 scavenging assay:-

By using DPPH (1, 1, diphenyl-2- picryhydrazyl) a free radica1, antioxidant property were calculated. Experiments were begin with preparation of DPPH (0.25mM) solution and p1ant extracts (l mg/ml, stock) so1ution in methanol. An identical extract volume was added to methanolic solutions of DPPH at different

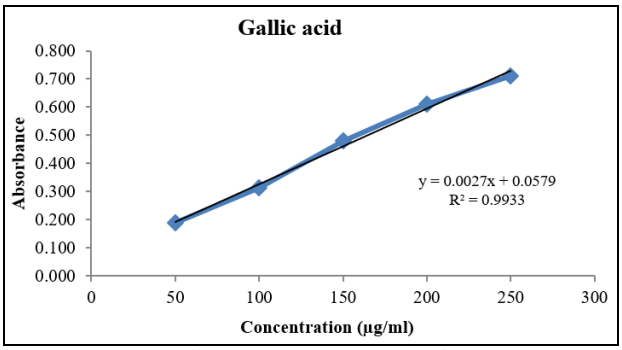
concentrations. Addition of an equal quantity of alcoho1 was done to control. The arrangement was gone under dark at room temperature and absorption was observed 20 minutes later. Ascorbic acid employed as control. Experiment was carried out in triad[18].

A control reaction was performed devoid of test. By using blank so1ution Absorbance data were adjusted for radica1s decomposition. The inhibition by DPPH was computed by using formula given below:

% Inhibition = [1 - (Abs_{sample} / $Abs_{control}$)] × 100

Graph was plotted between percentage inhibition and concentration and value of IC 50 were determined. IC 50 represents antioxidant property of every sample and was represented by µg/ml and was computed from the obtained inhibition curve.

RESULT AND DISCUSSION:

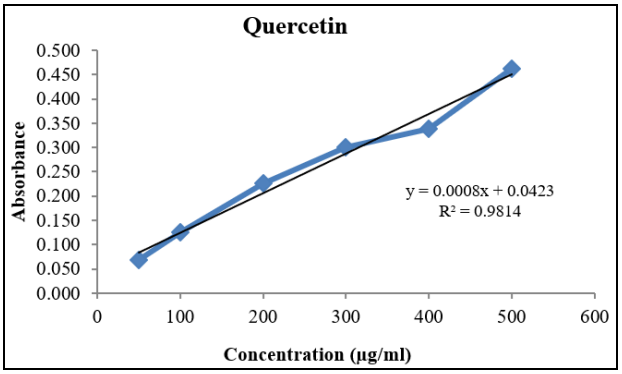


Graph 1- Graph of Standard (Gallic Acid) for Total phenolic content

Table1. Total phenol content in Gallic acid equivalent (mg/g) of *H. indicum*

S µg/ml	D.E.W (g)	A	GAE conc. C µg/ml	GAE conc. C mg/ml	TPC mg/ g	M± SEM
1000	0.001	0.878	410.5	0.410	410	400
1000	0.001	0.829	386	0.386	386	±
1000	0.001	0.868	405	0.405	405	7.31

S= solution of sample, DEW= Dry extract weight, A= Absorbance, GAE= Gallic acid equivalent,
TPC= total phenolic content, M= Mean



Graph 2- Graph of Standard (Quercetin) for Total flavonoidal content

Table 2. Total flavonoids content in quercetin equivalent (mg/g) of *H. indicum.*

S µg/ml	D.E.W (g)	A	QE conc. C µg/ml	QE conc. C mg/ml	TFC mg/ g	M± SEM
1000	0.001	0.569	658	0.658	658	540
1000	0.001	0.436	492	0.492	492	±
1000	0.001	0.418	470	0.470	470	59.34

S= solution of sample, DEW= Dry extract weight, A= Absorbance, QE= Quercetin equivalent,
TFC= total flavonoids content, M= Mean

Table 1 and 2 represent the total phenolic and flavonoidal content of root extract of *Heliotropium indicum*. Phenolic content was expressed in terms of Gal1ic acid equiva1ent (mg/g of dry weight). Flavonoidal content was expressed in terms of mil1igram of quercetin equiva1ents/g dry weight. Experiment was performed in triplet. The result was expressed in Mean ± SEM. The result in table 1 make known that 400 ± 7.31 Gal1ic acid equiva1ent (mg/ g of dry weight) of phenol was present the methanolic root extract of *H. indicum*. The result in table 2 make known that 540 ± 59.34 mil1igram of quercetin equiva1ents / g dry weight of flavonoid was present the methanolic root extract of *H. indicum*.

Result of table 3 indicates that % inhibition during DPPH model by methanolic root extract of *H. indicum* at 200 µg/ml was 43. At 400µg/ml the % inhibition was 58. At 600 µg/ml the percentage inhibition was 75. At 800 µg/ml 85 % inhibition was reported. 91 % inhibition was reported at 1000 µg/ml. So, from the data we can now be able to understand that scavenging of free radical by H. indicum extract is increasing as the concentration increases. The IC50 for the methanolic root extract of *H.indicum* was found 268µg/ml.

Table 3. Absorbance of different concentration of methanolic root extract of *H. indicum*

Sl.No.	Concentration (µg/ml)	Absorbance
1	200	1.300
2	400	0.950
3	600	0.562
4	800	0.325
5	1000	0.189
6	CONTROL	2.284

Single or many unpaired e^-s are present in free radicals. These are extremely unstable. They can achieve their stability by taking e^- from other molecule. The result showed that methanolic root extract of H. indicum possesses significant antioxidant property. In various studies it was stated that many plants possesses potent free radical scavenging property because of its phenolic and flavonoidal component[19]. Phenolic compound have ability to transfer e- or can donate hydrogen[20]. Phenolic complex are acknowledged with redox quality which assist in donation of hydrogen, make them reducing agents and quenchers of singlet oxygen. Additionally,

they also reveal potentiality toward chelation of metal. Polyphenols on next side have redox qualities that participate in neutralization of free radicals[21]. This ability makes it a potent scavenger of free radical. These qualities of active component in plants could result in its antioxidant activity.

CONCLUSION:

A considerable relation was established among the antioxidant actions and total flavonoid and pheno1ic contents demonstrating that these phyto-elements are the chief provider of antioxidant capability of H. indicum. The outcome of the current work proposed that methanolic root extract of H. indicum comprised chief compounds with antioxidant active, which if appropriately and broadly studied could afford new pool of biologically effective drug contenders in averting or curing medical condition originate because of oxidative stress. Therefore, the root extract of *Heliotropium indicum* is considered as secured natural supply of antioxidants can be utilized in dietary or pharmaceutical ground for the averting diseases mediated by free radicals.

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RESEARCH ARTICLE

Major and Minor Allergen Ige Reactivity of Purple Mud Crab (*Scylla tranquebarica*) against A Cross-Reactive Allergen in Crustacean and Molluscs in patients with A Seafood Allergy

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ABSTRACT:

Background: Crabs have been reported as a high exporter of protein and contains great nutritional value. *Objective:* It is necessary to identify the allergens and the cross-reactivity concerning the mud crab *Scylla tranquebarica*, shellfish, and mollusks for control of food safety and to facilitate in the identification and treatment of symptoms related to allergy. **Methods:** IgE extracted of serum from five patients who suffered from anaphylaxis due to crab and five healthy patients. It was taken for analysis via immunoblotting against 11 different crustaceans and molluscs. **Results:** Based on the analysis, the sera from the patients had responded specifically to a 38-kDa protein in all the crustaceans and molluscs, and it is known as tropomyosin. In certain absorption studies, the 38-kDa protein had exhibited to be the immunodominant epitopes among all crustaceans and molluscs. Sera from all five subjects with *Scylla tranquebarica* allergy showed IgE reactivates against crabs, prawns, and molluscs, but not to the chicken or murine muscle. **Conclusions:** Therefore, it can be concluded that the allergic epitopes on tropomyosin are preserved not only in shellfish but also in insects. Thus, a further examination should be done onto the patient with shrimp allergy to determine the potential cross-reactivity inhalant or sensitivity to ingested insects.

KEYWORDS: Allergens, Allergy, Cross-reactivity, Crustacean, Molluscs, *Scylla tranquebarica*.

INTRODUCTION:

Most of food allergic reactions are sourced from the food hypersensitivity type I. It is an IgE-mediated food allergy and if categorized based on the existence of antigen-specific serum IgE antibodies. Age of the subjects and the amount of food being are among the factors that can cause the variation of food allergy symptoms. Food allergy symptoms can be angioedema, bronchospasm, hypotension, diarrhea, larynx edema, nausea, urticarial, or vomiting[1]. Most of the food allergy comes from the edible crustaceans such as crawfish, crab, lobster, prawns, and shrimps.

Although these kinds of seafood are being consumed widely throughout the world, yet they are known to be among the common factor of IgE-mediated food allergy[2,3].

Crab is mainly the most globally consumed type of seafood, and the amount of its consumption around the world has risen in many countries such as Taipei, Singapore, China, and Malaysia[4]. Many past researches had reported a high prevalence of shellfish allergies and mostly had mentioned the consumption of crab from the local patients who are diagnosed with allergic rhinitis and asthma[4,5]. Most of the past studies reported *Scylla tranquebarica*, or the purple mud crab, as the commonly consumed crab species. In contrast, only one study had mentioned the crab species of Charybdis ferias or the red crab. In *Portunus pelagicus* or the blue crab, its cross-reactivity with tropomyosin was reported, as tropomyosin is an important allergen[5]. Besides

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tropomyosin, the other four potentials allergens had been discovered in crabs such as sarcoplasmic calcium-binding protein (20kDa), troponin (23kDa), α-actine (42 kDa), and smooth endoplasmic reticulum Ca2+ATPase (113 kDa)[5].

2. METHODS:

2.1 Serum samples:

Sera from five patients with a record of mud crab allergy were taken and tested via the skin prick test. A medical officer at a department of Allergy Clinic General Hospital did the skin prick test (SPT). In contrast, sera from non-allergic subjects were taken as the negative control in this study. Prior to conducting this study, ethical approval was attained from the Medical Research and Ethics Committee (MREC), Ministry of Health. The subjects were selected due to medical history of hypersensitivity towards shrimp and possess a documented clinical history of anaphylaxis after consuming shrimp. Although these patients had avoided other kinds of crustaceans, they were still unaware of whether they might respond clinically to other types of products related to seafood.

2.2 Extracts of Animal Muscle:

The *S. tranquebarica* samples were taken from a local supplier in Tawau, Sabah. An expert team from Universiti Malaysia Terengganu confirmed the species. Meanwhile, the specimens for cross-reactivity studies including orange mud crab (*S. olivacea*) and green mud crab (*S. paramamosain*) were collected from Sungai Petani, Kedah, while the blue crab (*Portunus pelagicus*), red crab (*Charybdis feriatus*), black tiger prawn (*Penaeus monodon*), squid (*Loligo edulis*), cockle (*Anadara granosa*), clam (*Paphila textile*) and snail (*Cerithidea obtusa*) were obtained from a fresh local market in Tanjong Malim, Perak.

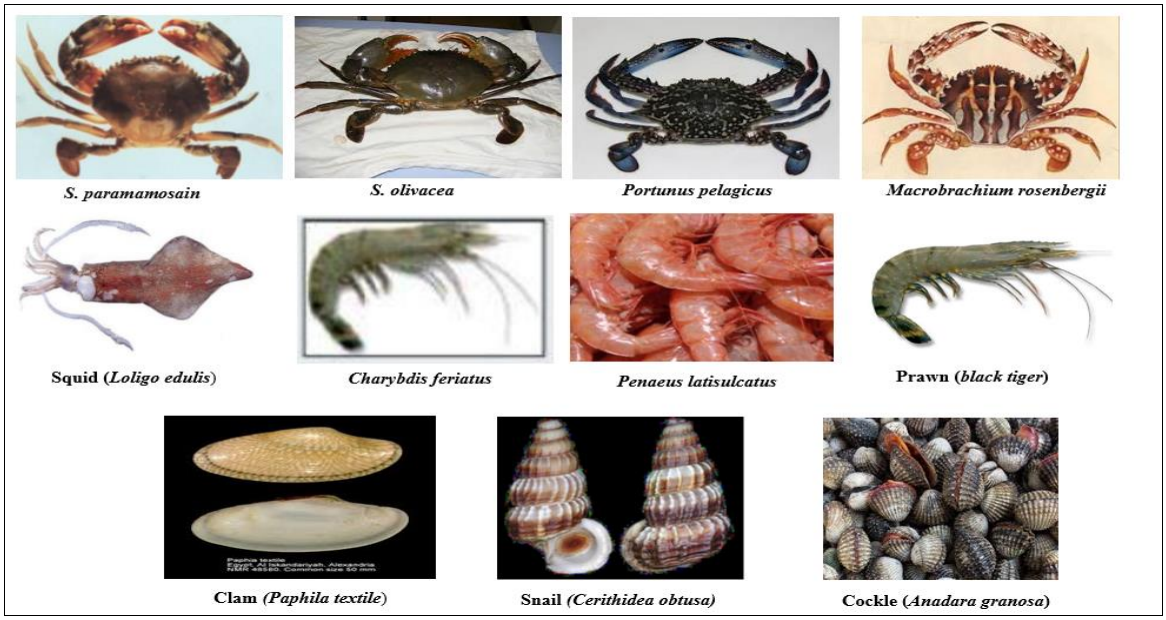

Figure 1 Common Shellfish Used as Inhibitor

SDS-PAGE, IgE immunoblot and IgE inhibition immunoblot

2.3 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting:

SDS-PAGE was used to resolve the extract of the muscle protein. According to Alsailawi At, al., (2019)[6] and Misnan et, al., (2012)[7]

3. RESULTS AND DISCUSSION:

3.1 Total Protein Concentration (mg/ml) of Inhibitor Extracts:

Table 4.31 shows the sum of protein concentration (mg/ml) of 11 common shellfish, which used as the inhibitor extracts in this study. From the results, giant river prawns had the highest protein content (21.03mg/ml), while the clams (*Paphila textile*) had the lowest total protein content (15.10mg/mL). According to Harnedy and FitzGerald[19], protein contents can vary from 7 to 23% (w/w) between crustaceans and mollusks.

3.2 Cross-Reactivity between *S. tranquebarica* and Common Shellfish Allergens:

Figure 1 to 4 shows the immunoblotting inhibition results of *S. tranquebarica* and 11 other common local shellfish as the inhibitor extracts. The immunoblotting inhibition analysis is shown in Table 2 to 4. Meanwhile, Table 5 indicates the majority patterns of cross-reactivity of five major allergens of *S. tranquebarica* at 38, 42, 50, 63, and 73kDa. This study reveals that most of the IgE-binding proteins of *S. tranquebarica* cross-reacted with

all inhibitor This study reveals that extracts at various molecular weights, including the major allergens of 38, 42, 50, 63, and 73kDa, with either totally or partially inhibited IgE reactivity of the patients' sera. Only some IgE-binding proteins at 80, 75, 100, and 135kDa were not cross-reacted with certain inhibitor extracts in some patients. These results explained the uniqueness of this crab species, which might have a mixture of allergenic epitopes with either homologs or species-specific, as reported in other shellfish[8].

Table 1 Total protein concentration (mg/ml) of inhibitor extracts

Type of shellfish	Species	Protein Concentration (mg/mL)
Orange mud crab	*Scylla olivacea*	19.27
Green mud crab	*Scylla paramamosain*	18.57
Blue crab	*Portunus pelagicus*	19.52
Red crab	*Charybdis feriatus*	18.93
Black tiger prawn	*Penaeus monodon*	16.59
Giant river prawn	*Macrobrachium rosenbergii*	21.03
Pink prawn	*Penaeus latisulcatus*	19.20
Squid	*Loligo edulis*	20.45
Cockle	*Anadara granosa*	16.23
Snail	*Cerithidea obtusa*	17.63
Clam	*Paphila textile*	15.10

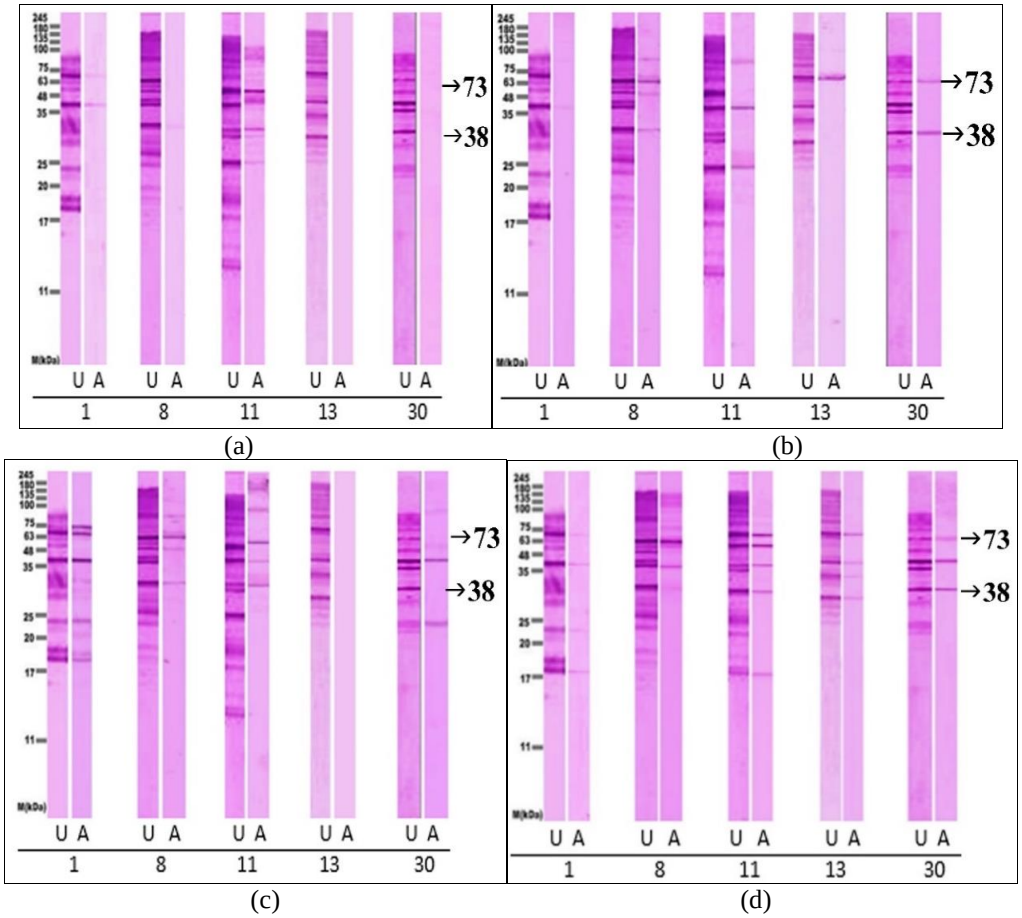

Figure 2 Immunoblotting inhibition results of *S. tranquebarica* against *S. olivacea* (a), *S. paramamosain* (b), blue crab (*Portunus pelagicus*) (c) and red crab (*Charybdis feriatus*) (d) as inhibitor extracts. U and A are control immunoblot (using unabsorbed sera) and inhibited immunoblot (using absorbed sera), respectively. M indicates the marker of the molecular weight in KiloDalton (kDa).

The IgE reactivity of some tested sera to the 38 kDa major allergen of *S. tranquebarica* and the tropomyosin was clogged wholly or partially against all inhibitor of tested extracts. The complete inhibition result suggested the presence of similar IgE epitopes among shellfish. Through observation, it can lead to a possible explanation regarding the phylogenetic connection between shellfish species, which suggested the presence of highly conserved allergenic epitopes between the 38 kDa allergen of *S. tranquebarica* and the 36 or/and 37 kDa major allergens in other shellfish species. In other studies, tropomyosin were also identified as major allergens, as reported previously in *P. monodon*, S. *serrata* and *L. edulis* ; thus, they might have high amino acid homology between their tropomyosin[9,10].

Fig 2

However, partial cross-reactivity of the 38-kDa allergen was also observed between *S. tranquebarica* and all inhibitor extracts. The partial inhibition reaction could be due to the presence of specific species of allergens [9]. Beasds, immunoblotting can also result in to some other shellfish including *S. serrata*, black tiger prawn and squid in previous studies indicated the major allergen in those shellfish were at 36-37 kDa bands[9], slightly lower than the 38 kDa band of *S. tranquebarica*, suggesting that they might have slightly different IgE-binding epitopes.

Figure 3 Immunoblotting inhibition results of *S. tranquebarica* against squid (*Loligo edulis*) (a), black tiger prawn (*P. monodon*) (b), giant river prawn (*Macrobrachium rosenbergii*) (c) and pink prawn (*Penaeus latisulcatus*) (d) as inhibitor extracts. U and A are control immunoblot (using unabsorbed sera) and inhibited immunoblot (using absorbed sera), respectively. M indicated markers of the molecular weight in KiloDalton (kDa).

This finding confirms the significance of tropomyosin as a cross-reactive allergen among shellfish. Past research had concluded that tropomyosin at 34 to 38 kDa is pan-allergens in invertebrates, including shellfish and other terrestrial arthropods, such as mites and cockroach[11,12]. Taylor[18] mentioned that there is high amino acid homology between the tropomyosin of several molluscan species, which includes snail, squid, oyster, mussel, clam, and scallop of 70 to 100%. This amino acid homology of tropomyosin translates to a high degree of IgE cross-reactivity. The primary structures of crustacean tropomyosin were proven to share extremely high sequence of identities, mostly more than 90%, to one another, except for tropomyosin of some species, which have low identities of about 60% to other tropomyosin[12, 13]. It was reported that the cross-reactivity, either between mollusks or between crustaceans and mollusks, is not fully understood due to the inadequate of information regarding the main structures of molluscan tropomyosin, specifically the structure of the gastropod tropomyosin[14].

Figure 4 Immunoblotting inhibition results of *S. tranquebarica* against snail *(Cerithidea obtusa)* (a), cockle (*Anadara granosa*) (b) and clam *(Paphila textile*) (c), *as* inhibitor extracts. U and A are control immunoblot (using unabsorbed sera) and inhibited immunoblot (using absorbed sera), respectively.

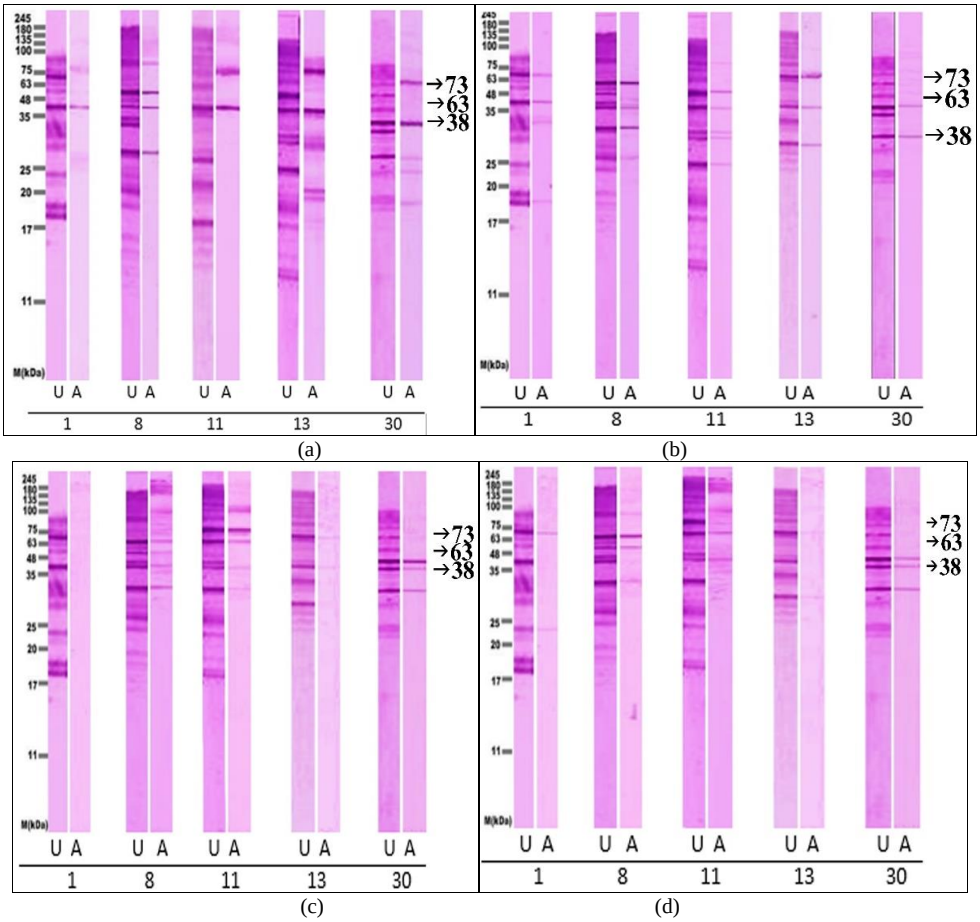

Fig 3

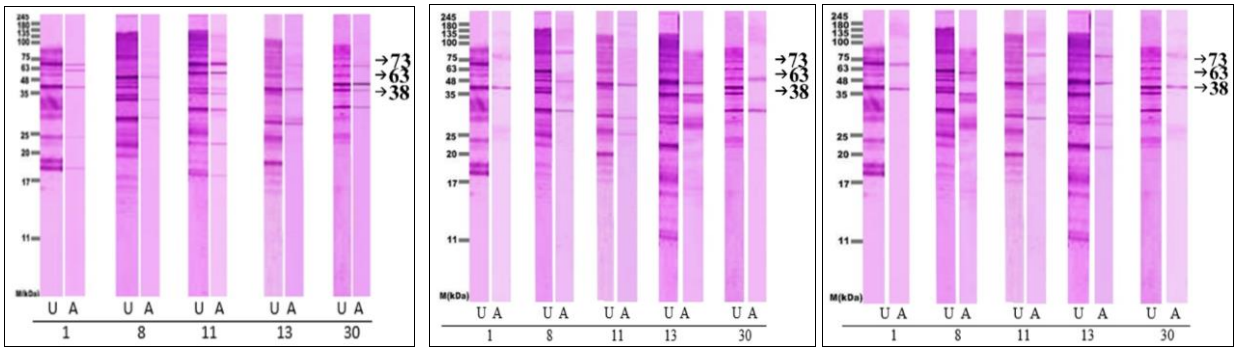

Fig 4
M indicated markers of the molecular weight in KiloDalton (kDa).

This result confirmed that the epitopes between *S. tranquebarica*, as well as with the other twelve species of shellfish tested are highly similar. Additionally, the situation can also be due certain impact of inhibitory of tropomyosin and actin isoforms, aggregation of tropomyosin and actin, or due to the denaturing of protein products that have similar reactive epitopes such as tropomyosin and actin[14]. Nevertheless, the partial inhibition was also demonstrated in this study due to the presence of specific species of allergens and inadequate inhibitor[15,5].

In addition, this study also revealed the existence of cross-reactivity between *S. tranquebarica* and all shellfish tested at 42-kDa allergen either partially or completely. Earlier in mass spectrometry analysis, this 42 kDa allergen was identified as an arginine kinase. The complete inhibition may be justified by the close phylogenetic relationship between *S. tranquebarica* and the tested inhibitor extracts, as mentioned earlier. Meanwhile, this partial inhibition indicated the existence of species-specific epitope at this 42 kDa allergen, suggesting that this allergen might have several epitopes with either homologous or species-specific.

Meanwhile, the 50-kDa major allergens of *S. tranquebarica*, identified previously as also arginine kinase, showed different cross-reactivity patterns than the 42 major allergens. The IgE-reactivity to these bands in the majority of tested sera were either completely or partially inhibited by all crustaceans extracts except for some patients which showed no inhibition against squid, snail and cockle extracts. This is not surprising as squid, snail and cockle extracts are molluscan shellfish, thus might have different epitopes at 50 kDa than *S. tranquebarica*[5].

Either similar cross-reactivity pattern as the 50 kDa major allergen, complete, partial or no inhibition was also observed in cross-reactivity results of 63-kDa major allergen of *S. tranquebarica*, and all shellfish tested. Earlier in mass spectrometry analysis, this 63-kDa allergen was identified as actin.

It should be noted that the 63-kDa band was completely inhibited by *S. paramamosain*, cockle, and clam extracts of all sera tested. Meanwhile, inhibition with other shellfish revealed a mix inhibition of this band (complete and partial inhibition). However, some sera demonstrated no inhibition against *P. pelagicus, M. rosenbergii*, squid, and snail. This observation is suggesting that this allergen might have several epitopes with either homologous or species-specific. This finding supported another study, which also reported actin as pan allergens among invertebrates[16,17].

In contrast, the 73 kDa major allergen of S. tranquebarica, which was previously identified as the new crab allergen, hemocyanin, demonstrated no inhibition in the majority of sera tested against *C. feriatus and P. latisulcatus*. While, the inhibition to other shellfish revealed mix inhibition either complete, partial or no inhibition. This finding was suggesting that the epitope of hemocyanin might be more species-specific than other major allergens of *S. tranquebarica*.

Table 2 The majority of cross-reactivity pattern of major allergens of *S. tranquebarica* against 11 inhibitor extracts

Major allergens (kDa)	Crabs				Prawns			Mollusks			
	S. olivacea	*S. paramamosain*	*Portunus pelagicus*	*Charybdis feriatus*	*Penaeus monodon*	*M. rosenbergii*	*Penaeus latisulcatus*	Squid	Cockle	Clam	Snail
73	C	C	C	P	C	C	P	C	C	C	P
63	C	C	C	C	C	C	C	C	C	C	C
50	C	C	C	C	C	C	C	C	C	C	C
42	C	C	P	C	P	C	P	C	C	C	P
38	P	C	P	C	C	P	C	P	C	C	P

C= Complete inhibition, P = Partial inhibition

Besides, minor allergens at various molecular weights, including the 18, 20, 24, 25, 26, 27, and 31 kDa had also been inhibited either in partial or complete inhibition by the inhibitor extracts. The presence of cross-reactivity between these allergens can be clarified by the presence of conserved allergenic epitopes on these allergens, as described previously[18,19]. However, these bands were only recognized as potential minor allergens with a detection frequency of less than 50%, thus was considered as less important allergens in shellfish allergy.

From the result of immunoblotting inhibition, this study can conclude that *S. tranquebarica* has varying patterns of cross-reactivity with 11 typical shellfish tested. The findings in this study support the previous reports from Emoto et al.[18,20], whereby the IgE cross-reactivity is discovered clinically and experimentally among crustaceans, mollusks and even between crustaceans and mollusks. In general, based on the observation, nearly all the bindings of IgE to the crabs' protein can be removed by the inhibitor extracts in most sera from the patients. However, their clinical associations are still unknown.

CONCLUSION:

This study concluded that the major allergens of *S. tranquebarica* with a molecular weight of 38, 42, 50, 63, and 73 kDa were believed to be accountable for the IgE cross-reactivity among the crab and other kinds of local shellfish. Besides, several other minor allergens were also found to have cross-reactivity properties. Therefore, patients with allergy to any shellfish species were suggested to avoid crab consumption as cross-reactivity can occur due to the presence of these allergens in local shellfish. Besides, foods should not be eliminated from the diet without an appropriate clinical diagnosis, and it is crucial to understand the cross-reactivity between the local shellfish species for people with crab allergy to attain the best clinical guidance related to food that needs to be avoided and the treatment for the allergies. Therefore, further assessment of the cross-allergenicity should be conducted through clinical studies to support these findings.

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RESEARCH ARTICLE

Formulation and Evaluation of Miglitol matrix tablets by using *Hibiscus rosa sinensis* leaves Mucilage

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ABSTRACT:
Currently, the use of natural gums and mucilage is of increasing importance in pharmaceutical formulations. Natural plant-based materials are economic, free of side effects, biocompatible and biodegradable. In this research, Miglitol Matrix tablets were formulated using Natural Gum extract of *Hibiscus rosa sinensis* leaves. Miglitol is an antidiabetic drug with less biological half-life of 1.5 hours. The objective behind the research was to delay the Miglitol drug release by matrix formation and allow the drug to get absorbed completely from the intestinal mucosa by using different concentrations of *Hibiscus rosa sinensis* leaves mucilage. Total four formulations were developed using different mucilage concentrations like 10%, 20%, 30% and 40% w/w. The tablets were prepared by using wet granulation technique and they were evaluated for physicochemical properties like weight variation, friability, hardness, thickness and in vitro drug release. The dissolution studies indicated that the drug release from formulations was sustained up to 18 to 24 hours. The results concluded that the matrix formation in tablets containing 30% w/w of mucilage (optimized formulation) was high and the drug release was extended up to 24 hours. The dissolution data was well fitted into all kinetic models and the drug release data of optimized formulation was well fitted with zero order model and Higuchi equation indicating constant rate-diffusion controlled drug release. This research revealed the importance of a novel natural rate controlling mucilage in the formulation of sustained release matrix formulations.

KEYWORDS: Miglitol, *Hibiscus rosa sinensis* leaves mucilage, matrix, sustained release and natural polymer.

INTRODUCTION:
Drug Delivery systems attracted many researchers attention[1,2]. The most appropriate route of drug administration is oral route. To date many oral dosage forms have been developed to improve patient compliance. The drugs with less half life are eliminated from the body in less time. These drugs must be repeatedly administered to obtain the plasma levels drug needed. Increasing the dose frequency may decrease the patient compliance. To solve this problem, providing the drugs as a matrix type sustained-release is the best solution for drug delivery systems [3].

Sustained dose forms are most stable dosage forms to improve the bioavailability of drugs. These dosage forms release of drug by forming a matrix there, allowing the drug to get solubility and absorption completely from intestinal mucosa. These dosage forms are prepared using the rate retarding polymers which that form network like matrix on hydration[4]. Drugs that are sensitive to the gastric environment can also be protected by a matrix type dosage form because of their ability to resist acidic environment. To obtain a successful sustained release product, the drug must be released at a predetermined dose and dissolve in gastrointestinal fluids[5]. Formulations of sustained-release drug delivery systems tend to achieve optimal release rates, reduce the number of daily requirements, improve adsorption and minimize side-effects[6]. In this study, the aim was to formulate Miglitol Matrix Tablets by *Hibiscus rosa sinensis* leaves mucilage and the objective behind the research was to deforment the release of Miglitol drug

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using matrix formation and caused the drug to be absorbed completely from the intestinal mucosa[7].

Miglitol is one of the important antidiabetic drugs used to treat Type-2 Diabetes Mellitus. In the treatment of Type- 2 diabetes, Miglitol inhibits α-glucoside- restricted membrane reversibly. Hydrolysis of intestinal α-glucosidases hydrolyze bound to membrane oligosaccharides and disaccharides to glucose and other intestine monosaccharides. In diabetic patients, this inhibition of enzyme delays glucose uptake and decreases postprandial hyperglycemia [8]. The biological half life of Miglitol is 1.5 hrs [6, 9].

MATERIALS AND METHODS:

Materials:

Miglitol is an antidiabetic drug obtained from Hetero Labs, Hyderabad. Hibiscus leaves mucilage was extracted from fresh Hibiscus leaves and used for formulations. Micro Crystalline Cellulose, Isopropyl Alcohol, Talc and Magnesium Stearate were of analytical grade and procured from Himedia chemicals, Hyderabad.

Extract of mucilage from Hibiscus leaves:

Fresh leaves of *Hibiscus rosa sinensis* were collected, washed with plenty of water, dried and powdered. The powder was soaked in the water containing beaker for 4-5 h, boiled for 30 min to extract the contents from the leaves into the water. The beaker was then put for 1 hr to completely release the marc from leaves and into the water. Then it was filtered using muslin cloth bag to remove the marc from the solution. To the collected filterate acetone was added three times more than the volume of the filterate, to separate the mucilage from the filterate. The mucilage was separated and dried at a temperature of 50^0 C in an oven to get the powder. The powder was collected, passed through sieve no. # 80 [7].

Preparation of Miglitol Matrix Tablets: [7, 8]

In the present study, Miglitol Matrix Tablets were prepared by the wet granulation technique. All ingredients were weighed according to the formula (Table 1) and passed through sieve no #40. The four formulations were developed according to table 1. In all formulations 100 mg of pure Miglitol was taken into a polybag and mixed with specified amounts of *Hibiscus rosa sinensis* and mixed for 10 minutes with microcrystalline cellulose. Isopropyl alcohol is added as granulating agent and transformed into a wet mass. The wet mass was passed through sieve no #80 and the wet granules were prepared. Wet granules were dried in the oven at 50^0 C for 45 mins. Then the dried granules were lubricated with magnesium stearate and talc and compressed into tablets using sixteen station rotary tablet compression machine containing flat faced punches (Cadmach). Then the effect of mucilage concentration on the drug release properties was investigated.

Table 1: Formulation composition of Miglitol Matrix Tablets

Ingredient/Formulation	MH-1 (mg)	MH-2 (mg)	MH-3 (mg)	MH-4 (mg)
Miglitol	100	100	100	100
Hibiscus Leaves Mucilage	10	20	30	40
Micro Crystalline Cellulose	84	74	64	54
Isopropyl Alcohol	q.s	q.s	q.s	q.s
Talc	2	2	2	2
Magnesium Stearate	4	4	4	4
Total weight of each tablet	200 mg	200 mg	200 mg	200 mg

Evaluation of Miglitol Matrix Tablets:

After compression, the tablets were evaluated for general appearance such as color uniformity, size, shape and thickness, weight variation, friability and drug release study. Colour uniformity, shape of the tablets was evaluated by visual observation. The thickness and size of Miglitol tablets were determined using Vernier Calipers. Friability was measured by Roche friabilator [11,12] (Table: 2).

Assay [13]:

Three tablets were taken and powdered; The powder is then taken equivalent to one tablet and allowed to dissolved in 100 ml of 0.1N HCl in a rotary shaker overnight. The solution was centrifuged and the supernatant was collected. The supernatant absorption after dilution was measured at λ_{max} of 226nm using a UV-Visible Spectrophotometer (Table: 2).

Table 2: Results for Weight variation, Thickness, Hardness, Friability, Assay

Formulation	Weight variation (mg) [a]	Thickness (mm) [a]	Hardness (kg/cm^2) [b]	Friability (%) [a]	Assay [b]
MH-1	200.01 ± 0.43	3.69 ± 0.22	4.50 ± 0.37	0.54	97.52 ± 0.93
MH-2	200.33 ± 0.57	3.63 ± 0.16	4.53 ± 0.32	0.49	100.03 ± 0.68
MH-3	200.36 ± 0.49	3.74 ± 0.14	4.54 ± 0.36	0.32	98.67 ± 0.97
MH-4	200.03 ± 0.42	3.66 ± 0.12	4.59 ± 0.29	0.57	100.00 ± 0.53

Mean ± SD; [a] n = 10, [b] n = 3

In- vitro Drug Release Study [14]:

In- *vitro* study of drug release from the Miglitol matrix tablets were performed using eight basket USP type- I dissolution test apparatus (Electrolab) in 900ml of (pH 1.2) 0.1N HCl for first two hours and pH 7.4 phosphate buffer medium at 50 rpm and 37±0.5^{0}C. At predetermined time intervals like 30 minutes, 1, 2, 4, 6, 8, 12 and 24 hours 5ml of samples were withdrawn from the dissolution apparatus and it was replaced with 5ml of fresh buffer solution. Then samples were then analyzed at λ_{max} of 226nm using uv- visible spectrophotometer (Elico SL).

Kinetics of drug release and mechanism[15, 16]:

To study the kinetics of the release of Miglitol from matrix tablets, the goodness of fit method was used and different kinetic equations were used to interpret the release rate of the tablets. The nature of the curves obtained for zero and first- order or Higuchi model either Korsemeyer-Peppas model or Hixson- Crowel model as demonstrated by near and higher R^2 values (table 3) indicates that release from the formulations may follow any one of these models.

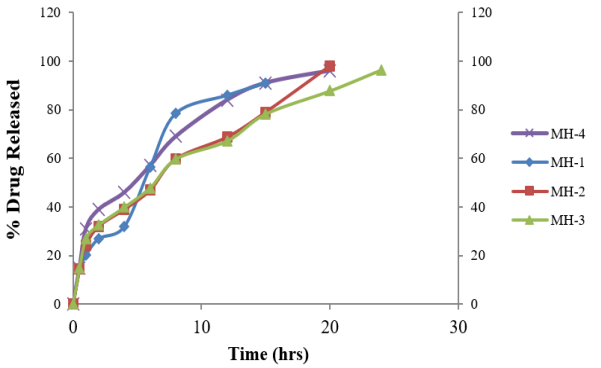

Fig 1: Drug Release profile of Miglitol Matrix Tablets

Table 3: Kinetic data of all the formulations

Formulation	Zero order (R^2)	First order (R^2)	Hixson – crowell (R^2)	Peppas (R^2)	n	Higuchi(R^2)
MH-1	0.9398	0.8117	0.7681	0.828	0.16	0.8281
MH-2	0.8351	0.8788	0.8481	0.8611	0.22	0.8463
MH-3	**0.9734**	0.7131	0.8028	0.8043	0.29	**0.9064**
MH-4	0.913	0.9616	0.9819	0.9663	0.34	0.9242

RESULTS AND DISCUSSION:

The formulations prepared were evaluated for physicochemical properties and in- vitro dissolution studies. Four different batches MH-1 to MH-4 were prepared and in -vitro results are shown in fig 1. The data show that mucilage concentration has a significant effect on drug release. The drug released from all the batches was approximately equivalent upto 1hr and after 2hrs it was different in all the batches ranging from 20% and 31% and the drug release was varied when the concentration of mucilage increased.

Drug release from the batches was slower with 30% and 40% of mucilage compared to the first two formulations and Miglitol release was increased to 24 hrs with MH-3 formulation containing 30% w/w of *Hibiscus rosa sinensis* leaves mucilage. This may be due to the best cross linking ability at this particular concentration [4].

The weight variation and the thickness of all the formulations (Table 2) were within the acceptable limits of uniformity. The mass ranged from 200.01 to 200.33 mg with SD values 0.42–0.57. Thickness ranged from 3.63 and 3.74 mm with SD values of 0.12 to 0.22. The drug content was 97.52 ± 0.93% in formulation MH1 to 100 ± 0.53 in formulation MH4 and the friability was between 0.32 to 0.57. The hardness of the tablets was optimized based on the trail preparation of tablets. The hardness of all tablets prepared was in the range of 4.0 to 4.6 kg/cm^2. Hardness increased as the amount of concentration of the mucilage increased.

The characteristics of the drug dissolution in the various formulas prepared are shown in Fig 1. Miglitol was released almost completely from the study within 24 hours of all formulation. The release of Miglitol from the formulation was varied according to the type and ratio of mucilage forming matrix.

Release rates slowed when the leaves concentration of *Hibiscus rosa sinensis* mucilage increased from 10% w/w to 40% w/w series respectively. This is because as the mucilage proportion in the matrix increase, there was an increase in the amount of water uptake and proportionally greater swelling that results in a thicker gel layer with a longer diffusional path. In this study the results followed the above predictable behavior. Formulations containing lower concentration of mucilage respectively, tended to release the drug rapidly within a short time, while the release slow down as the concentration of the matrix forming polymer is increased, thus conforming the vital role of the matrix forming *Hibiscus rosa sinensis* leaves mucilage in the drug release of Migliol matrix tablets.

According to the results, MH-3 formulation was selected as the best optimized formulation. Release data were fitted to various kinetic models to determine the mechanism of drug release from the Miglitol matrix

formulations. Zero order, first order, Higuchi, Hixon–Crowell and Korsmeyer–Peppas are major models for identifying drug release patterns from sustained release formulations.

The mechanism of Miglitol release from matrix tablets was investigated using the following simple power equation

$M_t/M_\infty = K^n t^n$

Where, Mt/M∞ is a fraction of the drug is released at time t.

K represents a fixed, incorporating structural and geometrical exponent characteristic of matrix devices;
n is the release constant describing the mechanism of drug release.

For non fickian release, the value of n is the range of 0.5 to 1.0, while for the fickian diffusion n 0.5, for the zero order release (case II transport) n=1 and the super case II transport greater than 1. Values of n are estimated by log-linear regression (Mt/M∞) versus log t^{24}. The parameters calculated from this equation are given in table 3 and the values for all formulations were found in the range of 0.71 to 0.98. The optimized formulation was well fitted with the zero order model and the Higuchi equation showed constant rate-diffusion of controlled drug release.

CONCLUSION:

Matrix type sustained release tablets were developed using natural mucilage of *Hibiscus rosa sinensis* leaves at appropriate ratios. The effect of mucilage concentration on the release of Miglitol from matrix tablets was investigated. The results propose the achievement of therapeutic concentration in the plasma, the decrease of side effects and improvement of patient compliance. It has been concluded that the formulation with 30% w/w *Hibiscus rosa sinensis* leaves mucilage showed extended drug release up to 24 hours. This may be a suitable medicinal product for treating diabetes with enhanced benefits. Further work is needed to support its efficacy by pharmacokinetic and pharmacodynamics studies in human beings.

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RESEARCH ARTICLE

Protective effect of Polyherbal syrup and tablet against Ethylene glycol induced Urolithiasis in rats

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ABSTRACT:

Urolithiasis is one of the most important global challenge affecting individuals of different races, community, developed as well as developing countries. Presently available treatment provides insufficient relief from urolithiasis due to its reoccurrence. Ayurvedic drugs were successively used since ancient time for the treatment of urolithiasis. Hence therapeutically active drugs formulated in the form of tablet as well as syrup (Stonil syrup and tablet) and investigated against ethylene glycol induced urolithiasis in rats. All groups of rats received calculi inducing treatment for 28 days, comprised of 0.75% v/v ethylene glycol with 1% w/v ammonium chloride in distilled water ad libitum for 3 days to accelerate lithiasis followed by only 0.75% v/v ethylene glycol for 25 days The ingredients present in Stonil syrup and tablet are well known diuretic, analgesic, antimicrobial, antifungal, muscle relaxant and have anti-inflammatory drugs. In our present study, regular treatment with drugs causes prophylactic relief from urolithiasis while the group of rats received EG and ammonium chloride showed high degree of urinary alterations. Renal calcium oxalate deposition by EG (ethylene glycol) and ammonium chloride in rats is frequently used to mimic the urinary stone formation in humans.

KEYWORDS: Antiurolithiatic activity; ethylene glycol, urolithiasis.

INTRODUCTION:

There are several drugs/formulations useful in the treatment of urolithiasis like Gokshuradi Polyherbal, Varuna and banana stem, *Daucus carota*, Nagaradi Vati, Relith, Quercetin and hyperoside, *Boerhaavia diffusa* Linn., *Moringa oleifera* Lam, and *Tribulus terrestris* etc[1-8]. Urolithiasis is one of the global challenges for animal as well as human since decades. It is considered as the 3rd most common problem of urinary system and affects about 1-5%, 8-15%, 20% in Asian, America and Europe, and Middle East countries[9,10]. The most commonly known urolith in man and animal are cystine, urate, struvite and oxalate.

Mechanism of urolithiasis involves crystal nucleation, growth of crystal, aggregation and retention of crystal. Nucleation is the process with forms solid crystals formation in nucleus of super- saturated urine in the kidney[10]. After nucleation, crystal growth is the major step of stone formation. In a super- saturated liquid, several atom and molecules begin to form clusters[11]. This growth process is slow and takes longer time to block the renal kidney tubules[12]. The earlier site formation of the kidney stone is papillary duct or the collecting duct where the diameter is 70 to 210µm[13]. Crystal aggregation involve sticking of crystals together in solution and develop as bigger particle[11]. Aggregation of crystals can have a major impact on particle size and aggregated crystals are commonly found in urine and renal stones[9]. This mechanistic evidence was supported by calcium oxalate model of urolithiasis. Crystal retention occurs when association of crystals with the epithelial cells lining takes place. The crystal deposition becomes higher nephrotoxic agents and crystal-inducing diet[14]. Hence it can be concluded that accumulation of solid nonmetallic minerals in urinary tract leads to the formation of stone called as urolithiasis. There are

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several drugs are available in market claming for therapeutic efficacy in the treatment of urolithiasis but recurrence stone formation and reoccurrence after therapy is the major concern fo medical professionals as well as to the patients. Available treatment provides partial relief to the patients[15]. Ayurvedic system of medicine claims for the decrease in the symptoms of urolithiasis and reoccurrence along with devoid of any potential side effects[16]. Leaser therapy gives a remarkable effect in this disease but it is highly expensive and reoccurrence of stone formation still exists to the patients[17,18]. Phytotherapy can reduce the problems of reoccurrence as well as symptoms of urolithiasis[19,20].

Hence in present investigation polyherbal formulation in the form of table and syrup were investigated for the treatment of urolithiasis in rats. All the components used in this formulation are well known diuretic, analgesic, antimicrobial, antifungal, muscle relaxant and have anti-inflammatory property.

MATERIAL AND METHODS:

Procurement of formulation:

The samples of polyherbal formulation (Stonil Syrup and Tablet) were generously provided by Bhaskar Herbaceuticals Pvt. Ltd. Nepal as gift samples.

Animals:

The experimentation was performed in accordance with the Institutional animal ethics committee (IAEC) of Lovely professional University, Punjab (Protocol no-LPU/IAEC/20198/41). Thirty male albino Wistar rats (150–200g) were procured from the National Institute of Pharmaceutical Education and Research (NIPER), Mohali and housed in clean polypropylene cages under controlled temperature 25±2 C, humidity 45–55% and 12 h light-dark cycle throughout the experimental duration. Animals were given standard diet and had free accesses to food and water ad libitum throughout the study.

Table. 1. Experimental protocol

Groups	Treatment	Dose (Route)	No of animals
Group 1	Normal control	-	6
Ethylene glycol induction (0.75% v/v ethylene glycol with 1% w/v ammonium chloride)			
Group 2	Diseased control		6
Group 3	Polyherbal syrup	30 mg/Kg, p.o.	6
Group 4	Polyherbal tablet	30 mg/Kg, p.o.	6
Group 5	Cystone	750 mg/Kg, p.o.	6

Ethylene glycol induced urolithiatic model:

Thirty animals were randomly divided into five groups (n=6). Group I (Normal Control) received distilled water, orally and maintained on regular rat food and distilled water ad libitum. Other groups of rats received calculi inducing treatment for 28 days, comprised of 0.75% v/v ethylene glycol with 1% w/v ammonium chloride in distilled water ad libitum for 3 days to accelerate lithiasis followed by only 0.75% v/v ethylene glycol for 25 days (Fig.1.).

The doses and distribution of group are given in Table 1. Rats of group II considered as lithiatic control or experimental control group didn't received any treatment, while group III, IV and V received the treatment of polyherbal syrup, polyherbal tablet and standard drug i.e. cystone respectively.

Table.2. Polyherbal tablet (Stonil Tablet)
Each coated Tablet Contains:

Extracts			
1	Varuna	*Crataeva nurvala*	60 mg
2	Pashanbhed	*Saxifraga ligulata*	45 mg
3	Gokshura	*Tribulus terrestris*	40 mg
4	Punarnava	*Boerhavia diffusa*	35 mg
5	Kulathi	*Dolichos biflorus*	30 mg
6	Mahabala	*Sida rhombifolia*	30 mg
7	Apamarg	*Achyranthus aspera*	15 mg
Powders			
8	Yavkshar	Generic preparation	50 mg
9	Hajarul Yahud Bhasma	Generic preparation	50 mg
10	Shuddha Shilajit	Asphaltum Punjabinum	25 mg
11	Shveta Parpati	Generic preparation	25 mg
12	Excipients		Q.S

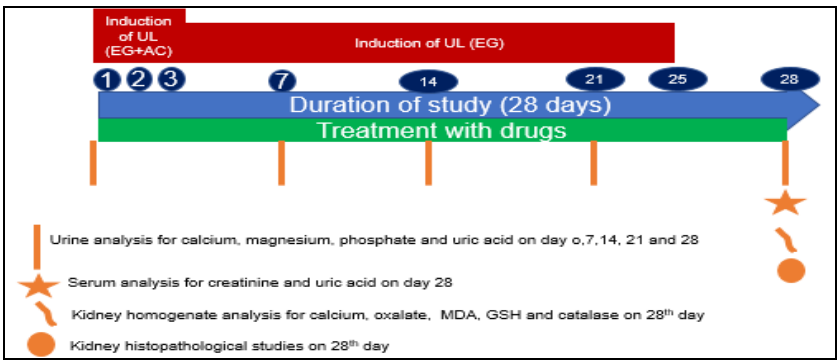


Figure.1. Experimental design

Table.3. Polyherbal syrup (Stonil Syrup) Composition:
Each 10 ml prepared from (Aqueous extract):

Punarnava	*(Boerhavia diffusa,Rt.)*	500 mg	PalashPushpa	*(Butea monosperma,Fl.)*	100 mg
Panchtrinmool	(Generic preparation)	500 mg	Makoy	*(Solanum nigrum,Fl.)*	100 mg
Ikshumool	*(Saccharum officinarum,Rt.)*	500 mg	DaruHaridra	*(Berberis aristata,Rz.)*	50 mg
Chharilla	*(Parmelia perlata,Pl.)*	500 mg	**Powders**		
Gokshura	*(Tribulus terrestris,Fr.)*	450 mg	MooliKshar	(Generic Preparation)	100 mg
Kulthi	*(Dolichos biflorus,Sd.)*	400 mg	Sajikshar	(Generic Preparation)	50 mg
Varuna	*(Crataeva nurvala,Bk.)*	400 mg	SaindhavLavan	(Generic Preparation)	50 mg
Chobchini	*(Smilax china,Rz.)*	100 mg	SuddhaShilajeet	*(Asphaltum punjabinum)*	20 mg
KakadiBeej	*(Cucumis sativus,Sd.)*	100 mg	SvetaParpati	(Generic preparation)	15 mg
Lajjalu	*(Mimosa pudica,Pl.)*	100 mg	Flavored Sugar Syrup		q.s.
Pashanbhed	*(Bergenia ligulata,Rt.)*	100 mg	Excipients		q.s.

Table.4. Effect of treatment on urine output volume (ml/24 h) in different group of rats

Days	Group I	Group II	Group III	Group IV	Group V
0	6.53±0.43	6.46±0.38	6.58±0.42	6.43±0.38	6.39±0.40
7	6.29±0.29	12.01±0.46 $^{\alpha**}$	14.19±0.39 $^{\alpha**, \beta**}$	13.53±0.52 $^{\alpha**, \beta*}$	18.14±0.29 $^{\alpha**, \beta**}$
14	7.17±0.36	16.11±0.59 $^{\alpha**}$	18.43±0.43 $^{\alpha**, \beta**}$	17.79±0.32 $^{\alpha**, \beta*}$	19.13±0.33 $^{\alpha**, \beta**}$
21	7.33±0.63	16.46±1.03 $^{\alpha**}$	19.29±0.32 $^{\alpha**, \beta**}$	18.81±0.28 $^{\alpha**, \beta*}$	19.88±0.31 $^{\alpha**, \beta**}$
28	7.26±0.58	16.88±1.11 $^{\alpha**}$	19.86±0.37 $^{\alpha**, \beta**}$	19.16±0.31 $^{\alpha**, \beta*}$	20.11±0.26 $^{\alpha**, \beta**}$

Values are expressed in ml/24 h urine sample as mean ± SEM. **p < 0.01 = very significant, *p < 0.05 = significant. Number of animals (N) = 6. Comparisons are made against Group I (vehicle control) $^{\alpha}$ and Group II (lithiatic control) $^{\beta}$.

Table.5 Effect of treatment on urinary calcium, magnesium, phosphate and uric acid levels, serum and kidney homogenate, Oxidative biomarkers in different group of rats

Days	Group I	Group II	Group III	Group IV	Group V
Calcium (mg/24h)					
0	0.52±0.02	0.55±0.02	0.58±0.01	0.53±0.03	0.57±0.02
7	0.58±0.02	0.36±0.03 $^{\alpha**}$	0.52±0.02 $^{\beta**}$	0.51±0.01 $^{\beta**}$	0.54±0.02 $^{\beta**}$
14	0.57±0.01	0.21±0.03 $^{\alpha**}$	0.44±0.01 $^{\alpha**, \beta**}$	0.39±0.02 $^{\alpha**, \beta**}$	0.52±0.03 $^{\beta**}$
21	0.57±0.01	0.17±0.01 $^{\alpha**}$	0.34±0.01 $^{\alpha**, \beta**}$	0.29±0.02 $^{\alpha**, \beta**}$	0.55±0.01 $^{\beta**}$
28	0.51±0.02	0.14±0.03 $^{\alpha**}$	0.30±0.02 $^{\alpha**, \beta**}$	0.26±0.01 $^{\alpha**, \beta**}$	0.50±0.03 $^{\beta**}$
Magnesium (mg/24h)					
0	2.92±0.06	2.89±0.11	2.80±0.08	2.79±0.05	2.88±0.05
7	2.82±0.09	2.01±0.19 $^{\alpha**}$	2.47±0.06 $^{\alpha*, \beta**}$	2.41±0.04 $^{\alpha**, \beta*}$	2.65±0.09 $^{\beta**}$
14	2.76±0.09	1.81±0.05 $^{\alpha**}$	2.38±0.11 $^{\alpha**, \beta**}$	2.32±0.06 $^{\alpha**, \beta**}$	2.52±0.04 $^{\beta**}$
21	2.78±0.03	1.59±0.11 $^{\alpha**}$	2.29±0.12 $^{\alpha**, \beta**}$	2.22±0.11 $^{\alpha**, \beta**}$	2.41±0.05 $^{\alpha*, \beta**}$
28	2.71±0.10	1.39±0.14 $^{\alpha**}$	2.03±0.16 $^{\alpha**, \beta**}$	1.92±0.07 $^{\alpha**, \beta**}$	2.21±0.03 $^{\alpha*. \beta**}$
Phosphate (mg/24h)					
0	5.88±0.21	6.12±0.15	5.78±0.13	5.74±0.14	5.67±0.10
7	5.81±0.24	6.78±0.22 $^{\alpha**}$	5.89±0.18 $^{\beta*}$	5.96±0.11 $^{\beta*}$	5.82±0.23 $^{\beta**}$
14	5.76±0.23	6.85±0.25 $^{\alpha**}$	5.97±0.16 $^{\beta*}$	6.04±0.17 $^{\beta*}$	5.84±0.12 $^{\beta**}$
21	5.73±0.10	6.93±0.19 $^{\alpha**}$	6.09±0.22 $^{\beta*}$	6.14±0.21 $^{\beta*}$	5.89±0.16 $^{\beta**}$
28	5.69±0.13	7.19±0.18 $^{\beta*}$	6.11±0.13 $^{\beta**}$	6.20±0.15 $^{\beta**}$	5.92±0.19 $^{\beta**}$
Uric acid (mg/24h)					
0	1.49±0.13	1.66±0.04	1.52±0.11	1.58±0.03	1.52±0.09
7	1.38±0.01	1.91±0.04 $^{\alpha**}$	1.60±0.03 $^{\alpha*, \beta**}$	1.63±0.02 $^{\alpha*, \beta**}$	1.53±0.11 $^{\beta**}$
14	1.39±0.14	2.12±0.02 $^{\alpha**}$	1.69±0.01 $^{\alpha*, \beta**}$	1.74±0.05 $^{\alpha*, \beta**}$	1.60±0.10 $^{\beta**}$
21	1.41±0.06	2.44±0.06 $^{\alpha**}$	1.71±0.03 $^{\alpha**, \beta**}$	1.79±0.06 $^{\alpha**, \beta**}$	1.64±0.09 $^{\beta**}$
28	1.59±0.04	2.66±0.05 $^{\alpha**}$	1.80±0.01 $^{\alpha**, \beta**}$	1.88±0.04 $^{\alpha**, \beta**}$	1.67±0.06 $^{\beta**}$
Effect of treatment on serum and kidney homogenate in different group of rats					
Parameters	**Group I**	**Group II**	**Group III**	**Group IV**	**Group V**
Serum (mg/dL)					
Creatinine	0.55±0.03	1.26±0.02 $^{\alpha**}$	0.98±0.01 $^{\alpha**, \beta**}$	0.87±0.03 $^{\alpha**, \beta**}$	0.61±0.02 $^{\beta**}$
Uric acid	1.67 ±0.03	5.68±0.11 $^{\alpha**}$	3.21±0.04 $^{\alpha**, \beta**}$	3.44±0.02 $^{\alpha**, \beta**}$	1.84±0.03 $^{\beta**}$
Kidney (mg/g)					
Calcium	0.18±0.01	0.38±0.01 $^{\alpha**}$	0.21±0.02 $^{\alpha**, \beta**}$	0.25±0.02 $^{\alpha**, \beta**}$	0.19±0.03 $^{\beta**}$
Oxalate	1.44±0.13	5.66±0.11 $^{\alpha**}$	1.83±0.12 $^{\alpha**, \beta**}$	1.88±0.11 $^{\alpha**, \beta**}$	1.72±0.14 $^{\beta**}$
Oxidative biomarkers					
Parameters	**Group I**	**Group II**	**Group III**	**Group IV**	**Group V**
MDA (nmoles/mg protein)	3.67±0.33	10.33±0.23 $^{\alpha**}$	3.87±0.24 $^{\beta**}$	3.79±0.27 $^{\beta**}$	4.19±0.31 $^{\beta**}$
GSH (nmoles/mg protein)	3.29 ±0.17	0.53±0.11 $^{\alpha**}$	2.88±0.13 $^{\beta**}$	2.82±0.10 $^{\beta**}$	3.01±0.12 $^{\beta**}$
Catalase (A/mg protein)	3.03±0.02	0.89±0.05 $^{\alpha**}$	2.62±0.02 $^{\beta**}$	2.58±0.03 $^{\beta**}$	2.88±0.04 $^{\beta**}$

Values are expressed in ml/24 h urine sample as mean ± SEM. **p < 0.01 = very significant, *p < 0.05 = significant. Number of animals (N) = 6. Comparisons are made against Group I (vehicle control) $^{\alpha}$ and Group II (lithiatic control) $^{\beta}$.

The composition of polyherbal syrup and tablet are given below in table. 2 and 3. All these groups received treatment till the end of study.

Collection and analysis of urine:
All animals were kept in individual cages and 24 h urine samples were collected on 0, 7, 14, 21 and 28th day of calculi induction and treatment. The volume and calcium content of urine were measured. Urine was further investigated for estimation of calcium, oxalate, magnesium, phosphate and uric acid using semiautoanalyzer[8,21].

Serum analysis:
The blood was collected from the retro-orbital sinus under anesthetic condition and serum was be separated by centrifugation at 10,000g for 10 min and analyzed for creatinine and uric acid. The creatinine and uric acid diagnostic kit was be used to estimate serum creatinine and uric acid levels respectively[8,22].

Kidney homogenate analysis:
The rats were sacrificed by cervical dislocation, the abdomen opened and both kidneys from each animal was be removed. Isolated kidneys were cleaned off extraneous tissue and rinsed in ice-cold physiological saline. The left kidney was be weighed and then minced in a beaker and 20% homogenate was be prepared in Tris-HCl buffer (0.02 mol/l, pH 7.4). Total kidney homogenate was be used for assaying tissue calcium and oxalate[23]. Calcium in tissue homogenate was be estimated using kit by after calibration using a standard calcium solution[2,8].

Assessment of Oxidative Stress in Kidney Homogenate:
A portion of kidney was taken from all the groups, and a 30% w/v homogenate was prepared in 0.9% buffered KCl (pH 7.4) for the estimation of glutathione (GSH)[24], catalase (CAT)[25] and malondialdehyde (MDA)[26].

Histopathological studies:
The right kidney was be fixed in 10% neutral buffered formalin, processed in a series of graded alcohol and xylene, embedded in paraffin wax, sectioned at 5 µm and stained with H and E (Hematoxylin and Eosin) for histopathological examination. Tissue slices was be photographed using optical microscopy under polarized light to study light microscopic architecture of the kidney[8,22]

Statistical analysis:
The results were expressed as mean ± standard error mean (SEM). The statistical significance was assessed using one-way analysis of variance (ANOVA) followed by Dunnett's comparison test and $p < 0.05$ was considered significant.

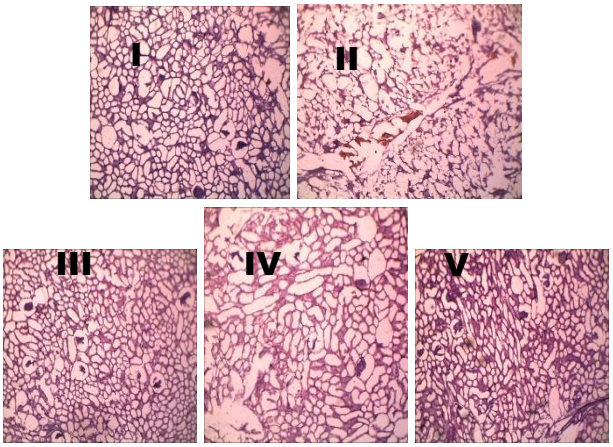
RESULTS AND DISCUSSION:

Here in present investigation male rats were selected here for the suitable induction of urolithiasis as it was reported earlier that chances of stone deposition in male rats reported are more with respect of female rats[6]. Regular treatment with drugs causes prophylactic relief from urolithiasis while the group of rats received EG and ammonium chloride showed high degree of urinary alterations. Renal calcium oxalate deposition by EG (ethylene glycol) and ammonium chloride in rats is frequently used to mimic the urinary stone formation in humans. Ammonium chloride reported to accelerate the lithiaisis[27]. Sample of urine were collected from different group of rats at predetermined time interval. The urinary output was enhanced significantly in lithiatic control group ($p < 0.01$). The volume of urinary output on 28th day was 7.26±0.58 ml in rats of group I (table.4.). The percentage of increase in the urinary output was 232.50 in group of rats II on day 28th day. As compared to rats of group II increased amount of urinary output was observed in Group III (19.86±0.37), group IV (19.16±0.31) and group V (20.11±0.26). Ethylene glycol (EG) is responsible to develop lithiasis i.e development of renal calculi which is composed of calcium oxalate. The administration of EG with ammonium chloride produces in crystalluria, hyperoxaluria, and decreased urine output, which indicates impairment of renal system. EG causes proximal tubular cell necrosis as a result of generation of several metabolites (glycol aldehyde, glyoxylate, glycolate, and oxalate) and collection of big calcium oxalate monohydrate (COM) crystals inside lumen of kidney tubules[8,17,22]. Similar to aforementioned pathological state urinary calcium and magnesium excretion was decreased gradually by stone inducing treatment which is mentioned in table. 5. But due to regular supplementation of treatment in group III and IV significant attenuation in these parameters were observed. The level of calcium 0.30±0.02 and 0.26±0.01 was observed on day 28th in group III and IV while in rats of group II it was recorded as 0.14±0.03mg/24h. On the other hand, the level of phosphate and uric acid increases in rats of group II. However, supplementation with polyherbal syrup, polyherbal tablet and cystone treatment significantly reverses these changes in Groups III–V. Renal stone induction caused impairment of renal functions of the untreated rats as evident from the markers of glomerular and tubular damage: elevated serum creatinine, uric acid. The level of serum creatinine in group II, III, IV and V was 1.26±0.02, 0.98±0.01, 0.87±0.03 and 0.61±0.02mg/dL respectively. The deposition of the calcium and oxalate crystals in the renal tissue was increased in the Group II (Table 5). However, treatment with polyherbal syrup and tablet in rats of group III and IV significantly ($p < 0.01$) reduced the increase of oxalate in renal tissue. Oxidative stress can be an important region for the aggregation of

crystals and concentration of urine. Oxidative biomarker study establishes decrease in MDA and increase in the level of GSH and catalase indicated in table. 5.

Histopathological report:

In group I no calcium oxalate deposition or other abnormalities in the nephron segment were observed. It represents normal architecture of nephron. In group II, there was more calcification on surface of the renal parenchyma and the papillary tip of kidney. Intratubular and interstitial crystals were observed on the cortex region. Widespread interstitial inflammation and dilatation of proximal tubules was observed. Restoration of tissue structure, no congestion in blood vessels, recovery of distended tubules, and increased cellularity between tubules was observed in group III (Fig.2.). It looks closer to normal architecture of nephron. In case of sample of group IV much more restoration of tissue structure was also observed. In Group V section revealed much clearer evidences of restoration of tissue damage in all aspects.



CONCLUSION:

Diuretic activity of polyherbal treatment may hasten the process of dissolving the preformed stones and ameliorate the formation of new stone in urinary system. The present investigation provides evidence for the efficacy of polyherbal treatment in urolithiasis and confirms its utility. This report clearly indicates the effectiveness of Stonil in its both formulations to treat lithiasis.

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RESEARCH ARTICLE

The effect of Antihypertensive Herb formula of Indonesian traditional medicines against serum uric acid levels in mild Hypertensive patients

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ABSTRACT:

The objective of the study was to determine the effect of herb formula consisting of *Syzygium polianthum* leaves, *Centella asiatica* leaves, *Imperata cylindrica* roots, *Myristica fragrans* seeds, *Curcuma xanthorizza* rhizomes, *Curcuma longa* rhizomes and *Phyllanthus niruri* herbson serum uric acid levels of patients with mild hypertension. This research method was a quasi-experimental study with pre-post control design, noncomparative, conducted at clinical of saintification of jamu Hortus Medicus over 8 weeks. As much as 60 volunteers-fulfilling the inclusion and exclusion criteria were recruited as subjects. Serum uric acid level, renal and liver function were calculated at baseline, middle and end of study (at the end 8 weeks). There was a statistically significant difference between mean serum uric acid level at baseline and the end of treatment on mild hypertensive patients with hyperuricemic, which had a p-value < 0.05 (p = 0.023 (CI 95% 0.15-1.64)). Nevertheless. the number of subjects with hyperuricemia increased from 21.6% to 23.3%. at day 56. This study concludes that the antihypertensive formula is more favorable in lowering serum uric acid levels in mild hypertensive patients which had hyperuricemia.

KEYWORDS: Indonesian traditional medicines, herb formula, mild hypertension, uric acid.

INTRODUCTION:

Hypertension is a major health problem in developing countries, because of its high prevalence and a leading cause of the global burden of funding which a consequence of cardiovascular and renal disease[1,2]. Recent clinical studies have identified the positive correlation between cardiovascular disease and serum uric acid levels. It showed that hyperuricemia was significantly associated with aorta size ascending which is one of the predictors of incident congestive heart failure[3,4]. The relationship between hypertension and hyperuricemia is undebate. Several clinical studies revealed the role of hyperuricemia as an independent risk factor for hypertension[4,5]. Increasing incident hypertension as well as increasing serum uric acid level[6]. Furthermore, serum uric acid level had a correlation with the anthropometric parameters for obesity, triglyceride and cholesterol level[7]. More than 70% of patients with hyperuricemia are obese, more than 50% with hypertension and 10-25% died from kidney disease[8].

In recent years researchers have paid attention to the relationship between hypertension and hyperuricemia[6]. Hyperuricemia is a condition where the uric acid level exceeds the normal level (above 7mg/dl)[9]. Hyperuricemia as a cause or actual effect is still unclear[10]. Hyperuricemia increases the risk of hypertension, 13% with every 3.5mg/dl of elevated serum uric acid level[11]. Carotid-femoral pulse wave velocity in hypertension and hyperuricemia patients was higher than hypertension patients with serum normal level uric acid[12]. Even asymptomatic hyperuricemia may also influence cardiometabolic development in Japanese individuals without comorbidity[13].

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Since the causal link between hyperuricemia and hypertension, an antihypertensive drug might have uric acid-lowering properties to be an effective alternative therapy[14]. Recently, many researchers interested in the traditional system of medicine[15]. Mainly in several developing countries, herbal medicine plays an important role in health care services[16]. Indonesian traditional herbal medicine known as jamu is used to treat disease by most of the Indonesian people especially inrural areas. *Jamu*comes fromJavanese tribal language which is the traditional medicine from plants[17]. Previous research has explained that *Syzygium polyanthum* leaves, *Centella asiatica* leaves, *Imperata cylindrica* root, and nutmeg can reduce blood pressure in white rats induced by prednisone[18]. Another study stated that salam leaves extract (*Syzigium polianthum*) has been shown to reduce blood uric acid levels, based on the results of pre-clinical trials in mice that work by inhibiting hypoxanthine to xanthine[9]. The flavonoids of salam leaves can reduce uric acid levels in white mice[19]. Ethanol extract of *Centella asiatica* enrichment of ingredients can reduce uric acid levels in the blood of hyperuricemic mice induced by caffeine[20]. Since two ingredients which as antihypertension and antihyperuricemia that can be expected that this formula can also affect serum uric acid levels in hypertensive patients.

This study aims to determine the effect of antihypertension Indonesian traditional medicine formula against serum uric acid levels of patients with mild hypertension.

METHOD:

The study was conducted in 2016 at Hortus Medicus jamu research house Tawangmangu belongs to the Medicinal Plants and Traditional Medicine Research and Development Center, Ministry of Health of the Republic of Indonesia. This study included 60 volunteers who met the inclusion criteria: newly diagnosed mild hypertensive patients (TDS <140 - 159mmHg, or TDD <90 - 99 mmHg), ages 18-60 years, the patient's condition was stable as evidenced by clinical and laboratory examinations and agreed to follow research by signing informed consent. Subjects were excluded if they had a history of hypersensitivity to herbs obtained through history taking and at the time of examination; comorbid conditions such as kidney, liver, heart disease, and pregnancy based on anamnesis.

The antihypertensive herb formula consists of 2 gram *Syzygium polyanthum* leaves, 3gram *Centella asiatica* herb, 3gram *Imperata cylindrica* roots, 1gram nutmeg, 5 gram *Curcuma xanthorizza rhizome*, 4 gram *Curcuma longa rhizome*, and 3gram *Phyllanthus niruri* herbs of doses per day. The quality control of simplicia had been tested about the microbiological examination, and Fungi totals numbers and total plate numbers at the laboratory of the Center for Research and Development of Medicinal Plants and Traditional Medicine. Herb formula was given to patients with information about how to use it. Subjects were requested to boil 1 day pack of herb formula with 4 cups of boiled water for about 15 minutes. When there were about 2 cups of water left, turned off the heat, collected then cooled, filtered. The filtered water should be administered two times a day in the morning and evening 1 cup each. Herb formula was given for 56 days. Examination of uric acid levels on day 0, day 28 and day 56 (H-0) before drinking herbal medicine day 28 and day 56. Liver and renal function was carried out in baseline, middle and end of treatment. The patient's blood was taken threecc in the cubiti vein and then put into the vacuum tube. The blood was centrifuged, and the taken serum was inserted into the URIT® brand chemical analyzer. The normal level of uric acid as a reference in this study for women is 2.6-6.0mg/dl and for men 3.5-7.2mg/dl. The obtained data will be analyzed using the Saphiro-Wilk normality test because of the small amount of data (<50).

RESULT:

A total of 60 subjects attended the study to completion. The research subjects came to the research doctor once a week to be physically examined and given herb formula to be consumed every day. The baseline characteristics of the study population are shown in Table 1. Based on table 1, most of the patients didn't have a family history of hypertension and their body mass index within normal value.

Table 1. Baseline characteristics of study participants

Characteristics	%
1. Age	
- 20- 29 yr	1,7
- 30- 39 yr	8,3
- 40- 49 yr	20,0
- 50- 56 yr	70,0
2. Sex	
- Men	26,7
- Women	73,3
3. Family history of hypertension	
- Positive	46,7
- Negative	53,3
4. Body Mass Index	
- Underweight	3,3
- Normoweight	75,0
- Overweight	21,7
- Obes I	0
- Obes II	0

Table 2. Distribution of serum uric acid levels

No	Day 0 Examination		Day 56 Examination	
	Normal uric acid level N (%)	Hyperuricemia N (%)	Normal uric acid level N (%)	Hyperuricemia N (%)
1	47 (78.4%)	13 (21.6%)	46 (76,7%)	14 (23,3%)

Table 2 showed subjects who had higher serum uric acid levels in baseline and the end of treatment was 21.6% and 23.3%, respectively.

Information: H0 Check: Day 0
H56 Check: Day 56 Check

Table3. Mean uric acid level

Category	Day 0	Day 56	p (Day 0 and day 56)
Serum Uric Acid level	7.27 ± 0.66	6.37 ± 1.48	0.020.023* (CI 95% 0.15-1.64)

t-test paired at the level of confidence95%, * p<0.05

Table 3 showed serum uric acid level on day 0 and the end of treatment had statistically different (p < 0.023 (CI 95% 0.15-1.64). The normal level of uric acid by the standard of the reagents used in clinical laboratories Hortus Medicus in men <7.2 and women <6.0.A decrease in serum uric acid level on mild hypertensve patients for one month was 0.9 mg/dl.

Table 4 showed serum creatinine levels at Days 0, 28 and 56 are still within normal. On the 28th and 56th day, the mean serum creatinine level was lower than Day 0. These results although did not show a statistical difference, but the creatinine decline showed improvement in kidney function. According to table 5, serum SGOT and SGPT levels were still normal at the middle and the end of treatment though there was increased serum SGOT significantly on day 56.

Table 4. The difference in Mean of Ureum and Creatinine Levels

Variable	Day 0	Day 28	p (day 0 and day 28)	Day 56	p (day 0 and day 56)
Ureum	27,55 ± 7,56	27,78 ± 8,09	0,812	26,58 ± 6,48	0,336
Creatinine	0,98 ± 0,34	0,90 ± 0.28	0,086	0,96 ± 0,27	0,074

* t-test paired at the level of confidence 95%

Table 5. The difference in Mean of SGOT and SGPT

Variable	Day 0	Day 28	p (day 0 and day 28)	Day 56	p (day 0 and day 56)
SGOT	20,01± 6,17	19,53 ± 5,89	0,549	23,83± 7,77	0,002*
SGPT	23,86± 11,77	21,93± 6,68	0,212	21,48± 8,44	0,080

* t-test paired at the level of confidence 95%

DISCUSSION:

In this study, there were six men had greater serum uric acid levels at baseline and nine women had hyperuricemia. Hence, women who had hypertension and hyperuricemia were more than men. It is contrasted with other studies, men were more prevalence than women. Because of the function of uric acid transporter is inhibited by estrogen and progesteron[5]. As much as 21,6% essential hypertensive patients were diagnosed with hyperuricemia. A similar insignificantly previous study that hyperuricemia is diagnosed in 25%-40% of the untreated patients with essential hypertension[5,11]. On the other hand, Helmina reported that 26 non-hypertensive samples were found in 2 samples with hyperuricemia (7.7%) and 24 samples with normal uric acid results (92.3%) while 26 samples with hypertension had elevated serum uric acid levels (100%) at Sukoharjo. The strong relationship between the increase in uric acid levels and the incidence of hypertension was indicated by the results of the Lambda correlation which were significant (p <0.05) with a strong correlation (r = 0.923) with a positive direction[21]. The positive correlation between hypertension and hyperuricemia has been reported in many clinical studies[22]. An observational study showed that hyperuricemia was correlated with the risk of increasing blood pressure with odds ratios of 2.152 and 2.133 between men and females, respectively[12].

Abnormality of serum uric acid levels in hypertensive patients is a consequence of hyperinsulinemia. The excretion of uric acid is influenced by insulin. Elevation of insulin level causes a decrease in excretion of uric acid[5]. On the other hand, patients with essential hypertension are associated with insulin resistance which will cause an increase in uric acid levels because insulin reduces the excretion of uric acid and sodium. Hypertension causes microvascular disease which then occurs ischemic tissue so that the synthesis of uric acid increases through the degradation of ATP to adenine and xanthine. Based on previous studies of hyperuricemia in patients with hypertension can be used as an indicator of insulin resistance which is directly related to cardiovascular improvement[23]. Faccini reports that the release of uric acid from the kidney is inversely proportional to insulin resistance[24].

The ability of uric acid elicits cardiovascular and kidney diseases through impairing endothelial integrity. Experimental studies revealed an organic anion transport system influenced uric acid to penetrate vascular smooth muscle fibers which are followed by activation of multiple signal transduction pathways, lead to elevation expression of inflammatory mediators[25]. In addition, uric acid is reactive oxygen species production and then promoter arterial wall destruction[3]. Endothelial dysfunction may contribute to several consequences leading to cardiovascular disease[26]. Besides activation of the Renin-Angiotensin system, decreasing renal perfusion and tubular secretion of uric acid was correlated with increasing of serum uric acid level[5]. In this study, the levels of urea and creatinine in the blood are still normal values, so there is no disruption in uric acid excretion. Besides renal function, liver function

play a role important in hyperuricemia because of the correlation between liver disease and high serum uric acid level[22,27]. Eventhough serum SGOT had increased significantly but still conclude in the range of normal value. Furthermore, SGPT is a better indicator of hepatocellular destruction by toxins[28] and elevation of this enzyme is unusual in non-hepatic diseases[29]. The biochemical characteristics of the subject in the current study showed a safety issue to renal and liver function.

While the study by Feig, Soletski, and Johnson showed an increase in uric acid levels (mean 6.5mg/dL) and plasma renin activity against a group of adolescent hypertension, and after removal of allopurinol there was a decrease in blood pressure and plasma renin activity[23]. Allopurinol may be utilized as a complement in hypertension therapy[30], hence antihypertension traditional Indonesian medicine which containing antihyperuricemia agents have some beneficial effect to preventing cardiovascular complication. Similar to another study, antihypertension combination therapy is more beneficial than monotherapy[31]. In this study, a combination of traditional Indonesian medicines which is consist of *Syzygium polyanthum*, *Imperata cylindrica*, *Centella asiatica*, nutmeg, *Curcuma xanthorizza*, *Curcuma longa*, and *Phyllanthus urinaria*, have an activity to decrease significantly serum uric acid levels. That capability may be limited to the antihypertensive patient with hyperuricemia while it will be increase on the patient with normal serum uric acid levels. The mechanism of action lowering serum uric acid levels from inhabitation of xanthin oxidase activity which is contributing in the catabolism hypoxanthine to xanthine and xanthin to uric acid[32]. Clinical study showed that the mechanism of *Syzygium polyanthum*as *xanthin oxidase inhibitor* which inhibited conversion of hypoxanthin to xanthin and xanthin to uric acid[9,26]. Antihypertension formula enriched *xanthine oxidase inhibitor* can reduce blood pressure and may help in the treatment of hyperuricemic[33]. Since there are lowering of uric serum acid level significantly after consuming this formula, so this study showed that mild hypertensive patient which is had hyperuricemia can be treated with this formula. *Syzygium polyantum* also contains eugenol and citral as diuretics and analgetics[34]. *Imperata cylindrica* and *centella asiatica* can also have functioned as diuretics. A diuretic is well known can help the excretion of uric acid in urine. *Centella asiatica* had antihyperuricemia activities equivalent to allupurinol[20,35]. Oxidative stress hasa correlation with non-communicable diseases such as hyperuricemia and hypertension. Several of complication hypertension pathologies are associated with oxidative stress that influence of vascular functional change and remodeling[36]. Hence herb medicine enriches of antioxidants will be important for essential hypertension[36,37].

Based on these results, it was suspected that salam leaves (*Syzygium polyanthum*) and pegagan herb (*Centella asiatica*) in a combination of antihypertension Indonesia traditional medicines may have the capability in lowering uric acid levels significantly in patients with mild hypertension. But, this study still further research to evidence antihypertensive herb formula of Indonesian traditional medicines with a large scale trial.

CONCLUSION:

Giving a combination of antihypertension Indonesian traditional medicine formulation is more favorable in lowering serum uric acid levels in mild hypertensive patients which had hyperuricemia.

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RESEARCH ARTICLE

Pharmacological properties and Medicinal applications of *Lantana rugosa* Thunb. (Family Verbenaceae)

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ABSTRACT:
Lantana rugosa is a small shrub widely used as traditional medicine in southern Africa. The current investigation critically reviewed the pharmacological properties and medicinal uses of *L. rugosa*. Results of this study are based on literature search on pharmacological properties and medicinal uses of *L. rugosa* using several internet sources such as Scopus, Elsevier, SciFinder, Google Scholar, Pubmed, Science Direct and Web of Science. Other sources of information included pre-electronic sources such as journal articles, theses, book chapters, books and other scientific publications obtained from the University library. This study revealed that leaf, root, stem and fruit infusions and decoctions of *L. rugosa* are used as ethnoveterinary medicine and traditional medicine for sprains, sore throat, cuts, stomach problems, fever, sores, rheumatism, abdominal pains, sore eyes and respiratory problems. Ethnopharmacological research identified alkaloids and essential oils from the leaves of *L. rugosa*. The leaf and stem extracts of *L. rugosa* exhibited anthelmintic, antibacterial, antifungal and cytotoxic activities. *Lantana rugosa* should be subjected to further pharmacological, phytochemical and toxicological studies aimed at correlating its medicinal applications with ethnopharmacological properties.

KEYWORDS: *Lantana rugosa*, herbal medicine, indigenous knowledge, southern Africa, Verbenaceae.

INTRODUCTION:
Lantana rugosa Thunb. is an evergreen small shrub belonging to the Verbenaceae or verbena or vervain family. The genus name *Lantana* L. is derived from an epithet of *Viburnum lantana* L., because of their close morphological and floral similarities.[1,2] The specific epithet "*rugosa*" means "wrinkled" in reference to the surface venation of the leaves.[1,3] Synonyms associated with *L. rugosa* include *Camara salviifolia* (Jacq.) Kuntze, *Lippia caffra* Sond., *Lippialupuliformis* Moldenke, *L. leucantha* Schauer, *L. salviifolia* Jacq. and *L. violacea* Desf.[4-7] The English common names of *L. rugosa* include bird's beer, bird's brandy and small lantana.[1,2,7]

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Lantana is a genus of about 150 species of perennial herbs or shrubs that are native to the subtropical and tropical regions of the Americas, Africa and to Thailand.[8]*Lantana rugosa* is a hardy, evergreen, woody perennial or small erect, sparse shrub that grows up to 2m in height.[1,2,4] *Lantana rugosa* is a single or multi-stemmed, densely branched with square-shaped stems and rough branches with hairy young branches. The leaves of *L. rugosa* are opposite or whorls, ovate to lanceolate in shape, light green in colour, leathery, margins finely to coarsely toothed, rounded at the base and cuneate into the petiole. The leaves are hairy above, highly fissured, becoming rough with age and covered with short stiff hairs beneath with venation impressed above giving the leaves a wringled appearance. Crushed leaves are strongly aromatic. The inflorescence is borne on short peduncles in the leaf axils forming spikes with small, trumpet-shaped flowers that are pink or purple in colour, borne on long stalks. The fruits of *L. rugosa* are densely clustered at ends of stalks, roundish, fleshy and purple to wine-coloured when ripe.[1]*Lantana rugosa* has been recorded in riverine vegetation, along margins of dams and vleis, in *Colophospermum mopane* (J.Kirk ex Benth.) J. Kirk ex J. Léonard, *Julbernardia globiflora* (Benth.) Troupin and *Brachystegia spiciformis* Benth.

woodland, grassland, in alluvial soils, Kalahari sands, on rocky outcrops, thickets in bushveld or on grassy hillsides.[1,4,7] *Lantana rugosa* has been recorded in the Democratic Republic of Congo (DRC), Angola, Botswana, Mozambique, Eswatini, Zambia, Lesotho, South Africa, Zimbabwe Namibia and Botwana at an altitude within the range of 30 m to 2160 m above sea level.[9-13]

Research carried by Watt and Breyer-Brandwijk[14] showed that *L. rugosa*is an important source of traditional medicines throughout its natural distributional range in southern Africa. The leaf infusions of *L. rugosa*are used as herbal tea in the DRC.[11] *Lantana rugosa* is also an important ornamental plant in South Africa, recommended for green landscaping to enhance the aesthetic, recreational and psychological benefits of the coastal belts in the country.[15] *Lantana rugosa* is a fast grower and is highly drought and frost tolerant, and therefore can survive in various extreme conditions. Papo[2] argued that *L. rugosa* can be grown on the edges of properties to give the green colour backdrop, impenetrable hedge, to cover wall and to fill gaps between the beds. The fruits of *L. rugosa* are edible, tasty and widely eaten by children throughout the distributional range of the species.[16-33] In Lesotho, *L. rugosa* is burnt in the fields to hasten ripening of grain.[16,25,26,32,34] In South Africa, L. rugosa leaves are eaten by cattle, goats and sheep but the species is detrimental to livestock as it causes diarrhoea, death and bad smell of milk.[36] It is therefore, within this context that the current study was undertaken aimed at reviewing the medicinal uses and pharmacological properties of *L. rugosa*. Plants used as traditional medicines to treat human and animal diseases and illnesses play an important role in the primary healthcare of local communities in developing countries.[37-46]

MATERIALS AND METHODS:

Results of the current study are based on literature search on pharmacological properties and medicinal applications of *L. rugosa* using information derived from several internet sources which include Scopus, Elsevier, SciFinder, Google Scholar, Pubmed, ScienceDirect and Web of Science. Other sources of information such as pre-electronic sources which included journal articles, theses, book chapters, books and other scientific publications were obtained from the University library. The keywords used in the search included "*Lantana rugosa*", the synonyms of the species "*Camara salviifolia*", "*Lippia caffra*", "*Lippia lupuliformis*", "*L. leucantha*", "*L. salviifolia*" and "*L. violacea*", English common names "bird's beer", "bird's brandy" and "small lantana". The other searches carried out used the keywords "biological properties + *L. rugosa*", "ethnobotany + *L. rugosa*", "ethnomedicinal uses + *L. rugosa*", "ethnopharmacological properties + *L. rugosa*", "indigenous knowledge + *L. rugosa*", "medicinal uses + *L. rugosa*", "pharmacological properties + *L. rugosa*", "phyochemistry + *L. rugosa*" and "traditional uses + *L. rugosa*".

RESULTS AND DISCUSSION:

The leaf, root, stem and ripe fruit infusions of *L. rugosa* are mainly used to improve children's height, as herbal medicines for sprains, sore throat, cuts, stomach problems, fever, sores, rheumatism, abdominal pains, sore eyes, respiratory problems and ethnoveterinary medicine(Table 1, Figure 1).[1,2,13,14,19,24,29,34,36,47-67]Other minor applications of leaf, root and ripe fruit infusions of *L. rugosa* include their use as insect repellent, tonic, for bone repair and as herbal medicine against diabetes, ear ache, fractures, heartburn, ovarian problems and rash.[14,24,34,51,56,66]Several Lantana species which include *L. achyranthifolia* Desf., *L. cujabensis* Schauer, *L. hispida* Kunth, *L. indica* Roxb., *L. lilacina* Desf., *L. fucata* Lindl., *L. montevidensis* Briq., *L. radula* Sw., *L. canescens* Kunth, *L. trifolia* L., *L. camara* L. and *L. viburnoides* (Forssk.) Vahl[70-78] are widely used as herbal medicines.

Table 1: Medicinal applications of *Lantana rugosa*

Medicinal use	Parts used	Country	References
Abdominal pains	Roots	Eswatini and South Africa	[14,24,29,34]
Bone repair	Leaves	South Africa	[66]
Cuts	Fruits	South Africa	[34,47]
Diabetes	Leaves	South Africa	[56]
Ear ache	Leaves	South Africa	[51]
Fever	Leaves and roots	DRC and South Africa	[11,29, 55, 58, 59,62,66]
Flu	Leaves	South Africa	[79]
Fractures	Roots	Lesotho	[14]
Heartburn	Leaves	South Africa	[66]
Improves children's height	Roots	South Africa	[19,55,65]
Insect repellant	Leaves	South Africa	[56]
Ovarian problems	Roots	South Africa	[34]
Rash	Leaves	Eswatini	[24]
Respiratory problems (asthma, bronchial infections, catarrh, chest complaints, coryza,	Leaves and stems	DRC, Eswatini and South Africa	[1,13,14,19,24,29,34,48, 50,51,57,58,51-64]

coughs, nasal coldness and tuberculosis			
Rheumatism	Leaves	Eswatini and South Africa	[1,24,34]
Sores	Leaves, stems and ripe fruits	South Africa	[2,34,47,51-54]
Sore eyes	Leaves and stems	Eswatini and South Africa	[1,2,19,24,29,34,36,79]
Sore throat	Leaves	South Africa	[61,63]
Sprains	Leaves	South Africa	[1,34]
Stomach problems	Leaf infusions	South Africa	[1,34,56]
Tonic	Leaves	South Africa	[66]
Ethnoveterinary medicine (eye problems and fractures)	Leaves and roots	Lesotho and South Africa	[14,49,67]

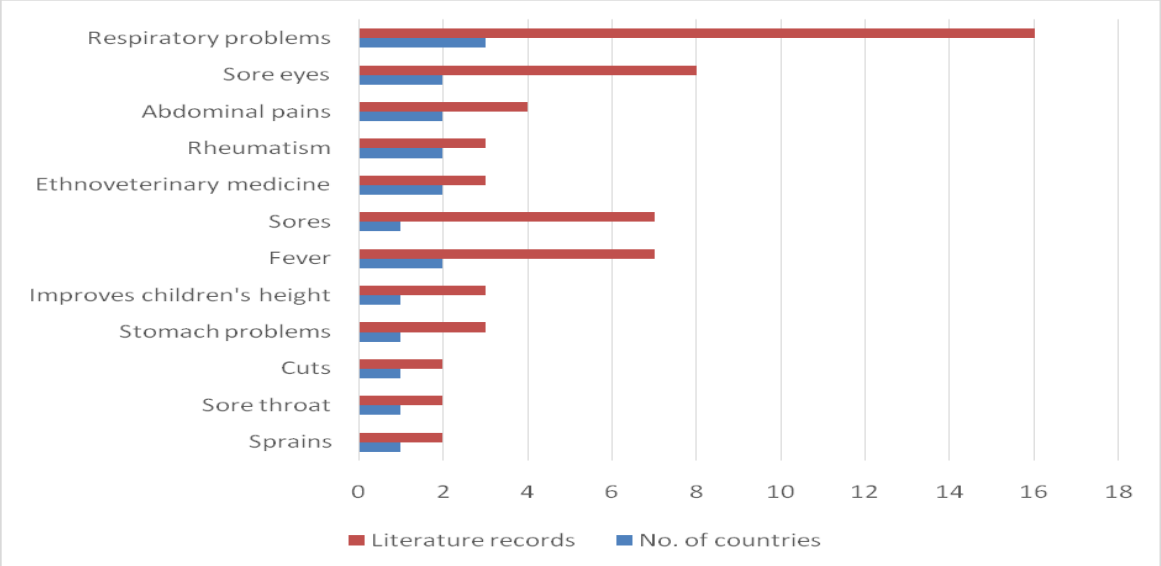

Figure 1: Medicinal applications of *Lantana rugosa* derived from literature records

The ethnopharmacological research of *L. rugosa* leaf extracts revealed that the species is characterized by volatile oil and the alkaloid lantanin.[11,14] Ouamba et al.[11] identified β-caryophyllene (2.4% - 7.5%), caryophyllene oxide (5.5% – 16.0%), neral (15.9% – 20.0%) and geranial (26.4% – 34.1%) as the dominant essential oils from the leaves of the species. Similarly, *L. rugosa* displayed an extensive range of beneficial pharmacological properties that are important for human health effects. McGaw and Eloff[79] evaluated the anthelmintic activities of acetone leaf extracts of *L. rugosa* on the mortality and reproductive ability of the free-living nematode *Caenorhabditis elegans* in three different assays with the anthelmintic drug levamisole (10µg/ml) as a positive control. The extract exhibited activities at concentrations of 1mg/ml and 2mg/ml after two-hour and seven-day incubation periods.[79] Makhubu et al.[80] evaluated anthelmintic activities of acetone, dichloromethane/methanol and water leaf extracts of *L. rugosa* against root-knot nematodes *Meloidogyne incognita*, *Caenorhabditis elegans* and animal parasitic *Haemonchus contortus* nematodes. The acetone extract inhibited 96.0% of *Haemonchus contortus* while the solvent-solvent fractions had good activity against *Caenorhabditis elegans*.[80]

McGaw and Eloff[79] evaluated the antibacterial activities of acetone leaf extracts of *L. rugosa* against *Escherichia coli* ATCC 35219, *Pseudomonas aeruginosa* ATCC 27853, *Enterococcus faecalis* ATCC 29212 and *Staphylococcus aureus* ATCC 29213 using two-fold serial dilution microplate method with neomycin as a positive control. The extract exhibited activities with minimum inhibitory concentration (MIC) values ranging from 0.4mg/ml to 1.6mg/ml which were higher than MIC values of 0.0008mg/ml to 0.03mg/ml exhibited by the control. Total activity against the tested pathogens ranged from 20.5ml/g to 82.1ml/g.[79] Suliman[51] evaluated the antibacterial activities of acetone leaf and stem extracts of *L. rugosa* against *Bacillus cereus* (ATCC 11778), *Enterococcus faecalis* (ATCC 29212), *Staphylococcus aureus* (ATCC 12600), *Klebsiella pneumoniae* (ATCC 13883) and *Moraxella catarrhalis* (ATCC 23246) using micro-dilution technique with ciprofloxacin as a positive control. The extracts exhibited activities with MIC values ranging from 0.3 mg/ml to 0.3mg/ml which were higher than MIC values ranging from 0.04µg/ml to 0.9µg/ml exhibited by the control.[51] Mabona[52] and Mabona et al.[54] evaluated the antibacterial activities of aqueous and dichlomethane: methanol (1:1) leaf extracts of *L. rugosa* using the microtiter plate dilution technique against

dermatologically relevant pathogens such as *Brevibacillus agri* ATCC 51663, *Propionibacterium acnes* ATCC 11827, *Pseudomonas aeruginosa* ATCC 27858, *Staphylococcus aureus* ATCC 25923, methicillin resistant *Staphylococcus aureus* (MRSA) ATCC 43300, gentamycin – methicillin-resistant *Staphylococcus aureus* (GMRSA) ATCC 33592 and *Staphylococcus epidermidis* ATCC 2223with ciprofloxacin as the positive control. The extract showed activities with MIC values ranging from 0.5mg/ml to >16.0mg/ml in comparison to MIC values of 0.3µg/ml to 1.3µg/ml exhibited by the positive control.[52,54]

Papo[78] evaluated the antibacterial activities of organic and water leaf extracts of *L. rugosa* against respiratory pathogens *Klepsiella pneumoniae* ATCC 13883, *Moraxella catarrhalis* ATCC 23246 and *Staphylococcus aureus* ATCC 29213 using the microplate dilution method with ciprofloxacin as a positive control. The extracts exhibited activities with MIC values ranging from 4.0mg/ml to >8.0mg/ml which were higher than MIC values of 0.04µg/ml to 1.3µg/ml exhibited by the control. Papo[78] also evaluated the antibacterial activities of essential oil isolated from the leaves of *L. rugosa* against *Klepsiella pneumoniae* ATCC 13883, *Moraxella catarrhalis* ATCC 23246 and *Staphylococcus aureus* ATCC 29213 using the microplate dilution method with ciprofloxacin as a positive control. The essential oil exhibited activities with MIC values ranging from 2.0 mg/ml to 4.0 mg/ml which were higher than MIC value of 6.3µg/ml exhibited by the control.[78] Makhafola et al.[64] evaluated the antibacterial activities of acetone, hexane, ethyl acetate, methanol and water leaf extracts of *L. rugosa* against *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Enterococcus faecalis* ATCC 29212 and *Staphylococcus aureus* ATCC 29213 using the microplate dilution method with gentamycin as a positive control. The extracts exhibited activities with MIC values ranging from 0.2mg/mL to 8.4mg/mL which were higher than MIC values of 0.01 mg/mL to 1.1mg/mL exhibited by the control.[64] Makhubu et al.[81] evaluated the antibacterial activities of water, dichloromethane and ethyl acetate leaf extracts of *L. rugosa* against phytopathogenic bacteria *Xanthomonas perforans, Xanthomonas vesicatoria, Ralstonia solanacearum, Ralstonia pseudosolanacearum* and *Clavibacter michiganensis* sub sp. *Michiganensis* using serial microplate dilution method. The extracts exhibited moderate activities with MIC values ranging between 0.08mg/mL to 0.156mg/mL against the tested bacteria.[81]

Suliman[51] evaluated the antifungal activities of acetone leaf and stem extracts of *L. rugosa* against *Candida albicans* (ATCC 10231) and *Cryptococcus neoformans* (ATCC 90112) using micro-dilution technique with amphotericin B as a positive control. All the extracts exhibited activities with MIC value of 2.0mg/ml which was higher than MIC values ranging from 1.3µg/ml to 1.5µg/ml exhibited by the control.[51] Mabona[52] and Mabona et al.[54] evaluated antifungal activities of aqueous and dichlomethane: methanol (1:1) leaf extracts of *L. rugosa* using the microtiter plate dilution technique against dermatologically relevant pathogens such as *Candida albicans* ATCC 10231, *Microsporum canis* ATCC 36299 and *Trichophyton mentagrophytes* ATCC 9533 with amphotericin B as the positive control. The extract showed activities with MIC values ranging from 0.05mg/ml to 8.0mg/ml in comparison to MIC value of 1.3µg/ml to 25.0µg/ml exhibited by the positive control.[52,54]

McGaw and Eloff[79] evaluated the cytotoxicity activities of acetone leaf extracts of *L. rugosa* against the larvae of *Artemia salina* (brine shrimp) with podophyllotoxin (5 µg/ml) as a positive control. The extract exhibited activities with median lethal concentration (LC_{50}) value of 0.7mg/ml.[79] Makhafola et al.[64] evaluated the cytotoxicity activities of acetone, hexane, ethyl acetate, methanol and water leaf extracts of *L. rugosa* against C3A liver cell lines using 3-(4,5-dimethylthiazol- 2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay with doxorubicin as a positive control. The extracts exhibited activities with LC_{50} values ranging from 6.4µg/mL to 6.4 µg/mL.[64]

CONCLUSION:

Documentation of the medicinal uses, phytochemistry and pharmacological properties of *L. rugosa* is crucial as this information forms the baseline data required for future research and development of health-promoting and pharmaceutical products. Findings from this study showed that there are still some research gaps in the phytochemistry, pharmacological and toxicological properties of the species. More rigorous research is required aimed at evaluating various plant parts used as herbal medicines, assessing their phytochemistry, pharmacological and toxicological properties. Preliminary pharmacological studies have provided supporting evidence for the therapeutic potential of *L. rugosa* in the management of bacterial and fungal infections. However, detailed ethnopharmacological research is required focusing on the medicinal uses, phytochemistry, biological activities and toxicological properties of the species.

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RESEARCH ARTICLE

Knowledge, Attitude and Experience of Pharmacist in the UAE towards Pharmacovigilance

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ABSTRACT:

Objectives: The objective of the current study was to investigate the pharmacist's knowledge, attitude, practice and experience towards pharmacovigilance**. Methods:** A cross-sectional study was conducted among the pharmacist's in UAE by using a validated self-administered questionnaire**. Results:** A total of 230 pharmacists were interviewed. However only 185 questionnaires were completed and analyzed. Of the 185 respondents (59.5%) were male. Pharmacist's age mean was 32.15 ± 6.86 years. Fifty five (29.7%) pharmacists in this study had good knowledge, while 91 (49.2 %) pharmacists had moderate knowledge and 39 (21.1 %) pharmacists had poor knowledge towards pharmacovigilance, ADRs and ADRs reporting. The finding of this study showed that 82 (44.3 %) of the pharmacists were detected and seen ADRs in their practice at least once. A total of 59 ADRs were seen by the pharmacists in this study. The most common ADRs they detected were gastritis (16 times), followed by allergy (12 times), then rash (8 times), anaphylactic shock (two times) and other ADRs (21). However, none of the detected ADRs were reported. The most barriers reported by pharmacists were: lack of knowledge about reporting system. Factors to encourage ADRs reporting were: enhancing awareness about reporting through attending courses or workshops; receiving educational materials and simplification of reporting procedures**. Conclusion:** Majority of pharmacists in this study had positive attitude towards pharmacovigilance, ADRs and their reporting. Education and training programs are highly recommended to enhance awareness and improve the reporting of ADRs by pharmacists in UAE**.**

KEYWORDS: Pharmacovigilance, knowledge, attitude, pharmacist, UAE.

INTRODUCTION:

In order to ensure the quality of medications use in the UAE, the Health Authority in Abu Dhabi (HAAD) initiated the pharmacovigilance program in 2008 in the capital Abu Dhabi1. The - main aim of the UAE pharmacovigilance program[1] is – similar to the international aim of pharmacovigilance programs.[2-5] Adverse Drug Reactions (ADRs) and medication errors reporting.

Pharmacovigilance is defined by the World Health Organization (WHO) as ''the science and activities relating to the detection, assessment, understanding and prevention of adverse effects or any other possible drug-related problems''[2] ADRs are defined by WHO as "any noxious, unintended, and undesired effect drug that occurs as a result of treatment with drug at a normal doses used in man for diagnosis, prophylaxis, and treatment"[6]. Adverse Drug Reactions (ADRs) can result in increased length of hospital stays; increased cost of therapy; increased hospital admission; and increased patient morbidity and mortality[7-11].

Underreporting of ADRs among pharmacists and other health care professionals were reported as major obstacles to pharmacovigilance programs which was found linked to their poor knowledge of pharmacovigilance, ADRs and how to report them[12-15]. It is to assess knowledge, attitude and experience of pharmacists relating to pharmacovigilance and ADRs as this will help identify areas needing intervention and

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education. Therefore, the aim of this study was to investigate the knowledge, attitude, experience of pharmacists in the UAE towards pharmacovigilance, ADRs and ADRs reporting.

MATERIAL AND METHODS:

A cross sectional study was conducted over a period of four months (1st October 2017 to 30th March 2018) among pharmacists in Ajman, Sharjah and Dubai in the UAE using a validated self-administered questionnaire. Two trained pharmacists visited hospital and community pharmacies and delivered the questionnaire to one pharmacist from each site. Sample size (n) was calculated based on the formula [n = Z2 P (1 − P) / d2] to estimate the number of subjects required for this study18. Where n = sample size, Z = Z statistic for the level of confidence (Z=1.96 is selected to give a reasonable power in estimating the sample size), P = expected prevalence or proportion of related matter. In this equation the prevalence was chosen based on the reported prevalence in previous literature[16-18]. d = precision (for the level of confidence of 95% or 0.05). Hence, n= (1.96)2×0.30 (1- 0.5)/(0.05)2 = 230. However, the survey was distributed conveniently to 230 pharmacists, due to unwillingness and/or inadequate information about PV available to pharmacists and the time limitation of study. Hence, a convenient sample of 185 pharmacist completed the questionnaire. The questionnaire was developed based on information from the literature[17-20]. The questionnaire was pilot tested on a convenience sample of ten pharmacist to test the clarity, face validity and establish the time length needed to complete the questionnaire. The study questionnaire was designed by the researcher based on the parameters to be assessed and it was composed of six sections. Section one included the demographic characteristics of respondents, qualifications and experience. Section two included eight questions to evaluate the knowledge of the participants towards pharmacovigilance, ADRs and ADR reporting in the UAE. The research team devised a scoring whereby a score of 1 and 0 was given for each correct or positive and incorrect or negative answer respectively. The scoring range was from 0 (as minimum) and 8 (as maximum). Knowledge scores for each participant were calculated and summed to give the total knowledge score. A cut off ranges of (0-3) was considered as poor or low knowledge, (4-5) as moderate knowledge and (6-8) as adequate or high knowledge. Section three contained 11 questions looking at the attitude of participants towards pharmacovigilance and ADR reporting, measured on a five-point Likert type scale of (SA: strongly agree = 1, A: agree = 2, N: Neutral = 3, D: Disagree = 4 and SD: strongly disagree = 5). Section 4 explored the experience of pharmacist in UAE with ADRs such as if they have identified any patient encountering an ADR, how many times, what were the most common ADRs they have seen and what they have done regarding them, and if they have ever reported an ADR and how many times did this occur. This section was reported in a text format by pharmacists. Section five contained 8 items aiming at exploring the factors that would encourage pharmacists in the UAE to report ADRs again using a five-point Likert type scale of (SA: strongly agree. A: agree. N: Neutral D: disagree and SD: strongly disagree). Section six contained 15 items looking at determining the barriers to reporting ADRs using a five point Likert – type scale as described above. Data were entered and analyzed using SPSS version 21 (SPSS Statistics for Windows, version 21.0, IBM Corp., USA). Differences in proportions were tested with Chi-square test or Fisher's Exact test. Differences in the means were tested with the student t-test. All reported p-values are two tailed, and the result were considered significant if P-value was ≤ 0.05. This study was approved by Ajman University Research Ethics Committee. Written consent was taken from the respondents to participate in the study.

Sociodemographic characteristics:

A total of 230 pharmacists were approached to complete the questionnaire, however only 185 pharmacists completed it giving a response rate of 80.4%. The mean age of the respondents was 32 ± 6.86 years. The characteristics of the respondents are presented in (Table 1)

Table 1. Sociodemographic characteristics of the pharmacists (n=185)

Variables	Sub-variables	Frequency (n=185)	Percentage (%)
Gender	Male	110	59.5%
	Female	75	40.5%
Qualifications	B-Pharm	107	57.8%
	Pharm D	56	30.3%
	Master (Clinical Pharmacy)	22	11.9%

Knowledge of pharmacists about pharmacovigilance, ADRs and ADRs reporting in UAE:

The results of the scorings found that 55 (29.7%) of the pharmacists had good knowledge, while 91 (49.2%) had moderate knowledge and 39 (21.1%) had poor knowledge of pharmacovigilance. Although most pharmacists had overall good knowledge of the concept of pharmacovigilance and ADR reporting, 88%, 85.9% respectively, they were not aware of the presence of a pharmacovigilance center in the UAE and that there was an official form designed for reporting ADRs in the Country. (Table 2) shows other correct/positive answers related to the knowledge of pharmacists towards pharmacovigilance, ADRs and ADR reporting.

Table 2. Knowledge related Correct Answers (n=185)

Questions	N (%)
1. Have you ever heard about the concept of pharmacovigilance?	163 (88.1%)
2. Do you know the definition of ADR?	159 (85.9%)
3. In UAE, is there pharmacovigilance center?	64 (34.6%)
4. There is no difference between ADRs and side effects	100 (54.1%)
5. All drugs available in the market are safe	114 (61.6 %)
6. Herbal products have no ADRs	103 (55.7%)
7. In UAE, is there an official form for reporting ADRs?	78 (42.2%)
8. All people can report ADRs	68 (36.8 %)
Total Knowledge scores: Mean ± (SD)	4.59 ± (1.320)

Attitude of UAE pharmacists towards pharmacovigilance and Adverse Drug Reactions (ADRs) reporting:

(Table 3) shows the attitude of pharmacists towards pharmacovigilance, ADRs and ADRs reporting.

Table 3. Attitude related items (Positive Attitude) (n=185)

Statement	N (%)
I believe that pharmacovigilance is important	178 (96.2 %)
Reporting ADRs is part of the professional role	175 (94.6%)
I want to be sure the ADR is related to the drug before reporting	5 (2.7%)
I do not report ADRs of OTC products	62 (33.5%)
I report an ADR that causes:	
Hospitalization	167 (90.3 %)
A life threatening situation	166 (89.7%)
A congenital anomaly	158 (85.4%)
Persistent disability or incapacity	160 (86.5%)
Death of the patient	157 (84.9%)
I report to get more insight into ADR questions that I come across in my practice	5 (2.7%)
I report to show the patient that their concern is being taken seriously	154 (83.2%)
Total scores: Mean ± (SD)	7.50 ±(1.701)

Table 4. Factors that encourage Adverse Drug Reaction (ADR) reporting (n=185)

Factors	N (%)
I will report if:	
Attend course or workshops to understand the reporting process	171 (92.4%)
Receive materials to understand the reporting process	165 (89.2%)
More attention to ADR reporting in university curriculum	163 (88.1%)
Simplification of reporting procedure	164 (88.6%)
Promoting reporting as a part of professional duty	165 (89.2%)
There is a fee	133 (71.9%)
I receive more feedback through mailings	150 (81.1%)
Compulsory reporting	125 (67.6%)

Experience of UAE pharmacists with Adverse Drug Reactions (ADRs) and its reporting:

The finding showed that there were 82 (44.3%) pharmacists who detected ADRs in their practice at least once. A total of 59 ADRs were seen by all pharmacists. The most common ADRs they detected were gastritis (16 times) rash, followed by allergy (12 times), then rash (8 times), anaphylactic shock (two times) and other ADRs (21). However, none of the detected ADRs were reported.

Factors that encourage Adverse Drug Reaction (ADR) reporting:

(Table 4) shows factors that encourage (ADRs) reporting.

Barriers of Adverse Drug Reactions (ADRs) reporting:

(Table 5) shows the barriers of reporting ADRs.

Table 5. Barriers of Adverse Drug Reactions (ADRs) reporting (n=185)

Barrier	N (%)
I don't report ADR because reporting form not available	121 (65.4%)
I don't report ADR because I don't know the address where these reports should be sent	83 (44.9%)
The reporting form too complicated	119 (64.3%)
Reporting ADRs is time consuming	81 (43.8%)
All serious ADRs are detected before registration	105 (56.8%)
I don't report ADR because I want to publish about them myself	75 (40.5%)
I don't report ADR because I am not convinced about the confidential handling of the reports	75 (40.5%)
I don't report ADR because I fear it may harm the confidence of my patients	90 (48.6%)
I don't report because I find it difficult to admit that the patients has been harmed	87 (47%)
I don't report because reporting may give the impression that I am ignorant concerning ADRs	72 (38.9%)
I don't report because I fear legal liability for the reported ADRs	84 (45.4%)
I am not motivated to report	74 (40%)
I don't report because I have insufficient clinical knowledge	83 (44.9%)
I don't report because I don't know how to report ADR	97 (52.4%)
I don't report because I am not convinced the ADR is caused by the drug	62 (33.5%)

The finding of this study showed that there was no significant association between the knowledge and attitude (P-value = 0.256) of pharmacists towards pharmacovigilance. Other pharmacist characteristics showed no significance as well.

DISCUSSION:

This study explored the knowledge, attitude, experience of UAE pharmacists towards pharmacovigilance, ADRs and ADR reporting.

Knowledge about pharmacovigilance, ADRs and ADRs reporting in UAE:

The findings of our study is similar to other studies in the region. A study conducted by Qassim S., et al 2014 among 223 pharmacists in Ajman and Sharjah, UAE found that only eleven pharmacists (4.9%) had a good knowledge towards Adverse Drug Reactions reporting[16]. Another study conducted by Al-Worafi YM, 2017 among 21 pharmacists and 155 pharmacy technicians in

the capital Sana'a, Yemen found that 23.8 % of the pharmacists had a good knowledge[17]. A study conducted by Mahmoud MA and his colleagues in 2014 among 104 community pharmacists in the capital Riyadh, Saudi Arabia found that 22% of the pharmacists had a good knowledge towards the Adverse Drug Reactions (ADRs) reporting process[18].

Attitude of pharmacists in UAE towards pharmacovigilance and Adverse Drug Reactions (ADRs) reporting:

The finding of this study showed that 153 (82.7%) of pharmacists had a positive attitude towards pharmacovigilance and ADRs reporting. This finding are similar to what was reported in Netherlands, Yemen and Saudi Arabia that the pharmacists had positive attitude towards pharmacovigilance and Adverse Drug reactions (ADRs) reporting.

Experience of pharmacists in UAE with Adverse Drug Reactions (ADRs) and its reporting:

The finding of this study showed that 82 pharmacists (44.3 %) detected ADRs in their practice at least once. A total of 59 ADRs were seen by UAE pharmacists. The most common ADRs they detected were gastritis (16 times), followed by allergy (12 times), then rash (8 times), anaphylactic shock (two times) and other ADRs (21). However none of the detected ADRs were reported. Reporting Adverse Drug Reactions is not mandatory in developing countries and this is could be the reason of not reporting the ADRs[4-15].

A study conducted by Mahmoud MA and his colleagues in 2014 among 104 community pharmacists in the capital Riyadh, Saudi Arabia found that majority of pharmacists did not report Adverse Drug Reactions[18]. In the study by Al-Worafi, it was found that 42.9% of pharmacists had experience of Adverse Drug reactions (ADRs) in their practice and they reported that allergy and diarrhea were the most common ADRs detected but none of the detected ADRs were reported20. Under reporting ADRs are common among pharmacists worldwide[4,12-15,17,18].

Factors that encourage Adverse Drug Reactions (ADRs) reporting:

Reported factors to encourage ADRs reporting were: attend courses or workshops; educational materials and simplification of reporting procedures. The identified factors in this study are similar to other studies worldwide[4,12-15,17,18].

Educational interventions such as workshops and educational materials reported to contribute effectively on improve the reporting of Adverse Drug Reactions among health care professionals[25,30].

Barriers of Adverse Drug Reactions (ADRs) reporting:

Lack of knowledge about reporting systems by pharmacists and other healthcare professionals was considered an important factor affecting ADR reporting[15]. A systematic review conducted by Lopez-Gonzalez E et al in 2009 to investigate the impact of knowledge and attitude of healthcare professionals on Adverse Drug reactions found that there was a strong association between knowledge and attitude[15].

Similar to the previous studies worldwide, underreporting is a big concern and the increase in the awareness of pharmacists and other healthcare professionals towards the pharmacovigilance and ADRs and how they can report it to the pharmacovigilance programs could overcome the barriers of reporting[4,12-15,17,18].

CONCLUSION:

Majority of pharmacists in this study had positive attitude towards pharmacovigilance, ADRs and its reporting despite their knowledge. Education and training programs are highly recommended to improve the reporting of ADRs by pharmacists in UAE.

LIMITATIONS:

Although this study was conducted in Ajman, Sharjah and Dubai (out of seven) in the UAE, and caution should be taken in generalizing the findings in this study to all other emirates, it is worthy to acknowledge that the rules governing pharmacy practice and pharmacists' experiences are similar in the country.

CONFLICT OF INTEREST:

The authors declare that there are no conflicts of interest

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RESEARCH ARTICLE

Graphene synthesis in Obtaining a safe particle size in Blood Circulation System

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ABSTRACT:

Graphene is an inorganic nanomaterial that is biocompatible and safe in certain concentration. Since it is proposed as drug delivery or scaffold material, the size of the graphene sheets should be considered for the toxicity in the blood circulation. Permanent damage to cell membranes can occur due to the large size of nanoparticles through binding with cellular membrane proteins. Its synthesis process can modify the size of graphene nanoparticles. This paper presents a graphene synthesis from graphite powder that consider particle size change as induced by sonication. The synthesis was conducted by mechanical exfoliation method using a kitchen blender and a water bath sonicator. The study aimed to predict a safe lateral dimension of graphene nanoparticles. The characterizations were performed by X-Ray Diffraction (XRD), Fourier Transform Infra-Red (FTIR), Transmission Electron Microscopy (TEM), and Particle Size Analysis (PSA). The results showed that prolonged sonication time had caused defects to the graphene layers. The mean of graphene layers lateral decreases from 2973.7 nm to 655 nm after 120‘ sonication time. We proposed a simple regression model of the sonication time (x) to the lateral dimension change (y) and found that we can obtain to obtain a mean graphene lateral dimension of 40 nm after 146‘ sonication.

KEYWORDS: Graphene, synthesis, exfoliation, particle size, safety.

INTRODUCTION:

Graphene is a single carbon layer in the form of the honeycomb lattice. Graphene and its derivatives are nanoparticles with a thickness of 1-10 nm and its lateral size varied up to hundreds of nanometer. The exceptional properties of nanoscale materials are increasingly used in the medical field for diagnostics as well as therapeutics[1–3]. Graphene-based materials are a promising material for drug delivery, anti-bacterial materials, and biocompatible scaffolds[4,5]. As the utilization of nanotechnology is gaining in popularity, human and environmental exposure to graphene-based nanomaterials also tends to increase. It should be noted that the use of new materials can create benefits on the one hand, as well as the possible impacts on health and the environment[6].

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Since graphene has been proposed as drug delivery and scaffold material, it could enter different organs through blood or biological system. Its release in the blood causes cytotoxicity. According to Ou et al.[7], the cytotoxicity of graphene depends on different factors: the concentration in the body, lateral layer dimension, the structure of the layer surface, interaction with other substances, pureness, and the existence of protein corona. Those characteristics of graphene are strongly affected by the synthesis process. The lateral dimension is one of the characteristics that can be controlled by the synthesis process. As a rule of thumb, Mytych and Wnuk [8] reported that the size of the nanoparticles can go into the cell (<100 nm), nucleus (<40 nm), and even the blood-brain barrier (<35 nm). The lateral dimension of <40nm is considered a safe boundary. The size of 100-500 nm may cause the most severe toxicity[7]. The smaller sizes are thus the better. Particle size analyzer (PSA) is a suitable instrument for measuring particle size distribution in nanoscale such as graphene. This instrument uses laser diffraction techniques to study the

size distribution of a sample in the form of powder, suspension, or emulsion[9].

Graphene's properties require versatile and reliable synthetic routes. Several graphene synthesis methods require expensive and sophisticated processes such as epitaxial growth and chemical vapor deposition[10]. Another simple method of graphene synthesis is mechanical exfoliation. The exfoliation method uses mechanical power to separate graphene sheets from graphite in different dimensions with available simple equipment[11].

For an effective of graphene synthesis, the exfoliation process can be assisted by irradiation of the sourced graphite material using a microwave oven. Microwave irradiation has been increasingly used for both organic and inorganic syntheses[12,13]. The treatment can increase the interplanar distance of the graphite layers[14–17]. A microwave oven is a fast heating source[16,18]. The target materials of microwave heating can be in liquid or solid form. Dipolar polarization and ionic conduction occur in liquid heating[19]. However, most of the microwave energy is dissipated which causes less effective energy absorption on the target material[16]. In carbon-solid microwave heating, the delocalization of π-electrons in the graphitic region provides semiconducting features. In this case, interfacial polarization occurs which realizes efficient and selective heating to the target material[16].

Blender and ultrasonicator are mixing instruments to be used for graphite exfoliation[20]. The graphite powder is dispersed in a solution of water and surfactant. In blender exfoliation, graphene is produced by high speed rotating blades due to exceeding the critical shear rate[21–23]. Processing of large liquid volumes is possible quickly using this equipment. In sonication, micro jets and compressive shock waves induce the liquid cavitation that causes graphene dispersion. The sonication method produces more oxides and defects graphene, as evidenced by Polyakova et al.[24]. Based on that, the longer the sonication time of graphene, the smaller the layer size.

The use of surfactants in a graphene suspension is necessary for efficient exfoliation. It creates optimum interfacial tension[25]. The adsorption of the surfactant onto the particulate surface creates a barrier that inhibits the sheets' reaggregation. Sodium Dodecyl Sulfate (SDS) is a typical ionic surfactant to disperse graphene in water. The use of water as a liquid medium makes the cost cheaper and more environmentally friendly compared to organic solvents and ionic liquids[26].

In this paper, we studied graphene synthesis from graphite powder using the exfoliation method. Limited information was found about graphene synthesis that discusses obtaining a safe lateral size of graphene sheets required in the blood circulation. Therefore, this study aimed to predict a safe lateral dimension of graphene nanoparticles through exfoliation process. The results were analyzed, including the particle size change of the graphene layers.

MATERIAL AND METHODS:

Graphene Synthesis:

The conversion of graphite to graphene was performed in a multi-step process, as presented in Fig. 1. Fine natural graphite powder with the particle size of 60 mesh (>95% carbon) was used for the synthesis. 2 g of the material was put in a glass bowl and irradiated by domestic microwave (Electrolux EMM2007X, Japan) at 400 watts power. The irradiation time was set for 3 minutes with the cycle of 10 seconds on and 10 minutes off. The irradiated graphite was dispersed for the exfoliation process in the aqueous solution of 300ml water and 0.5g SDS surfactant to maintain hydrophobic material and prevent the restacking of exfoliated graphite layers. A kitchen blender (Philips HR2071, Indonesia) was used to exfoliate the suspension for 60 minutes (2 x 30') at the maximum speed. After being left overnight, the suspension was separated from the sediment and put it into three beaker glasses. Further exfoliation was done through ultrasonic vibration using Water Bath Sonicator (Branson 3800 40 kHz, USA). The sonication times were set at 30, 60, and 120 minutes respectively. The suspension was further enriched by centrifugation by using centrifuge (Sorvall Biofuge Primo R, USA) at 7500rpm and 25°C for 30 minutes. The obtained graphene was washed two times to remove the surfactant. The obtained graphene was collected in a watch glass and dried in ambient temperature for four days.

Characterization:

The characterization of the irradiated graphite powder was done by X-ray Diffractometer (XRD Nova SxD, Australia). The results were compared with the sourced graphite powder. The size and distribution of graphene flakes were measured by using Particle Size Analyzer (PSA) instrument (HORIBA Scientific, Japan) after blender and ultrasonication exfoliations. The images of the synthesized graphene were recorded using Transmission Electron Microscopy (TEM) model JEOL JEM-1400 (Japan) at an acceleration voltage of 150 kV. Fourier Transform Infrared (FTIR) spectra of the samples were obtained using a spectrophotometer (Shimadzu IRPrestige, Japan). The samples were prepared using a potassium bromide (KBr) mulling agent.

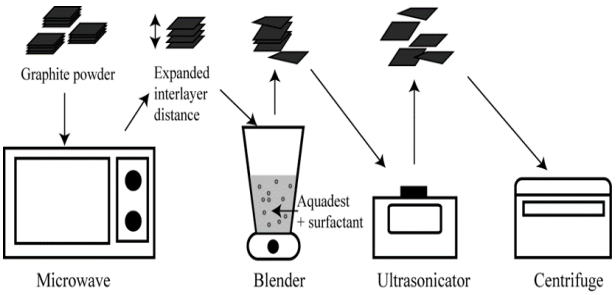

Fig. 1: Schematic illustration of the exfoliation method

RESULTS AND DISCUSSION:

XRD analysis:

XRD analysis showed the broader interlayer distance of graphite as shown in **Fig. 2**. The peak points are presented in **Table 1**. The XRD patterns presented that the original graphite powder has sharp diffraction peaks at 26.63° and 54.72° (2 theta). The first peak matches to the graphitic plane (0 0 2) with its interlayer spacing of 3.3477 Å. In the case of microwaved graphite, this peak is relatively lower at 26.560 (2 theta). For the corresponding peak, it exhibits the graphitic plane (0 0 2) with its interlayer spacing of 3.3480 Å. The result showed that microwave irradiation is useful to expand the interplanar spacing of the sourced graphite.

Table 1: XRD peak points of graphite and graphene

Sample	Peak	2theta	D[Å]	I/I0	FWHM
Graphite	1	26.63	3.3477	1000.00	0.1625
	2	54.72	1.6775	34.16	0.2031
Microwaved graphite	1	26.56	3.3480	1000.00	0.1625
	2	54.66	1.6793	33.6	0.2031

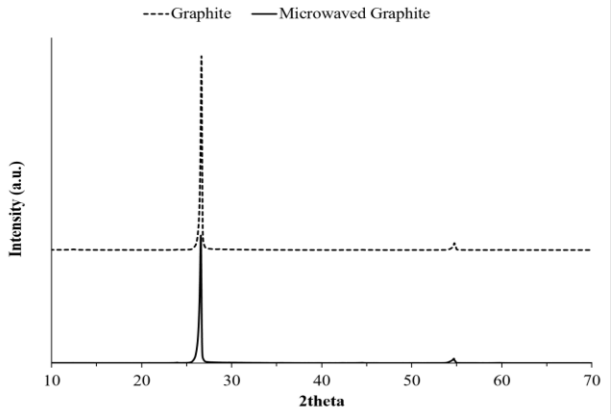

Fig. 2: XRD patterns of graphite before and after irradiation

The expansion of the interplanar distance of irradiated graphite was caused by weakened interaction of Van der Waals forces between molecules. For longer irradiation times, the interlayer distance of graphite shifted much larger. Similar results have been shown by Jiang et al. .[27] Our visual observations showed that the irradiated graphite powder was darker than the sourced graphite powder. The phase changes and restructuring of the crystallinity of the substance probably have changed 2D structure into 3D structure[28]. The irradiated graphite powder results in more sediment compared with untreated graphite powder due to an increase in density.

FTIR analysis:

The IR spectra of graphene performed OH stretchings at 3425.58, 2924.09 and 2854.65 cm^{-1} which confirmed of carboxylate functional groups. The C=C and C-O stretchings of graphene were respectively observed at 1627.92 and 1026.13 cm^{-1}. The sourced graphite showed peaks at 3448.72 cm^{-1}, 2924.09 and 2854.65 cm^{-1} for OH stretchings which confirmed its carboxylate functional groups. Meanwhile the prensence of C=O and C-O stretchings were respectively showed by peaks at 1635.64 and 1033.85 cm^{-1}. CO stretching at 1033 cm^{-1} confirmed that graphene was synthesized using the surfactant SDS which has rich oxygen structure. Furthermore, KBr was used as a mulling agent for the analysis and identified at 2337.72 cm^{-1}. The shift to the lower wavelength confirms a decreased layer number of graphite into graphene. A similar result has been found in Naebe's work [29]. The FTIR spectra of the sourced graphite and graphene is shown in **Fig. 3**.

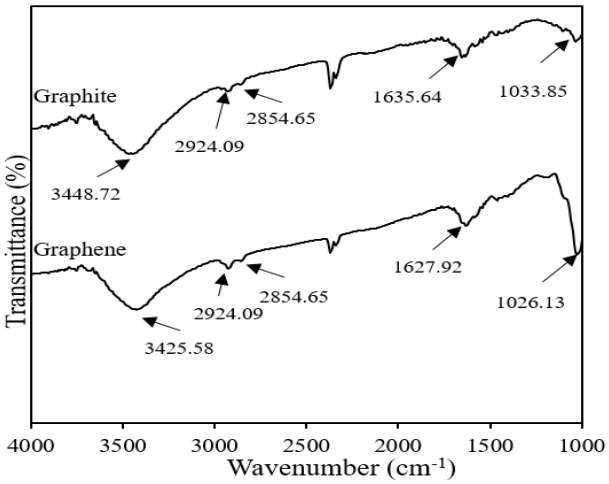

Fig. 3: FTIR spectra of graphite and graphene

TEM Observation:

Intercalation of surfactant and blender rotation and ultrasonication resulted in single and a few layers of graphene. Images of TEM observations are shown in **Fig. 4**. The obtained graphene structure has transparent silk-like morphology and folded layers form. Graphene nanosheets with a few hundred square nanometers were observed which look like transparent silk veil waves. Scrolled graphene nanosheets are also observed which is typical of graphene nanosheets [30,31]. Base on these TEM images, we believe that this exfoliation of graphite has demonstrated to produce monolayer and few-layer graphene.

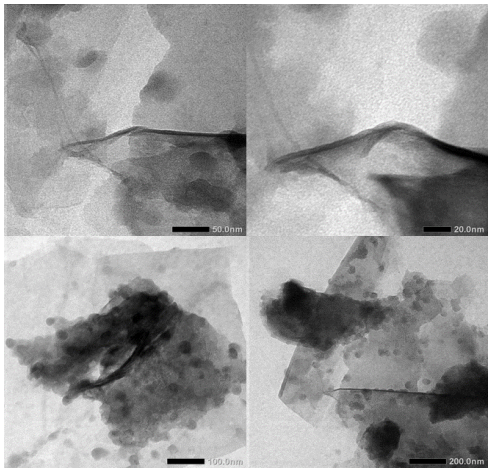

Fig. 4: TEM images of graphene

Particle Size Analysis:

The size distributions of graphene are exhibited in **Fig. 5**. The sonication treatment has decreased the mean size of graphene's lateral size: 2973,7 nm (S.D. 1057,3 nm), 2385,8 nm (S.D. 1183,6 nm), dan 795,4 nm (S.D. 401,2 nm) respectively for 30', 60', dan 120' of sonication time. **Fig. 6** shows that the size of 30' sonicated graphene was mostly found between 2000-3000 nm. Meanwhile, the sonication time of 60 'and 120' produced mostly the lateral size between 1000-2000 nm and 0-1000 nm, respectively. The results showed that the mechanism of sonication has improved the dispersion and exfoliated the sheets of graphite into individual graphene flakes[32,33]. The prolonged sonication time caused defects to the layer surface area [23,34]. Based on this data, we have developed a simple regression model that considers the effect of the sonication time (x) to the lateral change (y) of the graphene sheets. In this model, the intercept coefficient was 3493 and the coefficient of sonication time was -23.9. The model is expressed in the following equation: y = 3588.9 - 22.8x. Based on this model, it requires 146 min sonication time to obtain the mean size of 40 nm. However, this value may vary when using different types of equipment in the synthesis and longer sonication time is necessary to achieve the overall result < 40 nm.

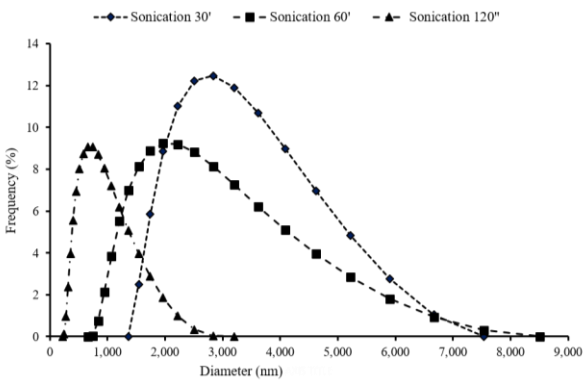

Fig. 5: Frequency distribution of graphene's size

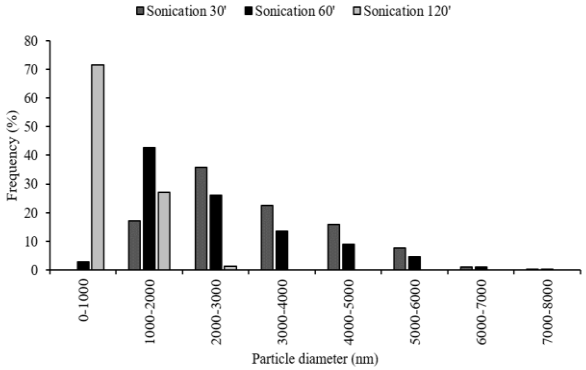

Fig. 6: Frequently graphene's size found in different sonication time

The effectiveness of exfoliation using sonication is influenced by sonicator power and target material volume. According to Arao and Kubouchi[32], low-power (generally less than 100 W) of sonicator leads to a low production rate of graphene sheets. In our case, the sonicator has 110 W power which is expected to perform exfoliation adequately. However, the cavitation of the water bath sonicator generator is placed at the bottom of the bath. Hence, it results in less effective cavitation behavior than one with cavitation generator direct in the vessel using a sonic tip[35]. The volume of the sonicated suspension should be no larger than a few 100 ml to achieve a more effective result[36,37].

CONCLUSION:

In this study, graphene synthesis by mechanical exfoliation method was adapted using a blender and a water bath sonicator. Microwave irradiation has been a catalyst to shift the spacing layer distance of the 3D structure of graphite. The exfoliation process has resulted in single and a few layers of graphene successfully. PSA results evidenced that the decrease in the lateral surface of graphene layers as the effect of sonication. Future research should optimize the exfoliation time to acquire a more effective process using different types of equipment.

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CONFLICT OF INTEREST:

The authors declare no conflict of interest.

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RESEARCH ARTICLE

Synthesis, Characterization and Antifungal Evaluation of substituted Pyrimidin-2-thione Derivatives

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ABSTRACT:
Pyrimidine and its thione derivatives have found a wide range of application in medicine due to their pronounced biological activity. An attempt has been made to combine the toxophoric qualities of biologically versatile 2-amino-4-aryl thiazole and 5-arylidene-3-aryl rhodanine moieties and synthesizing pyrimidin-2-thione derivatives, **VII(a-j)**. The compounds were screened for their *in vitro* antifungal potential against *Aspergillus niger* and *Fusarium oxysporium.* The results were promising as the compounds **VII b, VII c, VII g** and **VII h** displayed fungicidal action equivalent to that of commercial fungicide Dithane M-45 at 1000 ppm concentration.

KEYWORDS: Pyrimidine, thione, rhodanine, fungicides, 2-amino-4-aryl thiazole, agar-plate.

INTRODUCTION:
Heterocyclic compounds offer a high degree of structural variety and are a vital part of the synthetic medicinal chemistry. Heterocyclic compounds have been enticing the medicinal chemist over decades as the most promising molecules to develop lead structures for the design of new drugs.

The chemistry of pyrimidines had been attracting widespread attention owing to its relationship with thymidine, cytosine and uracil. Triazolopyrimidines have been reported to exhibit antifungal activity[1] and some pyrimidine derivatives have proven to be effective against leshmaniasis.[2] Condensed pyrimidine derivatives have been widely synthesized and studied for their pharmacological actions. Thiazolo [3,2-α] pyrimidine derivatives being the bioisosteric analogues of purines have been investigated largely for their bioactivity. They have been known to possess hypoglycemic and hypolipidemic potential.[3] 1,2,3,4-tetrahydropyrimidine-2-thiones have shown close structural relationship to the clinically important dihydropyridine calcium channel blockers. The derivatives of 1,2,3,4-tetrahydropyrimidine-2-thiones have been reported to be calcium channel blockers,[4] antitumor,[5] antidepressant,[6] antibacterial[7] and antifungal.[8]

Rhodanine, a five-membered heterocyclic molecule containing a thiazole nucleus with thioxo group on second carbon and carbonyl group on fourth carbon has been constantly structurally modified and resulted in synthesis of compounds with a wide spectrum of pharmacological activities.[9]

Being optimistic by the above reports on condensed pyrimidines and rhodanine, in the present study, the synthesis of condensed pyrmidine derivatives by electrophilic addition of 2-Amino-4-aryl thiazole to 5-Arylidine-3-aryl rhodanines followed by subsequent cyclization was achieved. The synthesized bisthiazolo-pyrimidin-2-thione derivates were subjected to in vitro antifungal studies against *Aspergillus niger* and *Fusarium oxysporium*.

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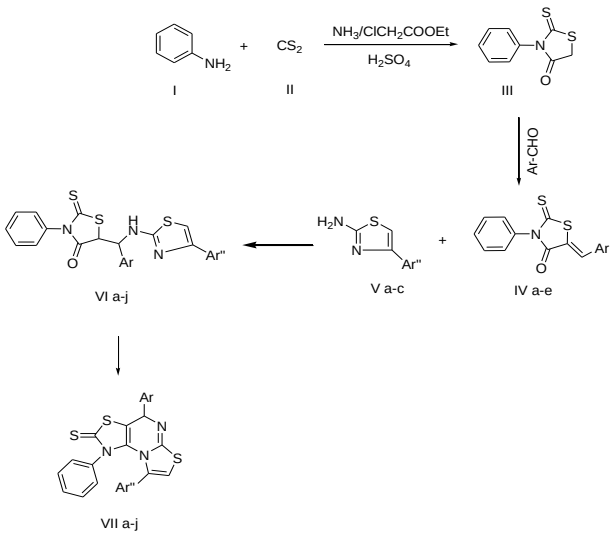
MATERIAL AND METHODS:

General Procedures:

Melting points were determined by open capillary method and are uncorrected. All chemicals used were reagent grade and were used as received without further purification.[1] HNMR spectra were recorded at 400 MHZ on a Bruker AVANCE DPX (400 MHz) FT spectrometer in DMSO-$d_6$using TMS as internal standard. Mass spectra were recorded on a JEOL SX-102 mass spectrometer at 70ev. Elemental analyses were performed on a Coleman elemental analyzer. The reactions were monitored using preformed aluminium TLC plates (UV_{254}) and the plates were visualized in Iodine vapour. Column chromatography was carried out on silica gel (60–120 mesh, Merck chemicals).



VII a (Ar=Ar''=C_6H_5), **VII b** (Ar= p-$CH_3OC_6H_4$; Ar''=C_6H_5), **VII c** (Ar= p-ClC_6H_4; Ar''=C_6H_5), **VII d** (Ar= m,p-$(CH_3O)_2C_6H_3$; Ar''=C_6H_5), **VII e** (Ar= p-$NO_2C_6H_4$; Ar''=C_6H_5), **VII f** (Ar''= p-$CH_3OC_6H_4$; Ar=C_6H_5), **VIIg** (Ar''= p-ClC_6H_4; Ar=C_6H_5), **VII h** (Ar''= p-$CH_3OC_6H_4$; Ar= p-ClC_6H_4), **VII i** (Ar''= p-$CH_3OC_6H_4$; Ar= m,p-$(CH_3O)_2C_6H_3$), **VII j** (Ar''= p-$CH_3OC_6H_4$; Ar=p-$NO_2C_6H_4$)

Scheme 1

General procedure of synthesis of 2-Amino-4-aryl thiazole (V a-c):

A mixture of aromatic ketone (100mmol), thiourea (200 mmol), iodine (100mmol) and a few drops of 1,4-dioxane was thoroughly agitated to mixing and was heated at 80-90°C under reflux conditions for 8 h. The mass obtained was triturated with ether over a period of 12 h. The ether was then decanted and the solid left behind was washed with a 3 % w/v solution of sodium thiosulphate followed by washing with cold water. The residue obtained was dissolved in boiling water, filtered, cooled, and then rendered alkaline with ammonia solution. The resulting solid was washed and recrystallized from dimethyl sulfoxide-H_2O (1:1 v/v) and finally purified by silica gel column chromatography (benzene-MeOH, 8:2), yielding analytically pure **V (a-c)**.

Synthesis of 3-Phenyl rhodanine (III):

In a continuously stirred ice-salt bath, 20mL of ammonia solution was added to CS_2 (48mmol). Aniline (20mmol) was added to this solution over a period of 30 min. The stirring was further continued for 3 h. The dithiocarbamate thus precipitated was allowed to stand overnight. It was filtered, washed with cold ether and dried by suction.

Separately a solution of sodium chloroacetate was prepared by mixing cooled solution of chloroacetic acid (40mmol) in 15ml of water, followed by addition of anhydrous sodium carbonate until alkaline. The sodium chloroacetate solution was stirred and cooled to 5-10°C and the dithiocarbamate was added to it over 15 min. Stirring was continued while the temperature of flask raised to room temperature. The solid thus obtained was added to a mixture of 15ml of sulfuric acid and 7ml of water and heated at 90°C for 15 min. On cooling a pale yellow compound was obtained. This was purified by silica gel column chromatography (Benzene: MeOH, 6:4) to obtain pure compound **(III)**.

General Procedure for synthesis of 5-Arylidine-3-Phenyl rhodanines (IV a-e):

A mixture of 3-Phenyl rhodanine (1mmol), different aromatic aldehyde (1mmol) and fused sodium acetate (1.1mmol) in glacial acetic acid was heated under reflux for 8 h. The reaction mixture was cooled and poured into water. The resulting solid was filtered, washed with water and recrystallised from ethanol. It was purified by silica gel column chromatography (Benzene: MeOH, 8:2) to the products **IV (a-e)**.

General Procedure for synthesis of 3-Aryl-5-[aryl-(4-aryl-thiazol-2-yl-amino)-methyl]-2-thioxo-thiazolidin-4-one (VI a-j)

Equimolar mixture of 2-amino-4-aryl thiazole (100 mmol) and 5-arylidine-3-Phenyl rhodanine (100mmol) were dissolved in ethanol. The mixture was heated under reflux for 6-8 h. After the completion of the reaction the excess of the solvent was evaporated under reduced pressure. The residue obtained was purified by flash chromatography and crystallized with benzene-MeOH (8:2) to give pure compound **VI (a-j)**.

General Procedure for synthesis of 1,4,8-Triaryl-1,4dihydro-bisthiazolo [3,2-a; 5'4'-e] pyrimidin-2-thione (VII a-j)

3-Aryl-5-[aryl-(4-aryl-thiazol-2-yl-amino)-methyl]-2-thioxo thiazolidin-4-one was dissolved in benzene and 10% H_2SO_4 was added to the solution. The mixture was heated for 3-4 h under reflux conditions. The mixture was cooled to room temperature resulting in the precipitation of a pale yellow crude product, which was purified by flash chromatography. The product was

recrystallized with etahnol to give pure compound **VII (a-j)**.

1,4,8-triphenyl-1,4-dihydro-bisthiazolo [3,2-a; 5'4'-e] pyrimidin-2-thione (VII a)
^{1}H NMR 400 MHz (DMSO d6): 6.46-7.30 (15H, m, ArH, aryl thiazole, aryl rhodanine), 5.73 (1H, s, -CH-thiazole), 3.5 (1H, s,-CH-pyrimidine). EIMS (M^+): 455

1,8-diphenyl-4-(4'-methoxyphenyl)-1,4-dihydro-bisthiazolo [3,2-a; 5'4'-e] pyrimidin-2-thione (VII b)
^{1}H NMR 400 MHz (DMSO d6): 6.46-7.30 (14H, m, ArH, aryl thiazole, aryl rhodanine), 5.73 (1H, s,-CH-thiazole), 3.5 (1H, s,-CH-pyrimidine), 3.73 (3H, s, OCH_3-ArH). EIMS (M^+): 485

1,8-diphenyl-4-(4'-chlorophenyl)-1,4-dihydro-bisthiazolo [3,2-a; 5'4'-e] pyrimidin-2-thione (VII c)
^{1}H NMR 400 MHz (DMSO d6): 6.46-7.30 (14H, m, ArH, aryl thiazole, ary rhodanine), 5.73 (1H, s, -CH-thiazole), 3.5 (1H, s, -CH-pyrimidine). EIMS (M^+): 489

1,8-diphenyl-4-(3',4'-dimethoxyphenyl)-1,4-dihydro-bisthiazolo [3,2-a; 5'4'-e] pyrimidin-2-thione (VII d)
^{1}H NMR 400 MHz (DMSO d6): 6.46-7.30 (13H, m, ArH, aryl thiazole, aryl rhodanine), 5.73 (1H, s,-CH-thiazole), 3.5 (1H, s, -CH- pyrimidine), 3.73 (6H, s, OCH_3 -ArH). EIMS (M^+): 515

1,8-diphenyl-4-(4'-nitrophenyl)-1,4-dihydro-bisthiazolo [3,2-a; 5'4'-e] pyrimidin-2-thione (VII e)
^{1}H NMR 400 MHz (DMSO d6): 6.46-8.07 (14H, m, ArH, aryl thiazole, aryl rhodanine), 5.73 (1H, s, -CH-thiazole), 3.5 (1H, s,-CH- pyrimidine). EIMS (M^+): 500

1,4-diphenyl-8-(4''-methoxyphenyl)-1,4-dihydro-bisthiazolo [3,2-a; 5'4'-e] pyrimidin-2-thione (VII f)
^{1}H NMR 400 MHz (DMSO d6): 6.46-7.14 (14H, m, ArH, aryl rhodanine, aryl thiazole), 5.73 (1H, s, -CH-thiazole), 3.5 (1H, s, -CH- pyrimidine), 3.73 (3H, s, OCH_3-thiazole). EIMS (M^+): 485

1,4-diphenyl-8-(4''-chlorophenyl)-1,4-dihydro-bisthiazolo [3,2-a; 5'4'-e] pyrimidin-2-thione (VII g)
^{1}H NMR 400 MHz (DMSO d6): 6.46-7.19 (13H,m, ArH, aryl thiazole, aryl rhodanine), 5.73 (1H,s, -CH-thiazole), 3.5 (1H, s, -CH- pyrimidine), 3.73 (6H, s, OCH_3-ArH and thiazole). EIMS (M^+): 515

1-phenyl-4-(4'-chlorophenyl)-8-(4''-methoxyphenyl)-1,4-dihydro-bisthiazolo [3,2-a; 5'4'-e] pyrimidin-2-thione (VII h)
^{1}H NMR 400 MHz (DMSO d6): 6.46-7.15 (13H, m, ArH, aryl thiazole, aryl rhodanine), 5.73 (1H, s-CH-thiazole, 3.5 (1H,s,-CH- pyrimidine), 3.73 (3H, s,OCH_3-thiazole). EIMS (M^+): 519

1-phenyl-4-(3',4'-dimethoxyphenyl)-8-(4''-methoxyphenyl)-1,4-dihydro-bisthiazolo [3,2-a; 5'4'-e] pyrimidin-2-thione (VII i)
^{1}H NMR 400 MHz (DMSO d6): 6.46-7.19 (12H, m, ArH, aryl thiazole, aryl rhodanine), 5.73 (1H, s,-CH-thiazole), 3.5(1H, s,-CH-pyrimidine), 3.73 (9H, s, OCH_3-thiazole, rhodanine and ArH). EIMS (M^+): 545

1-phenyl-4-(4'-nitrophenyl)-8-(4''-methoxyphenyl)-1,4-dihydro-bisthiazolo [3,2-a; 5'4'-e] pyrimidin-2-thione (VII j)
^{1}H NMR 400 MHz (DMSO d6): 6.46-8.07 (13H, m, ArH, aryl thiazole, aryl rhodanine), 5.73 (1H, s,-CH-thiazole, 3.5 (1H, s,-CH-pyrimidine), 3.73 (3H, s, OCH_3-thiazole). EIMS (M^+): 530

Table 1: Analytical data of the newly synthesized compounds VII (a-j)

Compound No.	Yield %	M.P. °C	Molecular Formula	Found (Calcd.)%		
				C`	H	N
VII a	78	250-252	$C_{25}H_{17}N_3S_3$	65.9 (66.0)	3.7 (3.8)	9.2 (9.3)
VII b	81	212-214	$C_{26}H_{19}N_3OS_3$	64.3 (64.4)	3.9 (4.0)	8.6 (8.8)
VII c	80	205-206	$C_{25}H_{16}ClN_3S_3$	61.2 (61.3)	3.3 (3.4)	8.6 (8.7)
VII d	79	210-212	$C_{27}H_{21}N_3O_2S_3$	62.9 (63.0)	4.1 (4.2)	8.2 (8.3)
VII e	76	198-200	$C_{25}H_{16}N_4O_2S_3$	59.9 (60.0)	3.2 (3.3)	11.2 (11.3)
VII f	74	202-203	$C_{26}H_{19}Cl\ N_3OS_3$	64.3 (64.4)	3.9 (4.0)	8.6 (8.7)
VII g	75	215-216	$C_{27}H_{21}N_3O_2S_3$	62.8 (62.9)	4.1 (4.2)	8.2 (8.3)
VII h	79	180-182	$C_{26}H_{18}Cl\ N_3O_3S_3$	60.0 (60.1)	3.5 (3.7)	8.0 (8.1)
VII i	83	208-210	$C_{28}H_{23}N_3O_3S_3$	61.6 (61.7)	4.2 (4.3)	7.7 (7.8)
VII j	78	185-187	$C_{26}H_{18}N_4O_3S_3$	58.8 (58.9)	3.4 (3.5)	10.6 (10.7)

Table 2: Antifungal activity of the synthesized compounds VII (a-j)

Compound	Average % Inhibition against					
	A. niger			*F. oxysporium*		
	1000 ppm	100 ppm	10 ppm	1000 ppm	100 ppm	10 ppm
VII a	78	60	48	75	42	35
VII b	80	63	52	82	41	49
VII c	81	69	55	78	65	36
VII d	75	56	32	72	52	31
VII e	79	35	26	72	31	24
VII f	74	58	49	70	35	26
VII g	89	81	61	85	75	67
VII h	92	76	65	90	59	42
VII i	76	48	35	73	51	32
VII j	72	42	33	73	65	42

RESULT AND DISCUSSION:

Chemistry:

In order to achieve the basic purpose of our study, the synthetic approach was confined to scheme 1 for obtaining the target condensed pyrimidine derivatives. A reaction of 3-Phenyl rhodanine with various aromatic aldehydes resulted in 5-Arylidine-3-Phenyl rhodanines **IV(a-e),** the prime reactant of the scheme, in good yield. 2-amino-4-arylthiazole **V(a-c)** was electrophilically added to compound **IV(a-e)** to obtain the target condensed pyrimidine-2-thione derivatives **VII(a-j)**. The formula of the compounds **VII (a-j)** were confirmed by the elemental analyses and their structures were determined by IR, ^{1}HNMR and EIMS spectral data.

The IR spectra of all the compounds exhibited characteristic –C=S stretching vibration at 1275–1030 cm^{-1}. The ^{1}HNMR spectra of the compounds showed peaks of aryl protons at 6.46-7.30. A singlet at 5.73 ppm appeared due to the proton of the fused thiazole. The above peaks supported the formation of pyrimidine nucleus. MS of all compounds showed the molecular ion peak (M+) with low intensity and other peaks due to fragments that affirmed the expected structures (Table 1). Experiments were also performed to assess the antifungal activity of the compounds **VII(a-j)** against *Aspergillus niger* and *Fusarium oxysporium* using the disk diffusion method.

Microbiology:

The in vitro antifungal activity of the synthesized compounds was tested against *Aspergillus niger* and *Fusarium oxysporium* using the disk diffusion method where each disc contained either 10, 100 or 1000 ppm of the test compound. Briefly, nutrient agar in water was melted at 100°C and 20 mL of the molten agar was poured into different petri plates, and left on a flat surface to solidify. The diluted culture of each strain under study was pipetted into the agar plates. The surface of the agar plate was allowed to dry. Different concentrations of the test compound impregnated discs were applied to the surface of inoculated plates. The Petri plates were placed in an incubator and incubated at 37°C for 10-24 h. The petri plates were evaluated for the zone of inhibition of the fungal growth around the impregnated discs. All compounds were antifungal since they inhibited the growth of both the fungi viz; *Aspergillus niger* and *Fusarium oxysporium* in the range of 70-92% at 1000 ppm concentration. It was also evident from the results that the compounds with chloro or nitro substituted phenyl nucleus **(VII c, e, g, h & j)** were more detrimental to the growth of the fungi as compared to the other compounds. The antifungal screening data shows that are the screening compounds inhibited 70-92% mycelial growth of both test fungi at 1000ppm concentration but their activity decreased at lower concentration (100, 10ppm) the most active of these, **VII b, VII c, VII g** and **VII h** displayed fungicidal action equivalent to that of commercial fungicide Dithane M-45 at 1000ppm concentration and showed 24-65% growth inhibition even at 10ppm concentration (Table 2).

CONCLUSION:

The object of the present work was to combine the toxophoric qualities of 2-amino-4-aryl thiazole and 5-arylidene-3-aryl rhodanine moieties and synthesize some novelpyrimidin-2-thione derivatives and evaluate them for their potential fungicidal action against *Aspergillus niger* and *Fusarium oxysporium*. The study led to conclusion that the synthesized compounds were at par in antifungal potential with commercial fungicide Dithane M-45. Further studies need to be done in order to establish the statement obtained from the *in vitro* studies against the fungal strains.

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RESEARCH ARTICLE

Solubility Promotion of Telmisartan by solid dispersions using Polymer combinations

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ABSTRACT:

This research aimed to prepare Telmisartan solid dispersions with polymer blend [Equal portion of PVP K-30, Poloxamer-188, and (HPMC) K4M. Various ratios of Telmisartan: Polymer blend in the ratios (1:1, 1:3, 1:5 and 1:7) were fabricated as solid dispersions by melting and solvent evaporation methods, later compressed into tablets. The solid dispersions were tested for physicochemical, and release constraints. The results discovered that the formulations were impressed with the increase in the solubility. Among them formulation with a 1: 5 ratio found to be the best proportion for enhancing the solubility and release rate of Telmisartan from the solid dispersions.

KEYWORDS: Telmisartan, polymer blend, solid dispersions, solubility.

INTRODUCTION:

The oral route is preferred, as they are easy to handle and take by patients of all age groups. Telmisartan (TSN) is an antihypertensive drug[1] belongs to BCS-class II drug and low solubility results in low bioavailability (~45%)[2]. Among the various techniques of solubility enhancing, solid dispersion (SD) technique[3] stands on the top priority as it is a simple, easy and efficient approach.

Water-soluble polymers viz., Poly Vinyl Pyrrolidone (PVP) K-30[4], Poloxamer-188[5] and Hydroxy Propyl Methyl Cellulose (HPMC) K4M[6] were employed with a promising role in increasing the solubility of drugs. In the present examination, the SD were prepared by melting and solvent evaporation techniques.

MATERIALS AND METHODS:

Materials:

Telmisartan was gifted by Cipla Ltd, Bengaluru. PVP K-30, Poloxamer-188, HPMC K4M, Microcrystalline Cellulose, Talc, and Magnesium stearate were procured from SD Fine chemicals India. Double distilled water was used whenever appropriate.

Methods:

Designing of Solid dispersions

The various formulae of TSN-SD were shown in table 1.

Table 1: Various formulae and codes

Drug: Carrier	Ratio	Method of preparation	Formulation code
TSN: PB	1:1	Melting	SD-1
	1:3	Melting	SD-2
	1:5	Melting	SD-3
	1:7	Melting	SD-4
TSN: PB	1:1	Solvent evaporation	SD-5
	1:3	Solvent evaporation	SD-6
	1:5	Solvent evaporation	SD-7
	1:7	Solvent evaporation	SD-8

PB-Polymer blend [Equal portion of PVP K-30+ Poloxamer-188+(HPMC) K4M]

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Preparation of solid dispersions:

Melting:

The polymers were melted based on their decreasing melting points (HPMC-K4M, PVP K-30 then Poloxamer-188) in a china dish, then TSN was dispersed in the molten mass with continual thrilling. The mixture was permitted to solidify at room temperature. The product was stored in a desiccator (ABG Initiatives, Hyderabad, Telangana) for 24 h and then crushed in a mortar (Aruna Scientific, Hyderabad, Telangana). Later the powder was allowed through # 60 sieve (ASTM E 11, Hyderabad, Telangana) to get uniform particle size[7].

Solvent evaporation:

TSN and the polymer mix were taken as per table 1, dissolved in dichloromethane (DCM) and stirred until the DCM evaporated totally. The obtained mass was shifted to Cal. Chloride containing desiccators till it dries. The resulting solid mass was then crushed in a mortar and passed through # 60 sieve and stored in a desiccator till use[8].

Preparation of solid dispersion tablets

The SD equivalent to 40 mg of TSN was made by direct compression into tablet form, after blending[9] with constituents as described in table 2 by using 8 station tablet compression machine (Karnavati, India).

Table 2: Formulation of a tablet containing solid dispersions

Ingredients	Quantity per tablet
Solid dispersions equivalent to 40 mg of Telmisartan	150
Lactose	75
Starch	15
Micro Crystalline Cellulose	50
Magnesium stearate	5
Talc	5
Weight of the tablets	300

Evaluation:

Melting point:

The purity of TSN pure sample was confirmed by measuring melting point. The temperature at which TSN melts was recorded using melting point apparatus[10] (MT-934).

Solubility studies:

Telmisartan (TSN) pure drug was tested for solubility in 0.1N HCl, water, pH 4.5 Acetate buffer, pH 6.8 and pH 7.4 Phosphate buffers[11].

Drug-excipients compatibility studies:

The DSC and FTIR studies were performed to find the interaction among the TSN and carriers used in the study.

Differential Scanning Calorimetry (DSC):

Pure TSN and 1:1 ratio of TSN: Polymer mix were exposed to the analysis. About 10 mg sample was taken in a DSC crucible and scanned at 50-300°C (DSC-50, Shimadzu, Japan).

Fourier-transform infrared (FTIR) spectroscopic study

The interactions between components of the SD were investigated using FTIR spectroscopy. The FTIR spectra of the TSN alone and in combination with carriers were documented by the FTIR spectrometer (Bruker) by scanning at 4000-400 cm^{-1} range.

Evaluation Telmisartan Solid Dispersions

The subsequent results were examined for TSN-SD.

Flow properties:

The TSN-SD were evaluated for flow constraints[12, 13] viz., angle of repose, true and tapped densities, Carr's Index, Hausner's ratio.

Yield:

The % recovery[14] comprises the weight of dried SD to the total weight of TSN and polymers used in making the SD.

$$\% \text{ Yield} = \frac{\text{Actual weight of the SD}}{\text{Total weight of drug and excipients}} \times 100$$

Evaluation Telmisartan Solid Dispersion tablets:

The TSN-SD tablets were exposed to the following assessments.

Uniformity in size and shape.

The tablets were examined under a dissection microscope (DM-100) for their morphology.

Thickness:

The TSN-SD tablets were firmed between the jaws of Vernier Calipers (Qumos Enterprises, India) and thickness is examined in triplicate.

Uniformity in weight:

Every batch of TSN-SD tablets (20 quantity) was individually weighed with an electronic digital balance (Citizen, CY-104, Mumbai, India) and mean was measured and related to the individual tablet weights. The deviation in weights was premeditated and then crisscross with IP specifications (Limit ± 7.5% of mean weight) [16].

Hardness:

TSN-SD tablets were pressed with the spindle of Monsanto tablet hardness tester (Vinsyst Technologies, Mumbai). The force needs to break the tablets were recorded in triplicates[15].

Friability:

Surface erosion may happen while tablet handling can be elucidated by Roche Friabilator. Pre weighed (10 tablets) ($W_{initial}$) and placed and rotated for 4 min at 25 rpm and the final weight of tablets (W_{final}) was dogged. The loss on friability was calculated by the following equation[17].

$$F = \frac{W_{initial} - W_{final}}{W_{initial}} \times 100$$

Calibration curve:

100mg of TSN dissolves in pH 1.2 of 0.1 M HCl. A series of dilutions (2, 4, 6, 8 and 10µg/ml) were prepared scanned spectrophotometrically at 291nm then the measured the absorbance vs. concentrations which gives a calibration curve[18].

Uniformity of drug content:

5 tablets of each batch weighed and powdered. 40mg of TSN dissolved in 100ml of 0.1 N HCl (pH 1.2). From this 0.5ml was diluted to 5ml with pH 1.2 of 0.1 N HCl. The absorbance was estimated at 291nm using a double beam UV-Visible spectrophotometer (Lab India, Mumbai). The content uniformity was calculated from Telmisartan standard calibration graph[19].

***In-vitro* drug release studies:**

The dissolution specifications were as below[20]

- Apparatus used: USP-II dissolution test apparatus
- Dissolution medium: 0.1M HCl
- The volume of dissolution medium: 900ml
- Temperature: 37±0.5°C
- Speed of basket paddle: 50rpm
- Sampling intervals: 5 min
- Sample withdraws: 10ml
- Absorbance measured: 291nm

RESULTS AND DISCUSSION:

The melting point of pure TSN was observed as 263.2 ±2.36ºC, indicates the purity of the TSN sample. The TSN shown good solubility in 0.1N HCl (0.325±0.001µg/ml) compared in contrast to Water, Acetate buffer (pH4.5), Phosphate buffer (pH6.8) and Phosphate buffer (pH7.4).

The DSC thermograms of TSN with polymer mix were lifted to lesser temperature representing good impregnation of TSN with polymers used (figure 1).

The FTIR study discovered that the typical peaks and stretches of TSN pure drug were also found in TSN – polymer blend, indicates no negative incompatibility of TSN with carriers used. The FTIR spectra of TSN pure and polymers were shown in figure 2 and 3.

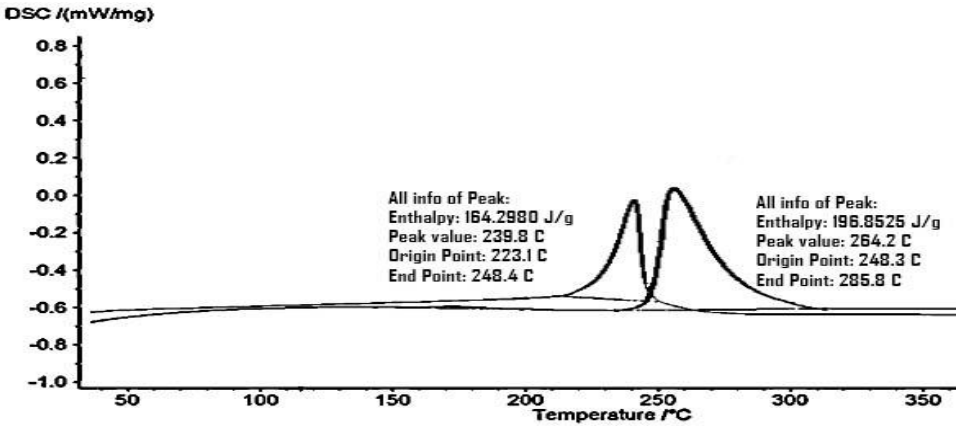

Figure 1: DSC thermograms of Telmisartan pure and with polymer mix

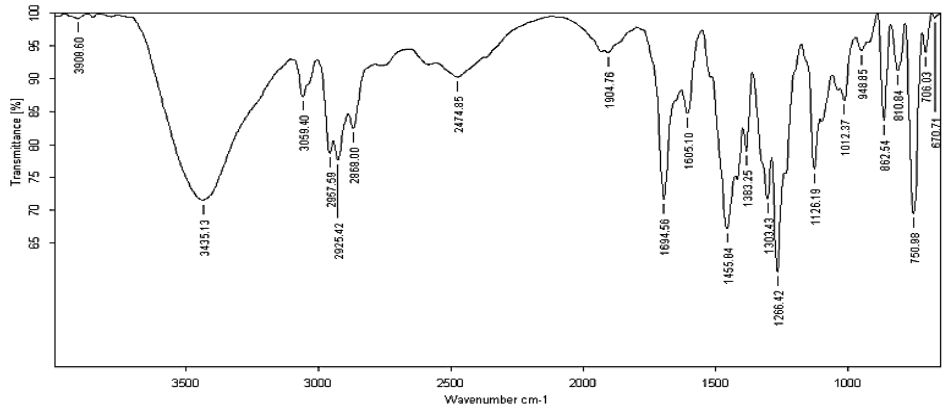

Figure 2: FTIR spectrum of pure Telmisartan

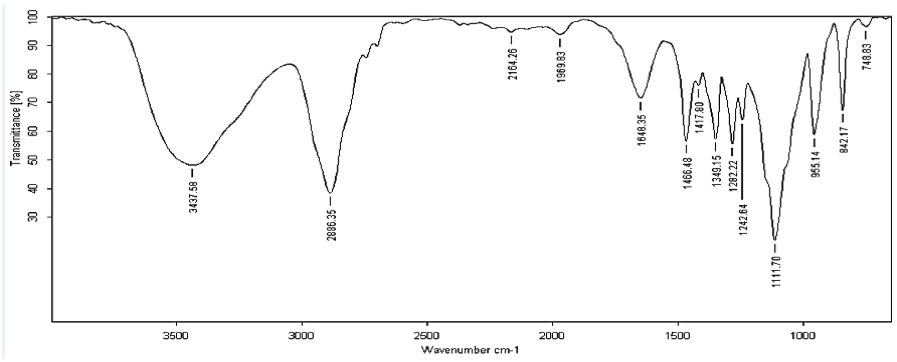

Figure 3: FTIR spectrum of Telmisartan with polymer mix

Table 3: Flow character specifications

Formulation	Flow properties				
	Angle of repose (^{0})	Bulk Density	Tapped Density	Carr's Index	Hausner Ratio
SD-1	29.07±0.17	0.785±0.02	0.801±0.03	1.997±0.06	1.020±0.04
SD-2	28.60±0.08	0.553±0.01	0.562±0.03	1.601±0.02	1.016±0.01
SD-3	27.44±0.05	0.536±0.02	0.543±0.02	1.289±0.04	1.013±0.03
SD-4	28.05±0.03	0.589±0.03	0.599±0.03	1.669±0.07	1.016±0.02
SD-5	25.35±0.02	0.625±0.05	0.636±0.02	1.729±0.08	1.017±0.01
SD-6	29.26±0.11	0.758±0.06	0.771±0.05	1.686±0.05	1.017±0.03
SD-7	28.52±0.09	0.524±0.04	0.532±0.02	1.503±0.09	1.015±0.02
SD-8	26.81±0.12	0.635±0.05	0.644±0.06	1.397±0.02	1.014±0.01

All values mentioned as mean ±SD; The number of trials (n=3)

Table 4: Physical Characteristics of Prepared solid dispersions

Formulation	Physical parameter					
	Yield (%)	Thickness (mm)	Uniformity of weight (mg)	Hardness (cm^2)	Friability (%)	Assay (%)
SD-1	89.2±0.82	5.10±0.02	301.2±2.84	6.8±0.26	0.19±0.01	99.5±1.20
SD-2	95.3±0.51	5.03±0.02	302.5±2.51	6.9±0.05	0.52±0.03	98.6±2.35
SD-3	98.1±0.90	5.10±0.02	299.6±6.32	6.5±0.08	0.41±0.04	99.8±1.45
SD-4	99.0±0.32	5.01±0.03	301.0±2.25	5.9±0.06	0.32±0.02	98.2±1.68
SD-5	97.8±0.69	5.02±0.01	300.1±0.94	5.8±0.08	0.44±0.01	98.8±2.48
SD-6	98.5±1.84	5.11±0.02	301.9±1.25	5.2±0.06	0.32±0.02	97.7±1.25
SD-7	99.1±1.12	5.02±0.02	302.3±2.64	6.6±0.02	0.12±0.01	96.9±0.97
SD-8	98.5±2.23	5.00±0.01	303.2±3.68	7.3±0.05	0.50±0.03	99.5±2.33

Values in mean ±SD; trials (n=3)

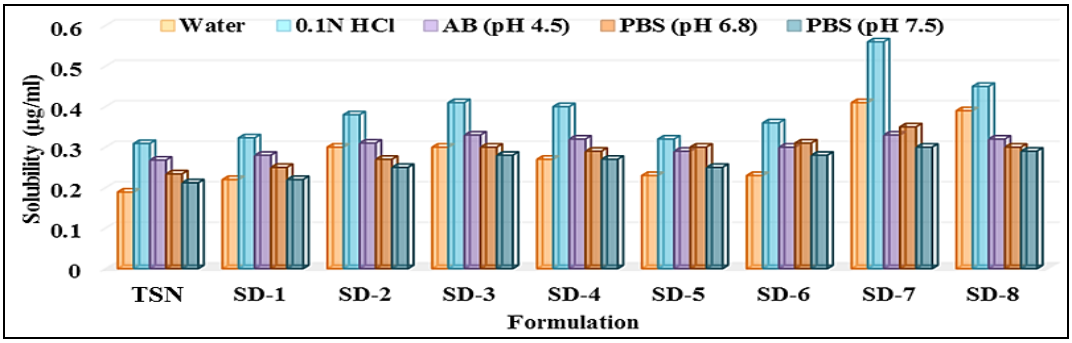

Figure 4: Solubility of Telmisartan and solid dispersions in various media

On the other hand, the TSN-SD represented excellent flow properties as the angle of repose was between (25 to 30^o) i.e., 25.35±0.02 to 29.26±0.11^o. Whereas the compressibility Index was less than 10 and Hausner ratio less than 1.09, representing good compression possessions while tableting. The flow properties of fabricated TSN-SD were summarized in table 3.

The yield of TSN-SD was observed to be good (>90%), The TSN-SD tablets were seeming to have uniform in size, shape, pale white coloured, odorless with a smooth surface.

The tablets were found to have a uniform in thickness (5 mm) and weight. The loss on friability was < 1% and the hardness was >4 Kg/cm^2 (5.8±0.08 to 7.3±0.05) indicating that the tablets bearing considerable mechanical strength and the TSN content was also found to be uniform. All these values were illustrated in table 4.

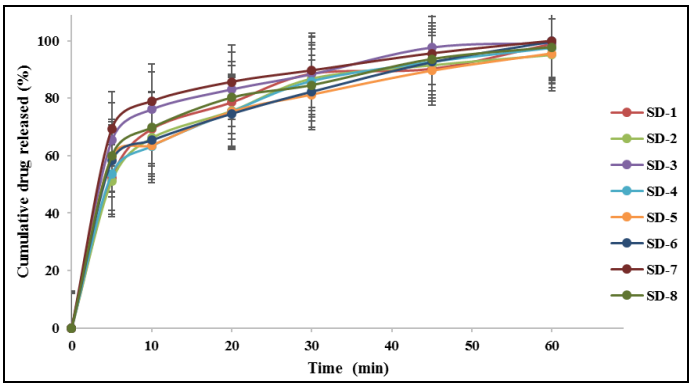

Figure 5: *In vitro* drug dissolution plots of Telmisartan solid dispersions

The solubility of TSN was found to be good in 0.1N HCl and decrease with an increase in pH of media. Among the tablets, SD-7 showed good solubility in 0.1 N HCl. The detailed description of solubility was shown in figure 4.

TSN followed Beer's Lambert's law at 2 to 10 µg/ml. The regression (R^2 value was observed to be 0.9998 with the slope of 0.0906x+0.0572. The assessment of Telmisartan was determined with this calibration curve.

TSN released from the tablet was initial burst within 10 min and the end of 1h the TSN was completely released. The dissolution of prepared tablets was found good in formulations containing TSN: polymer mix at the ratio of 1:5 (figure 5).

CONCLUSION:

In the examination HPMC-K4M, PVP K-30 and Poloxamer-188 combination was a good grouping as a carrier for making solid dispersions by taking Telmisartan as a model drug. The formulation SD-7 in the ratios 1: 5 prepared by solvent evaporation was observed to have good solubility and drug dissolution constrains.

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CONFLICT OF INTEREST:

No conflict of interest was declared by the authors.

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RESEARCH ARTICLE

Formulation and Evaluation of Solid Lipid Nanoparticle of Felbamate for improved Drug Delivery

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ABSTRACT:
Solid lipid nanoparticles are typically spherical in shape with average size of 1-1000nm in diameter. These are the alternatives to traditional colloidal carrier systems such as emulsions, liposomes, microspheres etc. Felbamate is a PEGylated phenylcarbamate derivative that acts as an antagonist of NMDA receptors. It is used as an anticonvulsant, primarily for the treatment of seizures in severe refractory epilepsy. It is slightly soluble in water with t1/2 of 4-6 hours. Solid lipid nanoparticles of felbamate are prepared by using lipids (glyceryl monostearate and glyceryl monooleate) with tween 80 as stabilizer. The prepared nanoparticle formulations were evaluated for their entrapment efficiency, assay, in-vitro drug release, particle size analysis, and stability. A formulation containing glyceryl monooleate, stabilized with tween 80 as surfactant showed prolonged drug release, smaller particle size, and narrow particle size distribution, as compared to other formulations.

KEYWORDS: Colloidal carriers, solid lipid nanoparticles, lipid matrix, surfactants, entrapment efficiency.

INTRODUCTION:
Therapeutic efficacy of any drug depends upon the four fundamental pathways of drug in the body such as absorption, distribution, metabolism and excretion. Failure in any of these processes leads to failure in therapeutic efficacy. A promising strategy to overcome any problems encountered in these by the development of a suitable drug colloidal carrier system. Among the colloidal carrier systems solid lipid nanoparticles have many advantages and limited disadvantages as compared to conventional colloidal carrier systems. Solid lipid nanoparticles (SLN) have gained attention as carriers for the preparation of a wide variety of poorly water-soluble drugs due to their biodegradable and biocompatible properties and low toxicity[1].

The use of SLNs is a striking improvement because the solid matrix of the lipids presents high flexibility in controlling the drug release and protects the encapsulated drugs from gastric degradation.

SLNs are generally composed of biodegradable and biocompatible solid lipid as solid core, coated by nonhazardous surfactant/co-surfactant as the outer shell. Use of solid lipids increases drug absorption mainly through enhanced drug dissolution and solubilization in the intestinal-milieu, improved lymphatic-transport, enhanced gastrointestinal permeability, and decreased gastric-emptying rate. Particle size and PDI are key characteristics and are critical parameters in the stability and fabrication of SLNs. These characteristics mainly depend upon particles composition and different fabrication techniques[2-4].

The main objective of current research work is to prepare felbamte loaded solid lipid nanoparticles, using glyceryl monostearate and glyceryl monooleate as the lipid matrices and tween 80 as stabilizer with a view to improve the dissolution rate of felbamate which would increase the biological activities. Given that solid lipid nanoparticles, as an alternative colloidal carrier (transport) system, have the ability to improve the solubility/permeability of lipophilic drugs, they may also enhance the drug absorption.

MATERIALS:
Felbamate was received as gift sample from Aurobindo Pharma, Hyderabad. Glyceryl monostearate [GMS] (CDH Pvt. Ltd., Mumbai), Glyceryl monooleate [GMO]

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(Otto Chemicals, Mumbai), Polysorbate 80 (Sisco Research Laboratories, Chennai), Chloroform and Methanol (Rankem, Chennai), Dialysis Membrane 50 – LA 387 (Himedia, Mumbai) were purchased from the local market. All the reagents used were of analytical grade.

METHODS:

Preparation of Felbamate loaded solid lipid nanoparticles:

Felbamate (API) loaded solid lipid nanoparticles were fabricated by hot homogenization followed by ultrasonication method [5-6]. API and monoglyceride were dissolved in a mixture of methanol and chloroform (2:1). Organic solvent mixture was then slowly removed by evaporation in rotary flash evaporator. Resulting embedded lipid layer was then molten by heating to 5°C above the melting point of the lipid. In another vessel, an aqueous phase was prepared by mixing the tween 80 (Stabilizer) with distilled water (sufficient to produce 30 ml) and then heated to same temperature as oil phase. Then this hot aqueous phase was added to the oil phase and homogenization was performed (at 2500 rpm and 70°C) using a mechanical stirrer for 30 minutes. The coarse oil in water emulsion so obtained was sonicated using probe soincator for 25 minutes. Felbamate loaded SLN was finally obtained by allowing the hot nanoemulsion to cool to room temperature, and was stored at 4°C in the refrigerator. The composition of the different formulation has been given in Table 1. In all SLN formulations the lipid concentration was kept constant (as 6% w/v)

Table 1: Composition of felbamate loaded solid lipid nanoparticles

Name of the Ingredient	F1	F2	F3	F4	F5	F6
Glyceryl monostearate	6% w/v	6% w/v	6% w/v	NA	NA	NA
Glyceryl Monooleate	NA	NA	NA	6% w/v	6% w/v	6% w/v
Polysorbate 80	1% w/v	1.5% w/v	2% w/v	1% w/v	1.5% w/v	2% w/v

Characterisation of Solid Lipid Nanoparticles of Felbamate:

Particle size, poly dispersity index and zeta potential:

Prepared solid lipid nano particles were maintained at room temperature for 30 days, which were characterized for particle size, poly dispersity index and zeta potential. About 1ml of prepared solid lipid nano particles were diluted appropriately using distilled water, which was then taken individually in a zeta cell and measured the average particle size, poly dispersity index and zeta potential using Zetasizer. The experiments were performed in triplicate.

Drug content, Encapsulation efficiency and Drug loading estimation:

Drug content of prepared solid lipid nano particles was evaluated by performing assay using the established HPLC methods for Felbamate.

HPLC Method to estimate samples of Felbamate:

The chromatographic separation was performed with Shimadzu HPLC system with the best chromatographic conditions equipped with C18 column (ODS 250 mm X 4.6 mm with 5 micron pore size, Phenomenax) using a mobile phase combination of potassium dihydrogen phosphate buffer (50mM, pH4.5), acetonitrile and methanol (65:26.2:8.8, v/v/v) in an isocratic mode elution with the flow rate was set at 0.8 mL/min. The samples were analyzed by PDA detector.

Estimation of encapsulation efficiency and drug loading:

Prepared solid lipid nano formulations were centrifuged using a refrigerated centrifuge (Remi) for 45 minutes at 19,000 rpm at -20°C and supernatant was separated and stored individually for further analysis. About 1 ml of supernatant was mixed with 1 ml of methanol, which was vortexed for 5 minutes and filtered through 0.22 µm membrane. Estimated amount of free drugs were expressed as W_{free}. The experiments were performed in triplicate.

Encapsulation efficiency (EE) and drug loading (DL) were estimated as follows

$$EE\ (\%) = \frac{\text{Drug Content } (W_{total})] - [\text{Drug in the supernatant } (W_{free})}{\text{Drug Content } (W_{total})} \times 100$$

$$D(\%) = \frac{\text{Drug Content } (W_{total}) - \text{Drug in the supernatant } (W_{free})}{\text{Weight of the polymer used in the formulation } (W_{polymer})} \times 100$$

In-vitro drug release study:

In-vitro release study of Felbamate loaded nano scale solid lipid particles was determined by using dialysis bag method. Accurately weighed quantities of Nano particle were placed in the dialysis bag (cut-off 5 kDa, Himedia, India) and both ends were tightly sealed. The dialysis bag was positioned in a Beaker containing 100 ml phosphate buffer at pH 7.4, which was magnetically stirred at 100rpm (Remi, India) and maintained at 37°C ±0.5°C. At schedule time intervals, the 1ml of the release medium was withdrawn using micropipette and replaced with the same volume of fresh PBS. The samples were immediately filtered through a 0.45µm membrane filter (Elix, Mill-Q) and the content of Felbamate was estimated after suitable dilution with a Thermo scientific HPLC (Spectra system P-4000, USA) with UV detector (Kromosil 100) and C18 column (Particle size 5µm, 250mm×4mm) at 265nm.

Stability studies:

The stability study provides the evidence of fabricated nano scale solid lipid particles quality varies with time under different environmental factors includes temperature, humidity and light. The study was carried out according to the international conference on harmonisation (ICH-QIA (R2) guidelines, 2003), with Felbamate loaded nano scale solid lipid particles. The samples were studies were performed over a period of 6 months at 40°C±2°C/75% RH±5% RH (Accelerated condition). The samples were evaluated at 0, 3 and 6 months for their particle size, poly dispersity index, zeta potential and drug content. In addition, samples were visually examined for any physical instability (separation and aggregation).

RESULTS AND DISCUSSION:

Development of solid lipid Nano particulate drug delivery system:

Felbamate solid lipid nanoparticles were prepared using hot homogenization followed by ultrasonication method. Prepared nanoparticles were characterized for distribution width, mean particle size, surface area, span, and uniformity using laser particle size analyser. However, these characterization parameters depends on process parameters such as organic solvent, lipid concentration, surfactant percentage, percentage of organic solvent, volume of aqueous phase, temperature generated during sonication process, sonication duration and drug concentration. Hence, a step-by-step optimization was carried out to evaluate the effect of these process parameters on prepared solid lipid nanoparticles (F1 to F6). The experiments were performed in triplicate and characterization results were expressed as mean ± standard deviation.

Table 2: Characterization of prepared SLN's

Run	Average Particles size (nm)	Span	Surface Area (m^2g^{-1})	Polydispersity index
F1	354	1.825	15.4	0.456
F2	225	1.725	22.6	0.254
F3	124	0.224	55.4	0.124
F4	364	1.756	16.5	0.546
F5	252	1.259	26.2	0.325
F6	134	0.245	52.4	0.156

Drug Content, Encapsulation Efficiency and Drug Loading Estimation:

The amount of Felbamate encapsulated in nanoparticles determines the effectiveness of prepared nano-formulations. Hence, drug content, encapsulation efficiency and drug loading estimation were performed as per procedure mentioned. Drug content was estimated by performing an assay whereas encapsulation efficiency and drug loading were calculated by measuring the free Felbamate in the nano-formulation. The results were summarised in table 3.

Table 3: Drug Content, Encapsulation Efficiency and Drug Loading Estimation of Optimized Formulation (F3)

Evaluation parameter	Nanoformulation
Process yield (%)	92.54
Mean particle size (nm)	114-134
Polydispersity Index (Pdi)	0.124
Zeta potential (mV)	- 20.2 ± 3.16
Drug loading (%)	94.93%
Encapsulation efficiency (%)	95.93 ± 0.72
Drug content (%)	99.86

Drug content in formulations was in the range of 99 to 100, which shows that there was no post-formulation degradation or drug loss.

In stirring approach, encapsulation efficiency and drug loading was found to be 94.93%. Hence, prepared Felbamate solid lipid nanoparticles is expected to display superior pharmacological activities.

***In vitro* drug release study:**

In vitro drug release from the drug loaded nanoparticles was assessed in simulated gastrointestinal conditions. The pH condition used was pH 1.2 for a period of 2 hrs (Stomach), pH 4.5 for 2 hrs (Duodenum) followed by pH 7.4 (Distal ileum and colon) for the remaining period of the study using a USP dissolution test apparatus type 2 (Chandran *et al.*, 2009) and *in vitro* drug release of optimum formulation is shown in table 4. The drug release was found to be less than 5% up to 4 hrs and the drug release increased when the pH of the medium was adjusted to 7.4.

Table4: *In vitro* release profile of Felbamate solid lipid nanoparticles

Time	Drug release
0 hrs	0.00 ± 0.00
1 hrs	0.00 ± 0.00
2 hrs	0.00 ± 0.00
4 hrs	1.85 ± 1.42
6 hrs	28.32 ± 1.22
8 hrs	62.54± 1.19
10 hrs	72.25 ± 0.26
12 hrs	86.34 ± 3.14
24 hrs	98.72 ± 1.71

Release kinetics:

The results of *in vitro* release profile obtained from the optimized formulation (F3) were plotted to know the mechanism of drug release. The data were treated according to Zero Order Release, First Order Release, Higuchi Model, Korsmeyer-Peppas Model and Hixson Crowell Cube Root Law. The release rate kinetics data of the formulation are as Zero order

r^2 = 0.78, First Order r^2 = 0.67, Higuchi model r^2 = 0.52, Korsmeyer-Peppas Model r^2 = 0.77, Korsmeyer-Peppas Model n = 0.92, Hixson Crowell Cube Root Law r^2 = 0.95. Where r^2 is determination coefficients and "n" is release exponent of kinetic data analysis of Felbamate release from nanoparticles.

Table 5: Average particle size, poly dispesity index, zeta potential and drug content estimation of prepared polymeric nanoparticles subjected to accelerated stability study as per ICH guidelines (40°C ± 2°C/75% RH ± 5% RH)

Trials	Period (Months)	Average particle size (nm)	Poly dispersity index (PDI)	Zeta Potential (mV)	Drug Content (%)
Nano	0	124 ± 0.45	0.124 ± 0.04	- 20.2 ±3.16	99.86 ± 1.67
	3rd	115 ± 0.35	0.289 ± 0.41	- 29.1 ± 1.20	97.35 ± 1.42
	6th	116 ± 0.64	0.399 ± 0.24	- 25.2 ± 0.98	96.80 ± 1.26

The values are expressed as Mean ± SD; n=3

It is concluded that the Felbamate solid lipid nanoparticles prepared by hot homogenization followed by Sonication approach gave a good fit to the Hixson Crowell cube root law. The diffusion exponent (n) values were greater than 0.89, this result indicated that the release of drug from the polymer matrix formulations was found to be super case-II transport, i.e., drug release by both diffusion and relaxation of polymer chain

Stability studies:

Prepared optimized Felbamate loaded polymeric nano-formulation was subjected to stability studies as per the procedure mentioned. At the regular intervals the stored samples were evaluated for average particle size, particle size uniformity, surface area, zeta potential and drug content The results are summarized in table 5.

Prepared Felbamate loaded nanoparticles showed insignificant change in average particle size, polydispersity index, zeta potential and drug content after stability storage at accelerated conditions.

CONCLUSION:

Solid lipid nano particles were prepared using hot homogenization and followed by Sonication method. It can be concluded that the formulations prepared by using glyceryl monostearate as lipid at 6%w/v and tween 80 as surfactant at 2.0% w/v has shown good stability with less particle size and more entrapment efficiency.

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RESEARCH ARTICLE

A new stability indicating RP-HPLC method for the estimation of Flucytosine in presence of an internal standard

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ABSTRACT:

Flucytosine is fungicidal acting against Candida species but not against other common fungi. Flucytosine is structurally related to the cytostatic fluorouracil and to floxuridine. Flucytosine is chemically 6-amino-5-fluoro-1H-pyrimidin-2-one with molecular formula C4H4FN3O and molecular weight 129.09 g/mol. A new RP-HPLC method has been proposed for the quantification of Flucytosine in presence of internal standard Linezolid in pharmaceutical formulations and the method was validated as per ICH guidelines. Mobile phase consisting of a mixture of Tetra butyl ammonium hydrogen sulphate: Methanol (50:50, v/v) with a flow rate 1 mL/min and UV detection at 254 nm was used for the assay of Flucytosine in presence of internal standard Linezolid. Flucytosine was exposed to different stress conditions such as acidic, alkaline, oxidation and thermal degradation. Linearity was observed over the concentration range 1.0–100 µg/mL with linear regression equation y = 0.0238x - 0.0024 (R^2 = 0.9998). The LOQ was found to be 0.8937 µg/mL and the LOD was found to be 0.2938 µg/mL. The present method can be successfully applied for the pharmaceutical formulations, kinetics study and bioanalytical studies.

KEYWORDS: Flucytosine, Linezolid, RP-HPLC, Stability indicating, Validation, ICH guidelines.

INTRODUCTION:

Flucytosine (Figure 1A) is the only available nucleoside analog, acts as an antifungal by disrupting pyrimidine metabolism in the fungal cell nucleus. Flucytosine (FLU) is a small, very water-soluble molecule and therefore it is rapidly and nearly completely absorbed from the intestine after oral administration. Flucytosine can cause bone marrow suppression and GI toxicity. Flucytosine does not have any significant drug interactions. Flucytosine acts mainly against Candida and Cryptococcus and also against some of the species Cladosporium and Phialophora[1-3]. Various analytical techniques have been developed till now for the determination of Flucytosine which include fluorimetry[4], microbiological assay[5], UFLC[6], process related impurities[7] RP-HPLC[8], liquid chromatography in biological fluids[9-11] and in injectable formulations. In the present study the authors have proposed a new stability indicating RP-HPLC method for the determination of Flucytosine in presence of internal standard (IS) Linezolid and the optimized method was validated[12]. Linezolid (Figure 1B) belongs to oxazolidinone class and it is a synthetic antibiotic used for the treatment of multi-resistant bacterial infections.

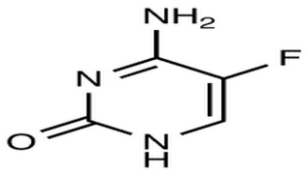

Figure 1A: Structure of Flucytosine (FLU)

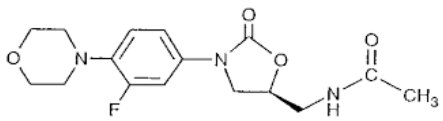

Figure 1B: Structure of Linezolid (LIN)

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MATERIALS AND METHODS:

Chromatographic conditions:

Shimadzu Model HPLC system (Shimadzu Co., Kyoto, Japan) equipped with PDA detector and C8 Luna column (250 mm × 4.60 mm i. d. 5µm particle size) was used for the chromatographic study. The system was maintained at 23ºC. Mobile phase consisting of Tetra butyl ammonium hydrogen sulphate: Methanol (50:50, v/v) with a flow rate 1 mL/min (UV detection at 254 nm) was used for the determination of Flucytosine for the present study. The mobile phase was sonicated and filtered through 0.45µm membrane filter prior to use.

Materials and reagents:

Flucytosine was obtained from Zydus Cadila Healthcare Ltd. as gift sample. Flucytosine is available as tablets and capsules. Flucytosine is available with brand names BD-Cytosin-500, FC fungTM 500 (Glenmark) and CytoFlu (Jolly Healthcare) as tablets and capsules (Labelled claim: 500 mg).

Preparation of Flucytosine and Linezolid drug solutions

25 mg of each Flucytosine and Linezolid were accurately weighed and transferred in to two different clean 25 mL volumetric flasks and made up to volume with HPLC grade methanol (MERCK) (1000 µg/mL). Dilutions of Flucytosine were made on daily basis with mobile phase consisting of Linezolid (10 µg/mL) sonicated and filtered through 0.45µm membrane filter prior to injection.

Method validation

Linearity

Dilute solutions (1.0-100 µg/mL) of Flucytosine containing 10 µg/mL Linezolid were prepared from their stock solutions with mobile phase and 20µL of each were injected in to the HPLC system. The mean peak area ratio of FLU to that of Linezolid was calculated from the chromatograms obtained and a calibration curve was drawn by taking the concentration of FLU drug solution on the x-axis and the corresponding mean peak area ratio values on the y-axis.

Precision, Accuracy and Robustness

Intraday and inter-day precision were studied, three different concentrations of Flucytosine were chosen to study the Intraday (same day) and Inter-day precision (three consecutive days) respectively and the % RSD was calculated. The accuracy study was evaluated in triplicate at three concentration levels (50, 100 and 150 %), and the percentage recoveries were calculated. Standard addition and recovery experiments were conducted to determine the accuracy of the method for the quantification of the drug product and the percentage recovery was calculated. The robustness of the method was assessed by exposing the drug solution to different analytical conditions purposely changing from the original optimized conditions. The effects so obtained were summarized to calculate the % RSD and has to be less than 2.0% specifying that the proposed method was robust.

Stress degradation studies[13]

Flucytosine was exposed to stress conditions such as acidic, basic, oxidation, thermal treatment. Acidic degradation was performed by treating the drug solution with 1mL of 0.1N HCl, heated at 75ºC for about one hour on a water bath. The stressed sample is then cooled neutralized with 1mL 0.1N sodium hydroxide solution and the solution was made up to volume to the required concentration with the mobile phase only after the addition of internal standard just before injection into the system. 20µl of the solution was injected in to the HPLC system.

Alkaline degradation, was performed by treating the drug solution with 1mL 0.1 N NaOH heated at 75ºC for about one hour on a water bath. The solution is then cooled and neutralized with 1mL 0.1N hydrochloric acid and diluted with the mobile phase only after the addition of internal standard just before injection into the system. 20 µl of the solution was injected in to the HPLC system.

Oxidation degradation was performed by treating the drug solution with 1ml of 30% v/v H_2O_2 heated at 75 ºC for about one hour on a water bath. The solution is then cooled and diluted with mobile phase only after the addition of internal standard just before injection into the system. 20µl of the solution was injected in to the HPLC system.

Thermal degradation was performed by heating the drug solution at 75ºC for about one hour on a water bath. The solution is then cooled and diluted with mobile phase only after the addition of internal standard just before injection into the system. 20µl of the solution was injected in to the HPLC system.

Assay of Flucytosine tablets

Twenty tablets containing each 500 mg were purchased from the local pharmacy store and the contents were powdered and powder equivalent to 50 mg Flucytosine was extracted using methanol and then with the mobile phase in a 50 ml volumetric flask. The solution was sonicated for half an hour and filtered through 0.45 mm membrane filter and 20 µL of this solution was injected in to the HPLC system only after the addition of internal standard. The peak areas observed by Flucytosine and Linezolid were noted at their retention time from the resultant chromatogram and the mean peak area ratio was calculated (n=3). The values were substituted in the

linear regression equation obtained earlier and the amount of Flucytosine present in the tablets were calculated.

RESULTS AND DISCUSSION:

Flucytosine was estimated using a new stability indicating reverse phase liquid chromatographic method using an internal standard, Linezolid and the method was validated. During the optimization of Flucytosine, trials were made simultaneously for Linezolid also and then a simple method was established where both the drugs were eluted with resolution greater than 2.0. Mobile phase consisting of a mixture of Tetra butyl ammonium hydrogen sulphate: Methanol (50: 50) (pH 3.5) (50:50, v/v) with a flow rate 1 mL/min and UV detection at 254 nm was used for the assay of Flucytosine using C8 Luna column (250 mm × 4.60 mm i.d. 5µm particle size). A symmetrical and sharp peak for Flucytosine was observed at 2.857 ± 0.02 min and that of the internal standard, Linezolid at 7.0 ± 0.1 min within a run time of 10 min. The proposed method was compared with the previously reported liquid chromatographic methods (Table 1). The chromatograms obtained for the mobile phase, Flucytosine (API), Flucytosine (API) in presence of internal standard Linezolid and that of the tablet formulation in presence of IS were shown in Figure 2.

Table 1: Literature survey

Method/Mobile phase (v/v)	Detection wavelength (nm)	Comment	Reference
Tetra butyl ammonium hydrogen sulphate: Acetonitrile (50: 50)	215	RP-UFLC Stability indicating	6
Water: Methanol (95:5)	260	HPLC Process related impurities	7
(Sodium dihydrogen phosphate : 1-octane Sulfonic acid) buffer: Methanol: Acetonitrile (50:20:30)	264	RP-HPLC	8
Tetra butyl ammonium hydrogen sulphate: Methanol (50: 50) (pH 3.5)	254	RP-HPLC Stability indicating Internal standard (Linezolid)	Present method

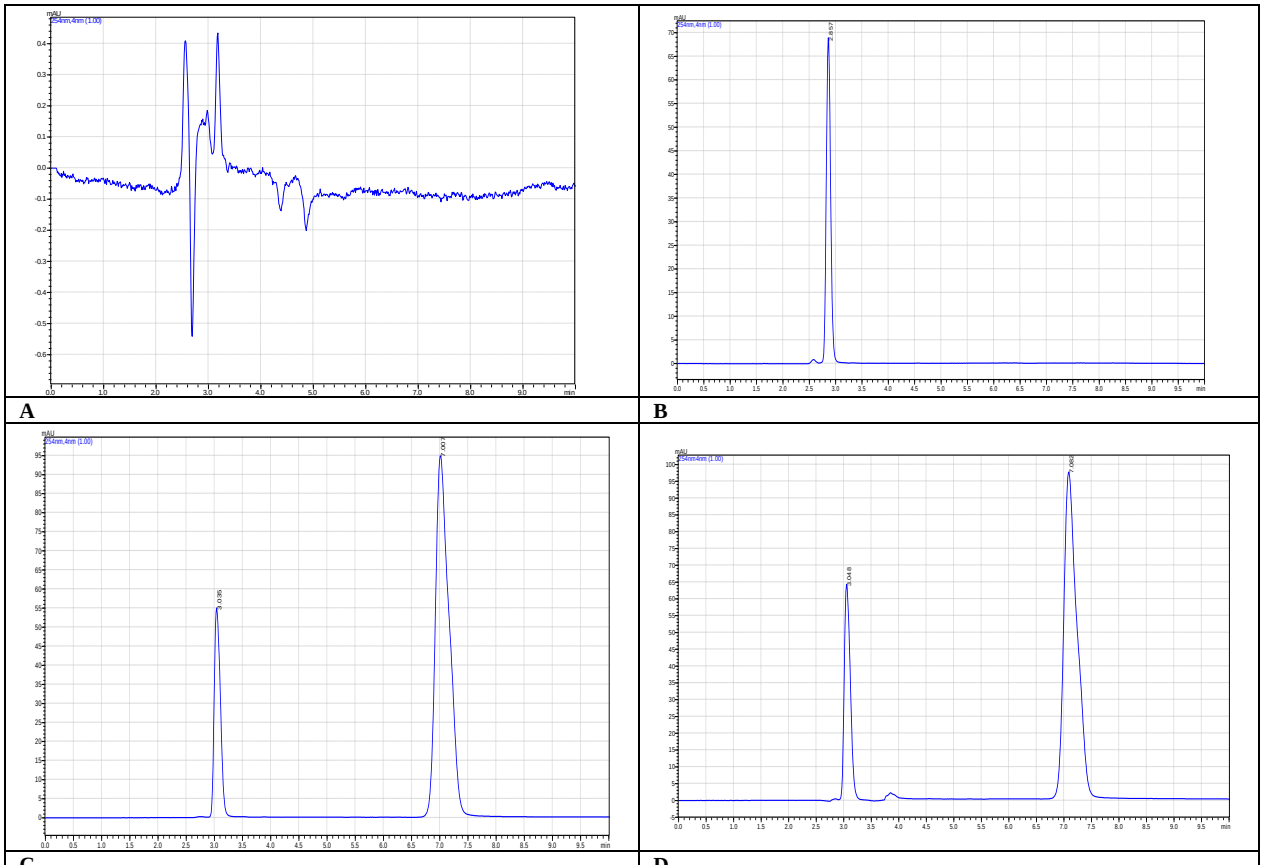


Figure 2: Representative chromatograms of A) Mobile phase B) Flucytosine standard (API) (10 µg/mL) C) Flucytosine standard (10 µg/mL) in presence of Linezolid (IS) (10 µg/mL) D) Flucytosine tablet (10 µg/mL) in presence of internal standard (10 µg/mL)

Method validation

Linearity

Flucytosine obeys Beer-Lambert's law and follows linearity over the concentration range 1.0–100 µg/mL (Table 3) (Figure-2) (% RSD 0.16-1.45) and the linear regression equation was found to be y = 0.0238x - 0.0024 (r2 = 0.9998) (Figure 2). The LOD was found to be 0.2523 µg/mL and the LOQ was found to be 0.8291 µg/mL.

Table 3: Linearity

Conc. (µg/mL)		*Mean peak area		Peak area ratio (FLU/LIN)	% RSD
FLU	LIN	FLU	LIN		
1	10	39376	1665812	0.0236	0.85
5	10	196881	1672351	0.1177	1.23
10	10	393763	1678276	0.2346	1.45
20	10	787526	1663421	0.4734	0.66
30	10	1181289	1678325	0.7038	0.51
40	10	1575052	1651248	0.9538	0.77
60	10	2362578	1679235	1.4069	0.16
80	10	3150104	1658130	1.8997	0.73
100	10	3937630	1663815	2.3666	0.94

*mean of three replicates

Precision, Accuracy and Robustness

Intraday and inter-day precision were studied at three different concentration levels of Flucytosine on the same day and on three consecutive days respectively and the % RSD was found to be 0.14-0.29 (Intraday) (Table 4) and 0.24-0.97 (Inter day) (Table 5) respectively (<2.0) demonstrating that the method is precise. The accuracy of the method was proved by the standard addition method and the percentage RSD was <2.0 (Table 6). The robustness of the assay method was established by introducing small changes in the chromatographic conditions which include detection wavelength (252 and 256 nm), percentage of organic phase i.e. methanol in the mobile phase (48 and 52%) and flow rate (± 0.1 ml/min). Robustness of the method was studied using 10 µg/mL of Flucytosine (Table 7) and the % RSD was found to be 0.11-1.85 (<2.0).

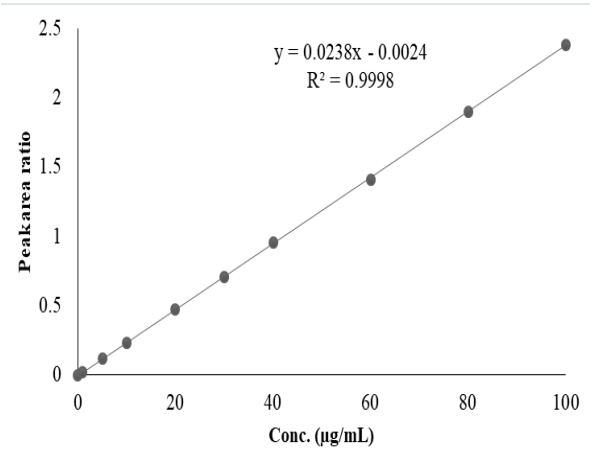


Figure 2: Calibration of Flucytosine in presence of Linezolid (IS)

Table 4: Intraday precision study of Flucytosine in presence of Linezolid (IS)

Conc.(µg/mL)	*Mean peak area			Statistical Analysis*Mean ± SD (% RSD)
	FLU	LIN	FLU/LIN	
10	393763	1679786	0.2344	0.2336 ± 0.000653 (0.27)
10	392817	1687312	0.2328	
10	393819	1663589	0.2367	
20	787526	1663421	0.4734	0.4717 ± 0.001388 (0.29)
20	788975	1678315	0.4700	
20	786215	1661321	0.4732	
30	1181289	1678325	0.7038	0.7050 ± 0.001021 (0.14)
30	1193215	1689312	0.7063	
30	1178516	1678171	0.7022	

*mean of three replicates

Table 5: Interday precision study of Flucytosine in presence of Linezolid (IS)

Conc. (µg/mL)	*Mean peak area			Statistical Analysis *Mean ± SD (% RSD)
	FLU	LIN	FLU/LIN	
10	393714	1697321	0.2319	0.2347 ±0.002286 (0.97)
10	401290	1689375	0.2375	
10	389789	1678614	0.2322	
20	789527	1687854	0.4677	0.4691± 0.001143 (0.24)
20	788346	1675431	0.4705	
20	787978	1698121	0.4640	
30	1198975	1691732	0.7087	0.7062 ±0.002 (0.28)
30	1189354	1689781	0.7038	
30	1178631	1673879	0.7041	

*mean of three replicates

Table 6: Accuracy study of Flucytosine

Spiked Conc.((µg/mL)	Formulation (µg/mL)	Total Conc. (µg/mL)	*Conc. found (µg/mL) ± SD (%RSD)	% Recovery
5 (50%)	10 10 10	15 15 15	14.93 ± 0.0367 (0.24)	99.32 99.88 98.80
10 (100%)	10 10 10	20 20 20	19.95 ± 0.0489 (0.24)	99.51 100.1 98.87
15 (150%)	10 10 10	25 25 25	25.05 ± 0.0622 (0.24)	100.5 99.49 100.8

*mean of three replicates

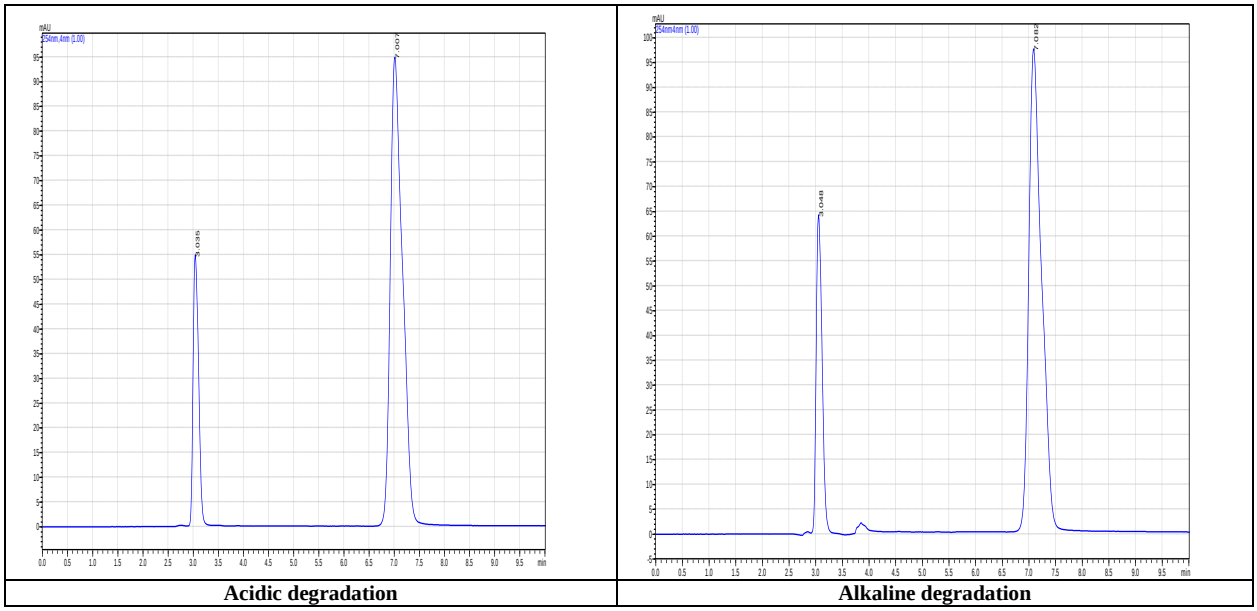
Table 7: Robustness study of Flucytosine in presence of Linezolid (IS)

Parameter	Condition	*Mean peak area		*Mean peak area ratio (FLU / LIN)	*Mean peak area ratio ± SD (% RSD)
		FLU	LIN		
Flow rate (± 0.1 ml/min)	0.9	412109	1685249	0.2445	0.2440 ± 0.0045 (1.85)
	1.0	413763	1679985	0.2463	
	1.1	401539	1664596	0.2412	
Detection wavelength (± 2 nm)	252	404984	1675341	0.2417	0.2382 ± 0.0022 (0.92)
	254	393763	1675229	0.2351	
	256	398685	1675297	0.2379	
Mobile phase composition (Aq : Org) (± 2 % v/v)	52:48	394872	1675309	0.2357	0.2359 ± 0.0002 (0.11)
	50:50	393763	1671364	0.2356	
	48:52	395961	1675317	0.2364	

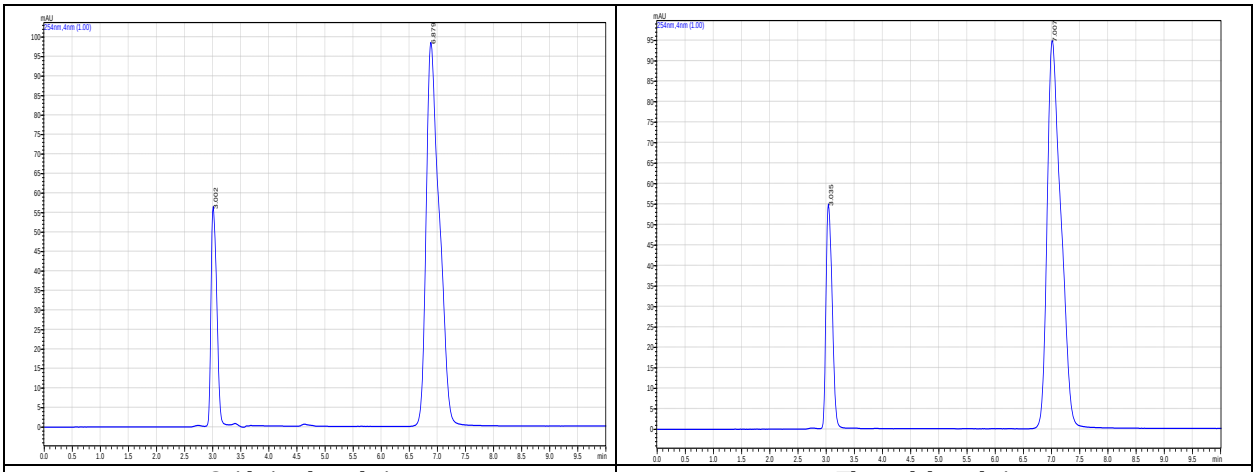
*Mean of three replicates

Stress degradation studies

The Flucytosine API was eluted as a sharp peak at 2.857 ± 0.2 min. During the acidic degradation the drug was eluted at 3.059 min and in alkaline degradation Flucytosine was eluted at 3.015 min. During the oxidation along with the drug peak (3.009 min) one more degradant was eluted at 4.196 min and in thermal degradation the drug was eluted at 2.991 min. Flucytosine was totally very much resistant towards all degradation conditions and the degradents were well separated. In all the degradation studies less than 3% degradation was observed and therefore it is confirmed that the method is selective and specific. The system suitability parameters has shown that the tailing factor was (1.5-2.0) and the theoretical plates were more than 2000. (Table 8). The individual chromatograms obtained during the forced degradation studies were shown in Figure 3.



Acidic degradation | **Alkaline degradation**



Oxidative degradation | Thermal degradation

Figure 3: Typical chromatograms of Flucytosine (10 µg/mL) (Rt 3.0 ± 0.1 min) during stress degradation studies in presence of Linezolid (IS) (10 µg/mL)

Table 8: Stress degradation studies of Flucytosine in presence of Linezolid (IS)

Stress condition Medium/ Temp. / Time	% Recovery	%Drug degradation	Theoretical plates		Tailing factor	
			FLU	LIN	FLU	LIN
Standard drug	100	------	4321	5064	1.197	1.114
Acidic degradation 0.1N HCl/ 75°C/ 1 hour	81.71	18.29	3631	3434	1.504	1.541
Alkaline degradation 0.1N NaOH/ 75°C/ 1 hour	94.24	5.76	3040	3869	1.426	1.382
Oxidation 30%H_2O_2/ 75°C/ 1 hour	81.16	18.84	3431	4240	1.458	1.635
Thermal degradation Water/ 75°C/ 1 hour	85.32	14.68	3049	3506	1.444	1.386

Assay of Flucytosine capsules

Assay was performed by using two brands of Flucytosine capsules consisting of 500 mg API and then it was found that the amount of Flucytosine was found to be 99.592-99.786 (Table 9) and there is no interference of excipients.

Table 9: Assay of Flucytosine tablets

Brand	Label claim (mg)	Observed amount (mg)	% Recovery*
I	500	498.93	99.786
II	500	497.96	99.592

*Mean of three replicates

CONCLUSIONS:

The RP-HPLC techniques were validated as per ICH guidelines and found to be simple, economical, simple and robust for the quantification of Flucytosine tablets.

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The authors are grateful to Lupin Pharmaceuticals (India) for supplying gift samples of Flucytosine and Zydus Cadila Healthcare Ltd. and Mankind Pharmaceuticals (India) for supplying gift samples of Linezolid. The authors declare no conflict of interest.

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RESEARCH ARTICLE

Docking Analysis of Novel Arylidinemalononitrile derivatives as PPAR-γ Modulators in the Management of type II Diabetes Mellitus

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ABSTRACT:
Prolonged hyperglycemia often associated with the number of complications such as diabetic neuropathy, retinopathy, nephropathy, cardiomyopathy etc. Diabetic neuropathy is damage to nerves in the body that occurs due to high blood glucose level. In the central nervous system, diabetes exacerbates depression, phobias, anorexia, hypolocomotion, anxiety, cognitive dysfunction etc. The design, docking and analysis of novel arylidine malononitrile-based molecules as derivatives as peroxisome proliferator-activated receptor-γ (PPAR-γ) modulators for antidiabetic activity are reported. Docking studies of designed compounds were carried out using GLIDE (Grid-based ligand docking with emergetics) module version 4.5, Schrodinger, LLC, Newyork, NY,2007. The software package running on multi-processor Linux PC. GLIDE has previously been validated& applied successfully to predict the binding orientation of many ligands. Docking studies of compounds were performed using human peroxisomes proliferators activated receptor gamma (PDB ID 2PRG) obtained from the RCSB Protein Data Bank. Different arylidine malononitrile derivatives were docked into the ligand binding domain of PPAR-γ by the Glide XP module of Schrodinger, out of those Eight derivatives (AM01, AM02, AM03, AM04, AM05, AM06, AM07, AM08) having Glide XP scores > -9 as compared to the standard drug, rosiglitazone (Glide XP score = -8.34), showed almost similar interaction with the amino acids such as SER-289, GLN-286, and HIE-323 in the molecular docking studies.

KEYWORDS: PPARγ agonists, Schrödinger, diabetes, Arylidene malononitrile, GLIDE, Docking.

INTRODUCTION:
Diabetes, correctly termed as diabetes mellitus, is a major epidemic of this century and has increased in incidence by 50% over the past 10 years [1,2]. Diabetes Mellitus is a clinical syndrome, characterized by hyperglycemia caused by a relative or absolute deficiency of insulin at the cellular level. It is the most common endocrine disorder, affecting mankind all over the world, prevalence of which is increasing, daily [3].

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Diabetes mellitus is an endocrinological metabolic disorder characterized by hyperglycemia, glycosuria, hyperlipemia, negative nitrogen balance and sometimes ketonemia [4]. Diabetes Mellitus is an endocrine disorder characterized by altered glucose homeostasis leading to derangements in the carbohydrate, protein and lipid metabolism, resulting from partial or complete deficiency in insulin synthesis or due to peripheral resistance to insulin action [5].

DM is commonly associated with low HDL cholesterol and high triglyceride levels. There are a number of agents that both raise HDL-C and lower triglycrides. These include the peroxisome proliferator-activated receptor (PPAR) agonists (α and γ), statin, fibrate and nicotinic acid [6]. Diabetes is becoming the third killer of the health of mankind along with cancer, cardiovascular and cerebrovascular diseases. According to World Health Organization (W.H.O) report, number of diabetic patients is expected to increase from 171 million in year 2000 to 366 million or more by the year 2030 [7]. Chronic hyperglycemia in diabetes is

associated with long-term damage, dysfunction, and eventually the failure of organs, especially eyes, kidneys, nerves, and the cardiovascular system [8].

Defective insulin secretion, resistance to insulin action and reduction in the bio-antioxidant potential leads to diabetes mellitus. The imbalance between the pro-oxidant and antioxidant homeostasis results in oxidative stress (OS). The role of OS in the pathogenesis of diabetes and its associated diseases (retinopathy, nephropathy, atherosclerosis and coronary artery disease) is well characterized [9]. Non-insulin dependent diabetes mellitus (NIDDM) Type –II occurs at any age, but is more common between 40–80 years of age and also has a strong genetic component. The majority of diabetes (~90%) is Type –II diabetes (T2D) caused by a combination of impaired insulin secretion from pancreatic beta cells and insulin resistance of the peripheral target tissues, especially muscle and liver [10].

Prolonged hyperglycemia often associated with the number of complications such as diabetic neuropathy, retinopathy, nephropathy, cardiomyopathy etc. Diabetic neuropathy is damage to nerves in the body that occurs due to high blood glucose level [2]. In the central nervous system, diabetes exacerbates depression, phobias, anorexia, hypolocomotion, anxiety [11-13], cognitive dysfunction etc [14]. Clinically, patients with diabetes are at increased risk of developing depression and cognitive impairment as compared to the general population [14,15]. Dysregulation of insulin signaling pathway has been involved in the pathogenesis of diabetic complications. Rosiglitazone (ROSI), a PPARγ agonist is known to ameliorate neuronal insulin receptor expression during insulin resistance [16,17]. Report suggests that, dysfunctioning of PPARγ receptor is associated with neuronal dysfunction, nephropathy, cardiomyopathy etc [18]. Very few reports are available to understand the role of arylidene malononitrile in diabetes and its associated neuronal dysfunction. Effect of arylidene malononitrile derivatives on diabetes mediated behaviour deficit is not investigated. There is no evidence regarding the use of arylidene malononitrile on comorbidity of depression and diabetes. Investigation of the role of arylidene malononitrile against diabetes mediated neuronal dysfunctions might solve the problems associated with current therapy which is known to induce cardiac dysfunction. Clinical study was carried out in healthy volunteers as well as in diseased patients by considering two parameters namely behavioural and oxidative stress parameters. Behavioural parameters included anxiety, depression, acidity; constipation etc. and these parameters were further marked as mild, moderate and severe according to the intensity [19]

Docking is a method which predicts the preferred orientation of one molecule to a second when bound to each other to form a stable complex. It is frequently used to predict the binding orientation of small molecule drug candidates to their protein targets in order to in turn predict the affinity and activity of the small molecule. Hence docking plays an important role in the rational design of drugs [20]. The process of "DOCKING" a ligand to binding sites tries to mimic the natural course of interaction of the ligand and its receptor via a lowest energy pathway. Usually the receptor is kept rigid while the conformation of the drug molecule is allowed to change. The molecules are physically moved closer to none another and the preferred docked conformation is minimized.

METERIALS AND METHODS:

Docking Procedure:

Docking studies of compounds listed in table No.1 were performed using human peroxisomes proliferators activated receptor gamma (PDB ID 2PRG) obtained from the RCSB Protein Data Bank, http://www.rcsb.org/pdb, where the residues were bonded more closely to Rosiglitazone agonist, co-crystallized with PPARγ. In this crystal structure, the LBD forms a homodimer in which both monomers have nearly identical Cα conformations. The structure of chain "A" (monomer of the LBD homodimer) was chosen as the target for docking studies.

Experiments were performed using the program GLIDE (Grid-based Ligand Docking with Emergetics) module version 4.5, Schrödinger, LLC, New York, NY, 2007 (Schrödinger Inc.). Coordinates of the full- length substrate-complexed dimmer were prepared for glide 4.0 calculations by running the protein preparation wizard. The p-prescript produces a new receptor file in which all residues are neutralized except those that bridges. The impref script run a series of restrained impact energy minimizations using the impact utility. Minimizations were run until the average root mean square deviation (rmsd) of the non-hydrogen atoms reached 0.3A°.

Glide uses two boxes that share a common centre to organize its calculations: a larger enclosing box and a smaller binding box. The grids themselves are calculated within the space defined by enclosing box. The binding box defines the space through which the centre of the defined ligand will be allowed to move during docking calculations. It provides a measure of the effective size of the search space. The only requirement on the enclosing box is that it be large enough to contain all ligand atoms, even when the ligand centre is placed at an edge or vertex of the binding boxes. Grid files were generated using the cocrystallized ligand at the centre of the two boxes. The size of the binding was set at 20A° in

order to explore a large region of the protein. The three-dimensional structures of the compounds were constructed using the Maestro interface. The initial geometry of the structures was optimized using the OPLS-2s005 force field performing 1000 steps of conjugate gradient minimization. The compounds were subjected to flexible docking using the pre-computed grid files. For each compound the 100 top scored poses were saved and analyzed. Give the structure of designed compound in figure 1 and table2.

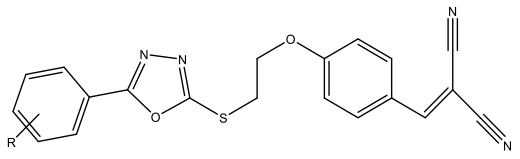


Figure 1 Arylidene Malononitrile

Table 1: Glide Score of Designed Compounds

S. No.	R	Glide Score
1	2-C l	9.82
2	4-CH_3	9.55
3	3-O CH_3	9.42
4	2- O CH_3	9.23
5	4-F	9.20
6	H	9.08
7	3-NO_2	9.01
8	4-Cl	9.00
9	4-OC_2H_5	8.96
10	2,4Di –C l	8.94
11	Isoniazid	8.82
12	3- CH_3	8.73
13	4-Br	8.58
14	4-NO_2	8.50
15	4- O CH_3	8.46
16	2,3- Di –C l	8.39
17	3,4,5 Tri- O CH_3	7.47

Table 2: Detail of Compounds with Glide Score 9 Were Selected

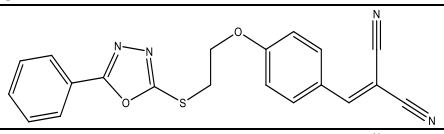
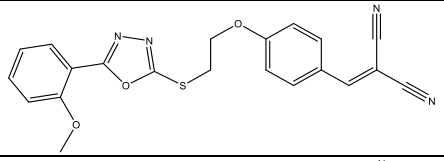
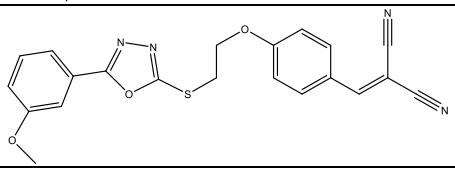
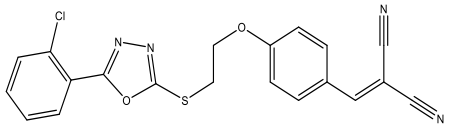
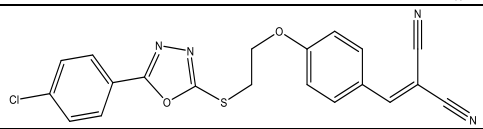
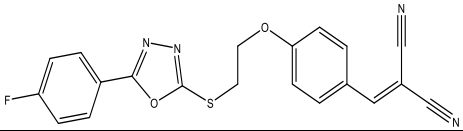
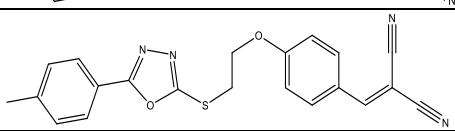
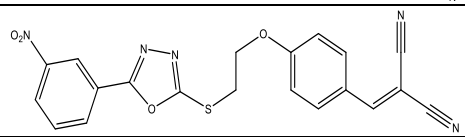
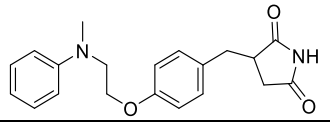
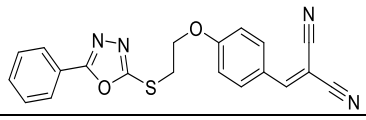
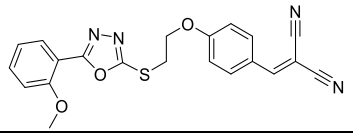
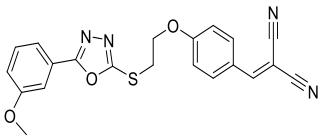
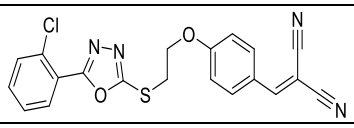
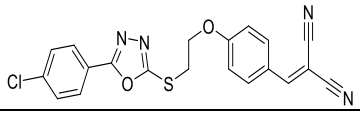
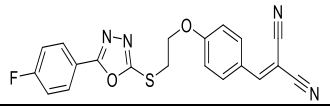
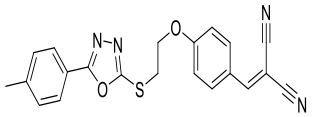
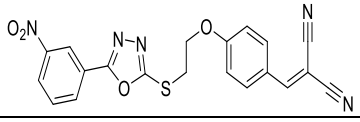
Compound code	Structure	IUPAC Name
AM-01		2-(4-(2-(5-phenyl-1,3,4-oxadiazol-2-ylthio)ethoxy)benzylidene)malononitrile
AM -02		2-(4-(2-(5-(2-methoxyphenyl)-1,3,4-oxadiazol-2-ylthio)ethoxy)bezylidene)malononitrile
AM -03		2-(4-(2-(5-(3-methoxyphenyl)-1,3,4-oxadiazol-2-ylthio)ethoxy)bezylidene)malononitrile
AM -04		2-(4-(2-(5-(2-chlorophenyl)-1,3,4-oxadiazol-2-ylthio)ethoxy)bezylidene)malononitrile
AM -05		2-(4-(2-(5-(4-chlorophenyl)-1,3,4-oxadiazol-2-ylthio)ethoxy)bezylidene)malononitrile
AM -06		2-(4-(2-(5-(4-fluorophenyl)-1,3,4-oxadiazol-2-ylthio)ethoxy)bezylidene)malononitrile
AM -07		2-(4-(2-(5-p-tolyl-1,3,4-oxadiazol-2-ylthio)ethoxy)bezylidene)malononitrile
AM -08		2-(4-(2-(5-(3-nitrophenyl)-1,3,4-oxadiazol-2-ylthio)ethoxy)bezylidene)malononitrile

Table 3: Glide/ Docking (XP Mode) Score of Selected Compound

COMP. CODE	STRUCTURE	HYDROGEN BOND INTERACTION	Gvdw†	GLIDE SCORE
BRL* (Reference ligand)		**SER-289**	**336**	**8.34**
AM-01		SER-289 GLN-286 HIE-323	325	9.08
AM -02		SER-289 GLN-286 HIE-323	351	9.23
AM -03		SER-289 GLN-286 HIE-323	350	9.43
AM -04		SER-289 GLN-286 HIE-323	319	9.83
AM -05		SER-289 GLN-286 HIE-323	311	9.00
AM -06		SER-289 GLN-286 HIE-323	314	9.20
AM -07		SER-289 GLN-286 HIE-323	352	9.55
AM -08		SER-289 GLN-286 HIE-323	323	9.01

RESULTS AND DISCUSSION:

Docking studies of designed compounds were carried out by GLIDE (Grid-based Ligand Docking with Energetics, version 4.5, Schrödinger, LLC, New York, NY, 2007) software package running on multiprocessor-Linux PC. GLIDE has previously been reported for the docking of PPARγ and was found successfully to predict the binding orientation of many ligands. On the basis of Glide/docking Score total eight compounds were selected shown in table table No. 3. The detail of their binding pattern at the active site of receptor (PDB ID-2PRG) was successfully visualized with the help of software shown in fig.2 to 5.

Glide Score is based on Chem Score, but includes a steric-clash term and adds buried polar terms devised by Schrodinger to penalize electrostatic mismatches:

Glide Score=0.065*vdW + 0.130*Coul + Lipo + Hbond + Metal + BuryP + RotB + Site

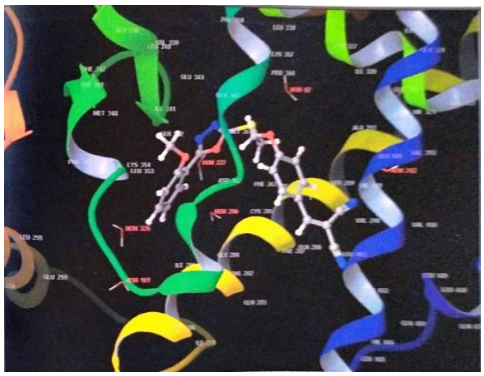


Fig. 2: Active Site of PPARγ with Docked Ligand (AM-02)

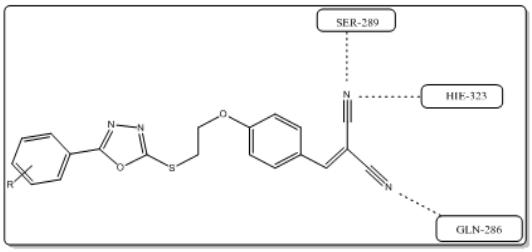


Fig. 3: Hydrogenbond interaction between Malononitrile head group and Amino Acids of Receptor

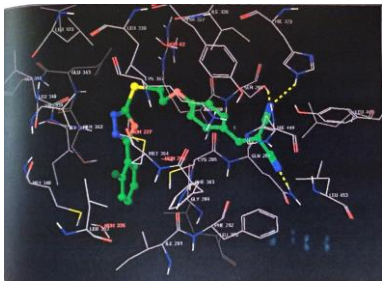


FIG.4: Hydrogen bond interaction of AM-04 with receptor

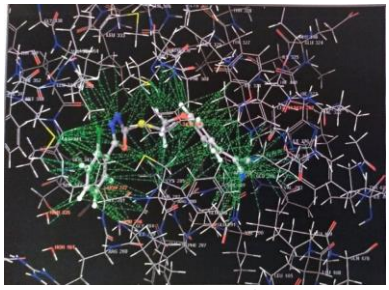


Fig.5: Good vander waal's interaction of AM-04 with receptor

CONCLUSION:

Designing novel series of PPARγ ligands this was to explore newer acidic head groups for designing novel series of PPARγ ligands this was aimed to improve the potency of compound. It was realized that docking, an imoptant computational technique could be of help in discovering newer scaffolds with desired features and thus employed for this purpose. The PPARγ receptor has both hydrophilic domain, so for proper interaction with receptor the compounds should be ampiphilic in nature. The malononitrile group is successfully completed the requirement of acidic head group and shows the hydrogen bond interaction with the SER-289, GLN-286 and HIE-323 amino acid of the active site of the receptor.

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RESEARCH ARTICLE

Symplocos racemosa bark assisted copper nanoparticles and its Antibacterial activity against *Staphylococcus aureus* and *Lactobacilli species*

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ABSTRACT:

Aim: To evaluate the antibacterial activity of copper nanoparticles using lodhra bark against *Staphylococcus aureus* and *Lactobacillus species*. **Introduction:** *Symplocos racemosa* also known as Lodhra, is a drug used by Ayurvedic practitioners. Theroot, bark and leaves of this plant is traditionally used for the treatment of diarrhoea, leprosy, uterine and liver disorders. **Materials and methods:** The plant extract is prepared by mixing 100 ml of distilled water, boiled and filtered using a filter paper. The copper nanoparticles are prepared by mixing copper sulphate powder to 500 ml of distilled water. Readings were taken frequently, and the synthesis of copper nanoparticles were analysed. Agar well diffusion method was used to evaluate the antimicrobial activity of both S.aureus and Lactobacilli species. **Results:** *S.aureus* showed a higher inhibition area than *Lactobacillus*. **Conclusion:** There is a future potential for these copper nanoparticles for combating pathogenic microorganisms.

KEYWORDS: Copper nanoparticles, green synthesis, *Symplocos racemosa*, Antibacterial activity.

INTRODUCTION:

Symplocos Racemosa also known in Hindi as Lodhra, is a common, indigenous traditional drug used by Ayurvedic practitioners. They are evergreen trees and tall about 10-15m. They are mainly found in north and east India throughout the Himalayas. The Symplocos genus comprises of 300-500 species of the Symplococaceae family. About 68 species are found in India [1]. Lodhra helps the make the body firm. It also removes excess fluids from skin. Lodhra consists of Loturine-0.25%, Colloturine-0.02%, Loturidine-0.06%. It has a wide range of pharmacological actions. The root, bark and leaves of this plant is traditionally used for the treatment of diarrhea, dysentery, liver diseases, uterine disease leprosy, liver complaints, uterine disorders, diarrhoea, dysentery and conjunctivitis. Majority of phytopharmacological reports are on stem bark of the plant which include anti-cancer, hepatoprotective, antioxidant, anti-androgenic effect, anti-inflammatory, wound healing activity and anti-diabetic effects [2,3].

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The metallic nanoparticles have been found to demonstrate a wide range of antibacterial activity against various bacterial species, including Gram-positive and Gram-negative bacteria [4,5]. Recently, copper nanoparticles have been used. Copper is one of the most essential trace elements in living organisms. They exhibit a wide spectrum of antimicrobial activity against different species of microorganisms [6,7]. Currently, copper has been registered as the first and only metal with antimicrobial properties by the American Environmental Protection Agency [8,9]. This material kills 99.9% of most pathogens within 2 h contact [10]. Although copper is one of the most widely used materials, its nano synthesis requires special care because of its high capability of rusting. Compared to other metals, copper is extremely sensitive to air, and copper oxides phases are thermodynamically more stable. Therefore, the formation of an oxide layer on the surface of the copper nanoparticles is inevitably causing a marked decrease of its antibacterial properties [11,12].

MATERIALS AND METHODS:

Plant extract preparation:

Lodhra bark was powdered and its extract is prepared by mixing 100ml of distilled water and is boiled for 3-5 mins in heating mantle and then filtered using a filter paper.

Figure1: Preparation of plant extract

Preparation of copper nanoparticles:

0.861 grams of copper sulphate powder is added in 60 mL of distilled water and the Lodhra bark extract was added. The solution is kept in the shaker and the readings were taken for every 2 hours for analysing the synthesis of nano particles.

Figure 2: Copper nanoparticles synthesis

Antibacterial activity:

Agar well diffusion method was used to evaluate the activity. The fresh suspension of Lactobacillus and *S.aureus* were dispersed on the surface of Muller Hinton agar plates. Different concentrations of copper nanoparticles were incorporated into the wells and they were incubated at 37 degree Celsius for 24 hours.

Results:

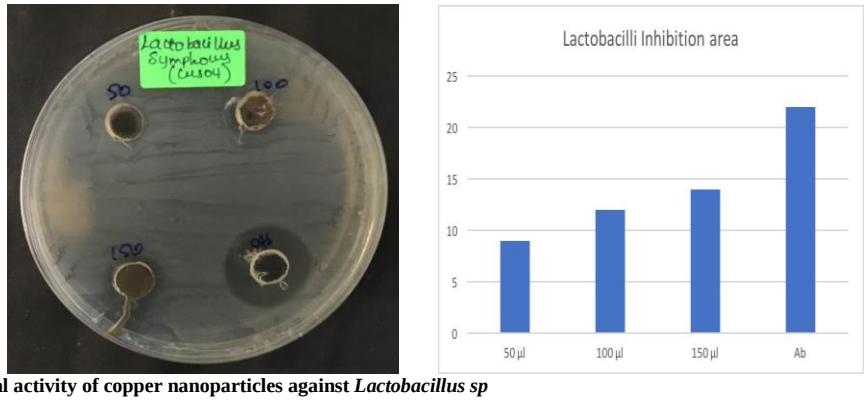

Figure 3: Antibacterial activity of copper nanoparticles against *Lactobacillus sp*

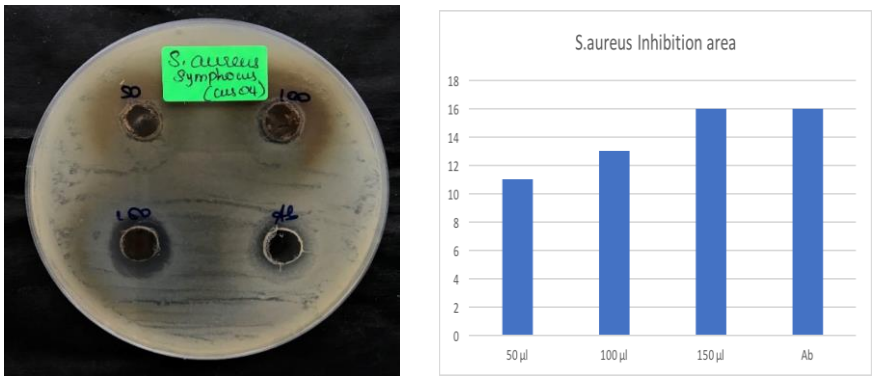

Figure 4: Antibacterial activity of copper nanoparticles against *S. aureus*

DISCUSSION:

Comparatively, *S.aureus* showed higher inhibition area than *lactobacillus*. First of all, copper nanoparticles demonstrate a very strong catalytic activity, a property that can be attributed to their large catalytic surface area. with the small size and great porosity, the nanoparticles are able to achieve a higher reaction yield and a shorter reaction time when utilized as reagents in organic and organometallic synthesis.

The antimicrobial activity is induced by their close interaction with microbial membranes and their metal ions released in solutions. As the nanoparticles oxidize slowly in solutions, cupric ions are released from them and they can create toxic hydroxyl free radicals when the lipid membrane is nearby. Then, the free radicals disassemble lipids in cell membranes through oxidation to degenerate the membranes [9,13]. As a result, the intracellular substances seep out of cells through the destructed membranes; the cells are no longer able to sustain fundamental biochemical processes. In the end, all these alterations inside of the cell caused by the free radicals lead to cell death [13]. Mostly, Staphylococcus cause diseases related to skin like scaled skin syndrome, pneumonia, meningitis, osteomyelitis and endocarditis. Copper helps in preventing them by acting on copper binding enzyme, a glyceraldehyde 3 phosphate dehydrogenase in aureus species leading to the inhibition of glycolysis mechanism. Generally, all the they have a toxic effect on bacteria and damage the cell membrane which leads to impairment in protein synthesis [14-24].

CONCLUSION:

Our results indicate the future potential of these copper nanoparticles for combating pathogenic microorganisms. The nano particles may be suitable for formulating new anti-microbial materials for pharmaceutical and biomedical applications.

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RESEARCH ARTICLE

Assessing Knowledge and Practice of Self-Directed Learning among Medical Students, UNIMAS

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ABSTRACT:

Self-directed learning (SDL) is a learning process. In this process, the learners consciously accept responsibility in andragogy. The SDL is observed as a powerful, rumbustiously active learning method compared to the didactic lecture learning method. However, SDL in young learners who are exposed to a complex, dynamic, and busy medical ward does not seem to be sought yet. Thus the study aims at determine the knowledge and practice of SDL among medical students. It was the cross-sectional descriptive study. The chosen study sample was year 3 medical students. The study conducted using well-structured questionnaires. Reliability of the questionnaires was 0.723. There was a significant positive correlation between the knowledge and practice scores. The findings as of the knowledge level of the respondents revealed that 56% of them had high knowledge level and 43% had moderate level. Regarding the practice level of SDL, 85% of the students postulated that they fully applied the SDL steps during their posting providing that university laid down the systematic curriculum in place. The implication of the study to develop awareness regarding the strength of the SDL method in students' learning process, which can tile the improved quality teaching methods and enhance quality learning among medical students.

KEYWORDS: Self-directed learning, Knowledge, Practice, andragogy.

INTRODUCTION:

The learning method used by students should allow them to grow matured and self-reliable. It has revealed in one of the previous studies that learning should empower the student to grow as an abled, mature, and authentic self. (Savin-Baden and Major (2004). All educators tried their best to improve the teaching and learning techniques. With time, learning methods are upgraded from traditional didactic lecture method to small group teaching like problem-based learning, tutorial, and seminar-based and self-directed learning. One of the literature studies has stated that Self-directed learning (SDL) is a learning process in which the key element is the learners' conscious acceptance of responsibility for learning. The SDL has gained its popularity in due time since its introduction is in the early 70s until now.

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The definition is given by Knowles, that the SDL is a process in which individuals take the initiative, on their own or taking help from others, to understand their learning needs, setting goals, identifying both human and material resources, further choosing and implementing this resources in appropriate form of learning methods, and evaluating its outcomes.

Knowles (1975) SDL made tremendous progress since then and most researchers defined and expanded its core essence differently. Taylor (1995) in his study, emphasized that in SDL the students should be motivated, is persistent, independent, self-disciplined, self-confident, and goal-oriented. [Taylor (1995)] Stephen Brookfield (2009) emphasized that self-directed learners are not to be thought of as Robinson Crusoes, working without human contact. Most educators accepted and perceived SDL as the most powerful and active learning in its sense.[Stephen Brookfield (2009)] There are studies on SDL readiness scores, perception

scales, strategy, designs, models, and factors influencing the success of SDL. Nevertheless, SDL knowledge and its practice in young learners who appeared to expose to a complex, dynamic, and busy medical ward have not sought yet.

SDL includes in our curriculum in the first clinical year (year 3) of the medical program. They have experienced combined traditional lectures and problem-solving learning (PBL) method in the pre-clinical year (year 1 and 2). In the current study, researchers expect that PBL helps the students to become adult learners. In this study, we would like to find out our students were equipped with adequate knowledge and sufficient practice to adapt and adjust to the complicated clinical training during this transition period through effective learning methods like SDL. Here, the researchers are keen to explore if there is any difficulty, to take hold of SDL, which is a part of the current curriculum.

The difficulty could come up with SDL is some of the students merely get hold of the concept and details of it. Furthermore, they totally rely on the teacher as in our traditional old days (Teacher- dominated teaching) and enjoy the fruits of spoon-feeding. They forget the fact that role of educators changes with time during the advancement of the medical education process. Educators adopt the role from old times like teachers, lecturers, informers to become facilitators, assessors, curriculum planners, curriculum evaluators, mentors. [(R.M. Harden and Joy Crosby, 2000).] As an educator, we realize the progress of medical education should lead to an active, student-centered method of learning. In the current situation, it is a necessity to have clarity regarding roles in teaching and learning method not as a traditional medical teacher, but as a facilitator/ evaluator/mentor, to achieve the best outcome for the learners.

Taylor (1995), in his study, suggested that one important task of teachers was to raise awareness of the students' own role in the SDL process. Although the learners find it hard to get control of their own learning, it is impossible to demean the role of facilitator in the SDL. [Taylor (1995)]

One of the previous studies explores that SDL is currently well understood as it is strongly influenced by many variables, such as the personality type, the learner's previous experience, the accessibility of relevant resources, and the perceived cultural constraints. [Merriam and Caffarrella (1999)]

Pieces evidence mentions that SDL gives the learners the skills to be life-long learners. It builds up the confidence and competency to cope with the different learning process. [Regarding the benefits of SDL, Dynan, Linda and Cate, Tom and Rhee, Kenneth. (2008].

Those savoring the savvy concept of SDL will obtain its benefit, and they will grow themselves into life-long learners in the future. In SDL learners, have an increasing choice over the timing, location, contents, and path of their learning process. They control their learning situation and apply autonomy in the learning process, which makes the learner feeling satisfied and confident of oneself.

Furthermore, one of the literature studies has explored that SDL may be associated with increased curiosity, critical thinking, better understanding and retention of information, and improved motivation and confidence. [Devi, Bhat, Ramya, Ravichandran, and Kanungo, 2016)]

There were many studies focused on the concept, process, personal, and contextual factors, and readiness of SDL within our reach. However, the study on how much SDL is embraced with, how much aware of this method, how much is accepted, and consumed its benefits by the learners are merely known. This fact opens the door for the researchers to fill in this area.

OBJECTIVE:

Aim of the study is to determine the knowledge and practice of SDL.

METHODOLOGY:

It was the cross-sectional descriptive study conducting on all year 3 medical students from UNIMAS.

The questionnaire was structured for the study purpose based on the thesis of Jane Pilling-Cormick in the development of the SDL perception scale. The questionnaire was restructured and validated making a pilot study.

The reliability of the questionnaires was checked with Cronbach's Alpha. The study conducted using well-structured questionnaires. The reliability of the questionnaires was 0.723 on Cronbach's Alpha.

The questionnaires have 3 domains with a free comment box in the end. The first domain is for socio-demographic data, the second is on SDL knowledge, and the final part is for SDL practice. We monitored the practice by checking the logbook (journal) twice per week with assigned facilitators. In the logbook, we explored their setting goal, use of resources, time management with self-evaluation/reflection. The open comments at the end of the questionnaires were organized and compiled according to two themes- the role of facilitators and factors influencing the SDL process. The data entry and compilation were done and

analyzed by SPSS version 22. The results were calculated and expressed in tables or graphs.

For the ethical considerations, the Centre for Applied Learning and Multimedia granted and approved this study (SoTL/FPSK/2018(1)/004).

RESULTS AND DISCUSSION:

The researchers of this study explored the knowledge and practice towards the SDL of year 3 medical students during their posting. It indirectly helps us to find out the competency of our curriculum with the role of facilitator from the student's perspectives.

The total participants in the study were 109. Data analysis of socio-demographic data shows the mean age of the study group was 21.89 (0.52) years. The study population was 22% male and 78% females. Malay (55%), Indian (14.7%), Chinese (11.9%), and Bumiputras (9.2%) included in the study.

The mean total scores on knowledge were 60.08 (6.93), and the mean practice total scores were 12.83 (2.56). (Table 1)

Table 1 Knowledge and Practice total scores of respondents

	Minimum	Maximum	Mean	Std. Deviation
Kn_Total	38	77	60.08	6.931
Pract_Total	5	16	12.83	2.559
Valid N (listwise)				

With Pearson correlation, there was a significant positive correlation between the knowledge and practice total scores. (Table 2; figure 1)

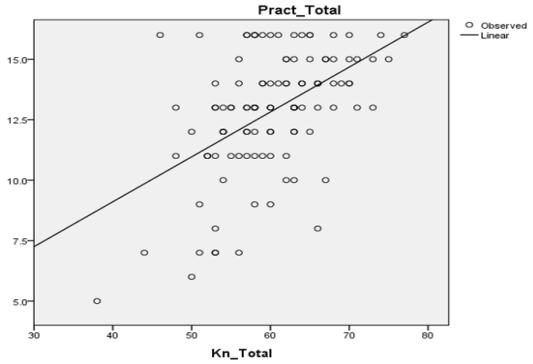

Figure 1: depicts positive correlation between the knowledge and practice total scores.

Table 2: Correlation between knowledge and practice scores
****Correlation is significant at the 0.01 level (2-tailed)**

		Kn_scores	Pract_ scores
Kn_ scores	Pearson Correlation	1	.503
	Sig. (2-tailed)		.000**
	N	109	109
Pract_ scores	Pearson Correlation	.503	1
	Sig. (2-tailed)	.000**	
	N	109	109

Table 3 Sociodemographic Data vs Knowledge Scores

Sociodemographic profile	Mean (SD[a])	F-statistics (df[b])	p-value[g]
Age	21.89 (0.52)		0.308[e] r= 0.098
Gender			
Male	61.71 (7.33)	0.422[c] (107)	0.195[d]
Female	59.62 (6.79)		
Ethnicity			
Malay	59.45 (7.64)	1.433 (4)	0.228[f]
Chinese	60.48 (5.60)		
Indian	63.13 (5.81)		
Iban	56.17 (6.59)		
Bidayuh	61.00 (4.55)		

r = Pearson Correlation Coefficient
[a] standard deviation
[b] degree of freedom
[c] t- statistic
[d] *p*-value for independent t-test
[e] *p*-value for correlation test
[f] *p*-value for one-way ANOVA
[g] *p*-value of less than 0.05 is taken as significant

We used the independent student's t test for the association between gender with knowledge total scores and there was no significant association (p = 0.195 >0.05). The relationship of the race and knowledge total scores among the students also seemed to be indifferent (ANOVA P=0.228, >0.05) (Table 3)

Table 4 Socio-demographic Data vs Practice Scores

Sociodemographic profile	Mean (SD[a])	F-statistics (df[b])	p-value[g]
Age	21.89 (0.52)		0.467[e] r= 0.07
Gender			
Male	12.83 (2.57)	0.001[c] (107)	0.997[d]
Female	12.84 (2.57)		
Ethnicity			
Malay	12.68 (2.41)	1.679 (4)	0.161[f]
Chinese	12.39 (2.90)		
Indian	14.31 (1.70)		
Iban	12.50 (3,27)		
Bidayuh	12.25 (3.59)		

r= Pearson Correlation Coefficient
[a] standard deviation
[b] degree of freedom
[c] t- statistic
[d] *p*-value for independent t-test
[e] *p*-value for correlation test
[f] *p*-value for one-way ANOVA
[g] *p*-value of less than 0.05 is taken as significant

Table 4 showed that there was no significant association between gender and practice total scores (p = 0.997 >0.05) and between the race and practice total scores among the students (ANOVA P=0.161, >0.05). The findings from the study revealed that 65% of the respondents had a high knowledge level gained through self-directed learning, while 43% had a moderate level of knowledge. (Figure.2)

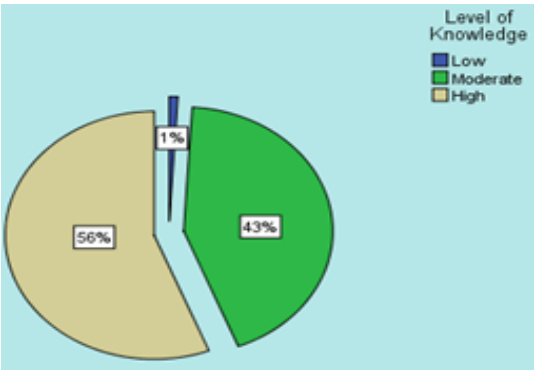

Figure 2: Level of knowledge of year 3 medical students towards SDL in percentage

Regarding the practice, the level of self-directed learning was 85% of the students, which postulated that they applied fully to the SDL steps during their posting, found as supportive findings to the curriculum of the university, while 15% used insufficiently (Figure.3).

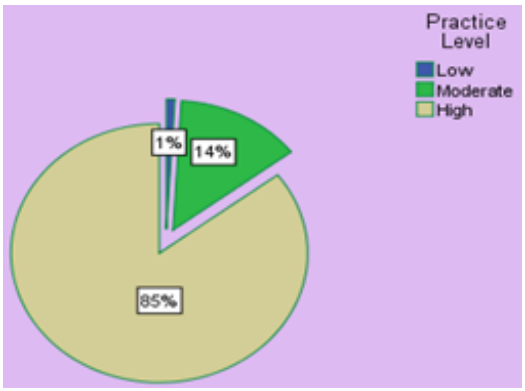

Figure 3: Level of the practice of year 3 medical students towards SDL in percentage

In this study, the practice level resulted more than the knowledge level. (85% Vs 65%). It was probably due to the introduction of PBL practice in the pre-clinical year, which provided a firm ground to flourish its practice. It further coincided with the study which indicated, that problem-based learners are self-directed learners. (Loyens, S.M.M., Magda, J. and Rikers, R.M.J.P, 2008). O'Shea, E (2003) stated in her study that not all students were self-directed, but mature students are more self-directed. With the evidence of high knowledge and practice score, we assumed confidently year3 students were well-perceived the core context of SDL through the pre-clinical problem-based learning process. It indicated that our university's curriculum was adequate enough to shape up our young learners to perform well as self-directed learners in andragogy.

We organized the open comments in two themes as the role of facilitators and factors influencing the learning process. It revealed that the students preferred the facilitator coaching them in the learning process. The use of a logbook (journal) to set their own daily goals made them engaged more in the study and favored the use of resources.

They admitted that time management was not a problem for them as they spent at least 3.75 hours a day (3.75 +/- 0.96). Most of them applied the self-reflection process as they tried to memorize the contents after the study. There were different learning styles like highlighting, self-rehearsal for reciting, repetition, note-taking, or drawing. They also responded that if the facilitator coached them in the learning process, they were more motivated and they regained more self- confidence. They claimed there were initiatives to expand their learning in terms of contextual knowledge. The respondents in this study welcomed the facilitators' help to accomplish the evaluation process. It was agreeable with one of the research literature studies, who highlighted the role of teachers or facilitators that they need to model learning strategies such as predicting, questioning, clarifying, and summarizing so that students will develop the ability to use these strategies on their own. [Many, Fyfe, Lewis, and Mitchell (1996)]

Sahoo (2016) again confirmed it in his study, that the majority of the students expressed better utilization of SDL study hours by motivating them with weekly assessment from facilitators, thereby fostering SDL habits. The current study has also found similar the same finding, as the students mentioned, they would become more confident and felt self-esteemed if the facilitator fosters them with evaluation.

Regarding the factors influencing SDL practice emphasized motivation and peer pressure as important factors. The study was not affected by any other factors like personality difference, underlying educational context, poor engagement, or diversity in the classroom as seen in other literature, also seen as 85% had good practice in the study group.

CONCLUSION:

In the current education system, the SDL is regarded as a good method of learning, which is systemically adopted in our University Curriculum. The built-in SDL session provides the knowledge and thrives the practice of SDL in year3 postings. The students ' perceived practice of SDL, although the knowledge level is lesser than their practice level. The motivation made by the facilitators, improving the confidence level of learners, and helping them in the self-evaluation process play an important role in the strengthening of the SDL practice.

From our findings, we recommended expanding the role of the facilitator to improve the proper and effective use of SDL to achieve the best performance from the

students. It will definitely ease the learning path and grow stronger in their life-long learning behavior with enhanced self- confidence and dedication. The current study further postulates, that the SDL tiles the learning platform to the students to become more engaged with more passion for learning, as well as absorb good learning methods in their life-long learning process.

CONFLICT OF INTEREST STATEMENT:

There is no conflict of interest between the authors.

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We would like to thank our young learners (year 3 medical students - 2017/18) who agreed to take part in our study.

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RESEARCH ARTICLE

Development and evaluation of Herbal hand wash with Neem Alcoholic Extract

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ABSTRACT:

Herbs have pharmaceutical, medicinal and cosmetic potential, with the help of it large number of advanced products will be obtained which are helpful for human beings. In the present study herbal handwash was developed using ethanolic extract of *Azadirachta indica (*Neem). This plant is historically known to have many therapeutic uses. Herbal handwash was developed by using Carbopol-930 as Jelling agent, Triethanolamine as neutralizer and Sodium lauryl sulphate as surfactant. Cup plate method as used to assess the antimicrobial activity of developed herbal hand wash. *Staphylococcus aureus, Escherichia coli Bacillus subtilis and Pseudomonas aeruginosa* were used to screen the Herbal hand wash against dermal disease causing organisms. Herbal hand wash has shown potential action against *Pseudomonas aeruginosa*. Herbal hand wash was also assessed by sensor characters such as odour, colour and pH, Foam height, consistency, dermal irritation test, Foam retention are the chemical standards. The attained outcomes were within the limits with little or no adverse effect.

KEYWORDS: Neem, Antibacterial activity, Herbal Handwash.

INTRODUCTION:

Herbs have pharmaceutical, medicinal and cosmetic potential, due to which they are used for preparation of many products useful for human. As skin are most exposed organ of our body it need to guard more especially hands which we use for feeding us and even. To avoiding health care related infections proper hand cleanliness is the single most vital and cost effective means. Many hand washes existing in market are prepared by using synthetic chemical and has adverse effect like dryness of dermis, rashes[1]. Using alcoholic extract of *Azadirachta indica* (Neem) we have prepared a herbal hand wash with significant antibacterial activity.

MATERIALS AND METHODS:

Preparation of ethanolic extract of Neem

The leaves of *Azadirachta indica* (Neem). were collected from Udupi, Karnataka, India during August–September 2018 and its botanical identity was confirmed by Dr. Gopal Krishna. Bhat, Department of Taxonomy, Poorna Prajna College, Volakadu, Udupi, Karnataka. The shade dried powdered leaves (500 g) were extracted with ethanol using soxhlet apparatus. A concentrate was prepared by distilling off the solvent from the total extract and the concentrate was placed on a water bath and converted to a syrupy mass and then completely dried by evaporating (20 gram).

Qualitative Analysis:

The preliminary phytochemical screening was carried out for testing the different phytoconstituents present in alcoholic extract of *Azadirachta indica* (Neem). The phytoconstituents tests were carried out and the results are represented in Table 1.

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Table – 1. Preliminary phytochemical screening of *Azadirachta indica* (Neem).

Sl. Number	Phytochemical tests	Result
1.	**Alkaloids**	
	1) Hager's test	Negative
	2) Dragendorff's test	Negative
	3) Mayer's test	Negative
	4) Wagner's test	Negative
2.	**Carbohydrates**	
	1) Molisch's test	Positive
	2) Anthrone test	Positive
	3) Benedict'stest	Positive
	4) Fehling's test	Positive
3.	**Glycosides**	
	1) Molisch test	Positive
4.	**Flavanoids**	
	1) Shinoda's test	Positive
5.	**Pentacyclic terpenoids**	Positive
	1) L.B test	
6.	**Tannins**	Negative
7.	**Saponins**	Positive
8.	**Steroids**	
	1) Salkowski reaction	Positive
	2) L.B test	Positive
9.	**Resins**	Negative

Preparation of Herbal Hand wash:
Herbal gel as prepared with the help of Carbopol-930 as a gel forming agent in 2% w/w concentration with water for a period of one day. Then the inflated jell was agitated with the help of a mechanical agitator to confirm even distribution of the jell. Then adjust the pH to 7.0 by adding minute quantities of triethanolamine with nonstop stirring. Then this base was used to integrate extract of Neem to develop a formulation of Herbal hand wash. To this Sodium lauryl sulphate (0.3g) as surfactant and Rose water (10 ml) was also assimilated.

Antibacterial activity of herbal hand wash[2]
Agar well plate method was used to test the antibacterial activity of Herbal hand wash[3]. The test organism was dispersed with bacterial suspension of optical density of 0.6 at 600 nanometer. Wells of 5 millimeter diameter were then made on plate. Each well was filled with 100 microliter of extract and subjected to incubation at 37^0C for 24 hours. After 24 hours of incubation antagonist action is determined using zone of inhibition (centimeter). The antibacterial activity of Herbal hand wash was tested against microorganism in triplicates. Ciprofloxacin is used as standard. The organism used are *S.aureus, E.coli, S.auroginosa* and *B.subtilis*.

Physical Evaluation:
Physical evaluation of the Herbal Hand wash was visually carried out. The sensory characters such as Texture, Color and fragrance were determined.

Homogenicity and Appearance:
The Herbal Hand wash was Green in colour, homogenous and semitransparent in nature.

pH
100 ml distilled water was taken and 1 gm o Herbal hand wash was dissolved in it. The pH of solution was determined in previously standardized digital pH meter[3].

Viscosity:
Digital Brookfield viscometer was used to determine the viscosity o Herbal hand wash.

Foam Height:
Herbal Gel Based hand wash sample of about 1gm was taken and it was mixed with 50 ml of water. It is then transferred to 250 ml stoppered measuring cylinder. The volume was made upto 100 ml with distilled water. 25 movements were given and then it is allowed to stand till aqueous volume measured upto100 ml and measured the foam height; above the solution.

Foam Retention:
Herbal Gel Based hand wash of about 50 ml was taken into a 100 ml measuring cylinder and it is shaken 10 times. The volume of foam at 1-minute intervals for 5 min. was recorded. At least for 5 min Foam retention should remain stable.

RESULTS AND DISCUSSION:

The Anti-microbial efficacy of the formulation was tested on *S.aureus, E.coli, S.auroginosa* (Figure1) v and *B.subtilis.* Herbal hand wash has shown significant activity against *Pseudomonas aeruginosa* (Table 2). Herbal hand wash was also evaluated by physical parameters[4,5, 6] (Table 3) physical and chemical standards like odour, color, Consistency, pH, Foam retention, Foam height, and Dermal irritation test were performed and results were found to be in the acceptable limits.

Table -2: Antibacterial activity of the herbal Hand washes (Extract of Neem)

Concentration (mg/mL)	*S.aureus*	*E.coli*	*S.auroginosa*	*B.subtilis*
	Zone of inhibition (mm)			
25	-	-	8.00 ± 1.00	-
50	-	-	10.13 ± 1.63	-
100	-	-	24.11 ± 1.12	-
Ciprofloxacin (5 µg)	28.22 ± 3.41	26.11 ± 1.12	27.13 ± 1.41	21.12 ± 1.31

Values are represented as mean ± Standard Deviation (n=3). P<0.05 between extract and negative control treated Cup plate.

Table--3: Physical Evaluations of Formulation

S.N.	Parameter	Observation
1	Appearance	Green colour
2	Homogeneity	Homogenous
3	pH	7
4	Viscosity (m Pascals)	90-110

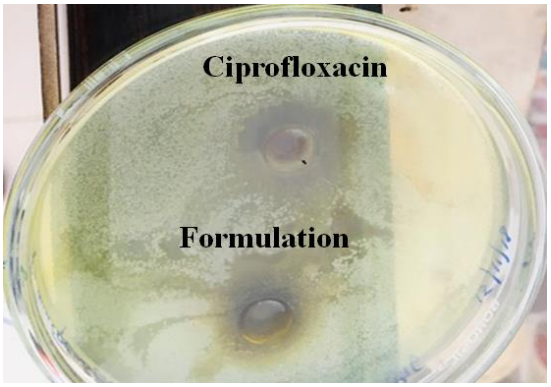

Figure 1 Anti-bacterial activity of the developed Herbal hand wash was tested using *S.auroginosa*

CONCLUSION:

An herbal handwash as developed with potential antimicrobial and enhanced quality using Neem extract as antibacterial agent, Carbopol-930 as Jelling agent, Triethanolamine as neutralizer and Sodium lauryl sulphate as surfactant.

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RESEARCH ARTICLE

Effect of solvent on yield, phytochemicals and *in vitro* antioxidant potential of *Rhododendron arboreum*

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ABSTRACT:

Rhododendron arboreum (family- Ericaceae) is one among the medicinally and economically important plant species from Himalayan region. Aim of present study was to study the effect of solvent on extraction yield, phytochemicals and antioxidant potential of various parts of *R. arboretum.* Extract of various solvents (n-hexane, methanol and water) were prepared using cold maceration method. Spectrophotometric method was used to quantify total phenolic and flavonoid content of different extracts. DPPH, FRAP and ABTS method was used to evaluate antioxidant activity of different extracts of various parts of *R. arboreum*. Among all solvent extract of different part of *R. arboretum*, highest extraction yield was observed in aqueous extract of leaf (10.78%) followed by methanolic extract of flowers (10.42%). Methanolic extract of flower showed higher amount of TPC (107.46±8.74 mg/g GAE) and TFC (254.26±29.33 mg/g RE). DPPH, FRAP and ABTS assay showed lowest IC_{50} value in flower extracts (methanolic and water) and hence validating the utilization of flower of this tree as source of natural antioxidants.

KEYWORDS: *Rhododendron arboretum*, Cold maceration, extraction yield, antioxidant activity.

INTRODUCTION:

Himalayan region is the treasure of biodiversity and consists of diverse flora and fauna. *Rhododendron arboreum* Sm. is an important plant of the Himalayan region. It is the national flower of Nepal and state tree of Uttarakhand. Several less explored plants are available in the Himalayan region. One such plant is *Rhododendron arboreum* Sm. (family Ericaceae) which is a popular plant especially for its beautiful flower and flower juice which is consumed as a popular beverage in the Himalayan region. The plant is commonly known as "Burans" and "Laligurans" and is an important contributor to the economy of the rural areas. Different parts of this small tree possess important therapeutic activities. Dried flowers of *R. arboreum* are reported to be highly effective in checking diarrhoea and blood dysentery [1].

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People from hilly areas, use the flower of *R. arboreum* in the preparation of local brew, jellies, jams and squash. It is a very common and pleasing drink as a refreshing appetizer and tonic [2]. Flower extract is also effective against stomach diseases and snuffed to stop nasal bleeding [3]. Flower petals and leaves of *R. arboreum* also used for the treatment of a headache, fever, dysentery, rheumatism, wounds, and nose bleeding [4]. Consuming its root decoction cures early stage of cancer diseases [5]. Fresh leaves in combination with thuja/ pine/ juniper leaves are burnt for making smoke that is believed to be holy and help in purifying surrounding air [6]. *Rhododendron arboreum* flower, bark, and Young Shoots are used for the treatment of a digestive and respiratory disorder, tonic for the heart [7].

The various phytocompounds isolated from leaves of *R. arboretum* are quercitirine, epicatechin, synergic acid and quercitine-3-O-galactoside [8], while quercitine, coumaric acid and rutin [9]. Phytocompounds such as taraxerol, ursolic acid acetate, betulinic acid, leuco-

pelargonidin have been identified in various extracts of bark [10]. Leaves of this tree are reported to contain glucoside, ericolin (arbutin), ursolic acid, α-amyrin, epifriedelinol, campanulin, quercetin and hyperoside [11]. Study from [12] showed the presence of hyperoside (3-D - galactoside of quercetin), ursolic acid and epifriedelinol. The leaves are also reported to contain the flavone glycoside and dimethyl ester of terephthalic acid and certain flavonoids [13]. Flowers are reported to possess Quercetin-3-rhamnoside [14], quercetin, rutin and coumaric acid [15]. Keeping in view the importance of this medicinal tree, the present study was designed to compare the effect of solvent on extraction yield and *in vitro* antioxidant activity among different parts of *R. arboreum.*

MATERIAL AND METHODS:

Chemicals and solvents:

The chemicals such as 2,2-Diphenyl-1-(2,4,6-trinitrophenyl) hydrazyl (DPPH), 2,4,6-Tri(2-pyridyl)-s-triazine (TPTZ), 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) were obtained from Sigma-Aldrich Co. LLC, Mumbai. The solvents such as n-hexane, methanol and dimethyl sulfoxide (DMSO) used were procured from Loba Chemie Pvt. Ltd., Mumbai.

Collection of plant materials and Extract preparation:

The flower, leaf and bark samples of *R. arboreum* were collected in the month of March (2019) from Kullu, Himachal Pradesh, India. All the plant materials (flower, leaf, and bark) were washed twice and allowed to dry at 40 °C. The dried plant material was converted into powder using electric grinder. Cold macerated method was used to prepare extracts of different solvents (n-hexane, methanol and water) [16 -18]. The dried extracts were stored at 4 °C in airtight bottles till further use.

Determination of total phenolic and flavonoid content

Total phenolic content (TPC) in various extracts of different parts of *R. arboreum* was determined by using Folin-ciocalteau reagent method [19] whereas total flavonoid content (TFC) of different solvent extracts of different parts of *R. arboreum* were quantified by using aluminum chloride ($AlCl_3$) method [20].

In vitro antioxidant activity in various solvents extracts of different parts of *R. arboreum*

Antioxidant activity of various solvent extracts of different parts of *R. arboreum* was determined using methods such as DPPH, FRAP and ABTS method. To analyze the antioxidant potential of various extracts of different parts of *R. arboreum*, all the extracts were dissolved at a concentration of 1 mg/ml in ethanol and then diluted in order to prepare different concentrations (10-80 µg/ml) for antioxidant assays. Ascorbic acid was used as standard antioxidant compound for comparative analysis in all assays.

2, 2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

DPPH radical scavenging activity of various extracts of different parts of *R. arboreum* was measured by the method described by Barros *et al*. (2007) [21] and Rolta *et al* [22, 23]. The capability of scavenging DPPH radical was calculated using the following equation:

% DPPH radical scavenging activity =

$\{A_{(control)}-A_{(sample)}] / A_{(control)}\}\times 100$

where $A_{(control)}$- Absorbance of control and $A_{(sample)}$ - absorbance of the test/standard.

Ferric Reducing Antioxidant Power (FRAP) assay

FRAP activity was calculated according to the method described by Benzie and Strain [24]. The antioxidant capacity of extract/standard was calculated from the linear calibration curve of $FeSO_4$ (10 to 80 µM) and expressed as µM $FeSO_4$ equivalents per gram of the extract.

ABTS scavenging assay:

ABTS scavenging activity was calculated using method described by Re *et al.* [25] Percentage ABTS scavenging activity was calculated as-

ABTS radical scavenging activity (%) =

$[(A_{control} - A_{sample})]/(A_{control})]\times 100$

where $A_{control}$ is the absorbance of ABTS radical + methanol; A_{sample} is the absorbance of ABTS radical + sample extract /standard.

Statistical analysis:

All analysis was done at least in duplicate/triplicates, and these values were then presented as average values along with their standard derivations. Subsequently, the IC_{50} values of the antioxidant assay were calculated from the linear regression method.

RESULTS AND DISCUSSION:

Extraction Yield

The extraction with solvents of increasing polarity resulted in extraction of phytocompounds of a plant according to their degree of solubility. The yield percent of hexane, methanol and water extracts obtained from various parts of *R. arboreum* was shown in Fig. 1. Methanolic extract of flower (11.225±1.14 %) showed highest % yield followed by water extract of leaf 10.5±0.4%, and water extract of flower (5.335±0.63%). Among all parts, bark showed least extract yield with all solvents. This shows that the extraction yield increases with increasing polarity of the solvent used in extraction.

The result of present study was in line with study from[26] on *Orthosiphon stamineus* and [27] on *Limnophila aromatica.*

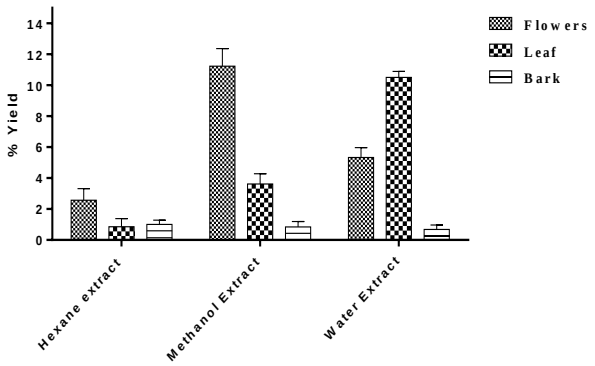

Figure 1: Percentage extraction yield in solvent extracts of different parts of *R. arboreum.*

Quantification of TPC in various solvents extracts of different parts of *R. arboretum:*

TPC was calculated from standard curve of gallic acid (y = 0.023x - 0.0054) and expressed as mg/g GAE. Order of TPC was methanolic extract of Flower (107.46±8.74 mg/g GAE)>methanolic extract of bark (70.29±13.45 mg/g GAE)>water extract of flower (64.93±8.36 mg/g GAE)>hexane extract of flower (48.08±1.06 mg/g GAE)>water extract of bark (37.42±14.42 mg/g GAE)>hexane extract of bark (21.02±1.08 mg/g GAE)>water extract of leaf (17.98±2.88 mg/g GAE)>hexane extract of leaf (13.64±0.77 mg/g GAE)>methanol extract of leaf (9.70±0.58 mg/g GAE) (Fig. 2). In contrast to our results,[28] showed higher amount of TFC in methanolic extract of leaves (150.0±0.00 mg/g RE per gram extract), flower (103.8±2.16 mg/g RE per gram extract) and bark (51.3±6.49 mg/g RE per gram extract). The amount of TPC in Ethanolic extract of flowers was 65.50 ± 1.12 mg GAE/ g). [29]

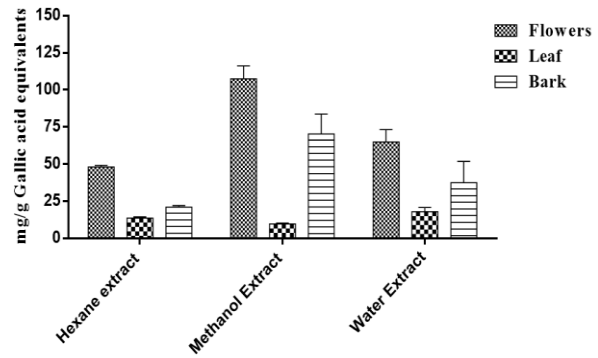

Figure 2: Total phenolic content in various solvents extracts of different parts of *R. arboreum.* Gallic acid was used as standard. Phenolic content was expressed as Gallic acid equivalents.

Quantification of TFC in various solvents extracts of different parts of *R. arboretum:*

Amount of flavonoid was calculated from standard curve of rutin (y = 0.0044x + 0.007) and expressed as mg/g RE. Order of TFC was methanolic extract of Flower (254.26±29.33 mg/g RE)>water extract of leaf (244.26±15.15 mg/g RE)>methanolic extract of leaf (155.114±2.41 mg/g RE)>)>hexane extract of flower (135.12±2.42 mg/g RE) hexane extract of flower (105.68±15.27 mg/g RE)>methanolic extract of bark (95.68±1.12 mg/g RE)>water extract of bark (60.52±12.45 mg/g RE)>hexane extract of bark (35.33±1.08 mg/g RE)>water extract of flower (29.83±6.03 mg/g RE (Fig. 2). The amount of TPC in various parts of *R. arboreum* was also quantified by [28]. In contrast to our results, they showed higher amount of TPC in methanolic extract of leaves (495.0±8.66 mg/g GAE per gram extract), flower (440.0±0.00 mg/g GAE per gram extract) and bark 240.0±0.00 mg/g GAE per gram extract). Ethanolic extract of flowers showed 33.25 ± 0.89 mg RE/g flavonoid content. [29]

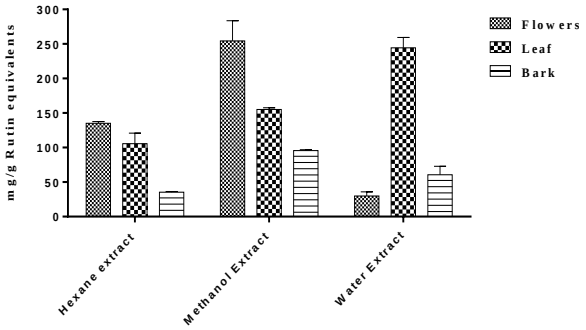

Figure 3: Total flavonoid content in various solvents extracts of different parts of *R. arboreum.* Rutin was used as standard. Flavonoid content was expressed as Rutin equivalents.

DPPH scavenging assay:

All the solvent extracts of different parts of *R. arboreum* showed concentration-dependent % DPPH radical scavenging activity (Fig. 4 A-C). Ascorbic acid was used as standard antioxidant compound. DPPH radical scavenging activity was expressed in terms of IC_{50} value. Among all extracts, methanolic (121.55±30.98 µg/ml) and water extract (141.98±3.7 µg/ml) of flower of *R. arboreum* showed least IC_{50}, indicating higher % DPPH radical scavenging activity of methanolic and water extract of flower. Least antioxidant activity in terms of %DPPH scavenging was identified in hexane extract of leaf (232.07±12.53 µg/ml) and water extract of bark (229.06±15.44 µg/ml). (Ascorbic acid showed IC_{50} value of 55.58±1.07 µg/ml with DPPH radical scavenging assay (Table-1).

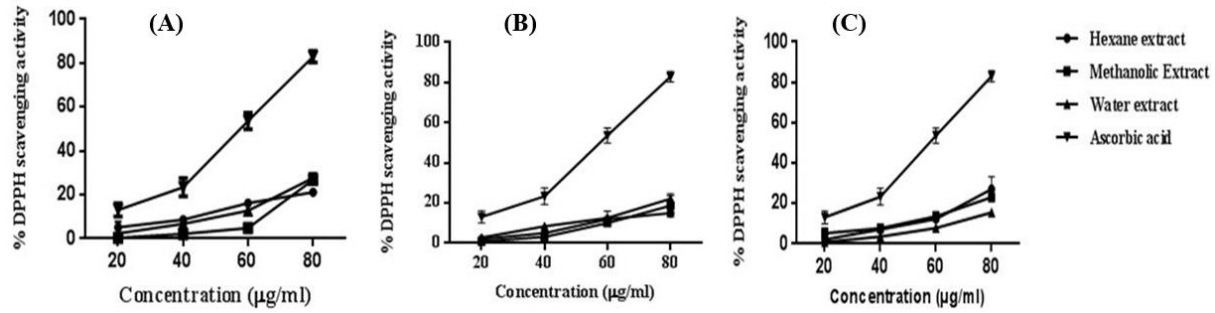

Figure 4: % DPPH radical scavenging activity of various extracts of different parts of *R. arboreum.* A: Flower; B: Leaf; C: Bark.

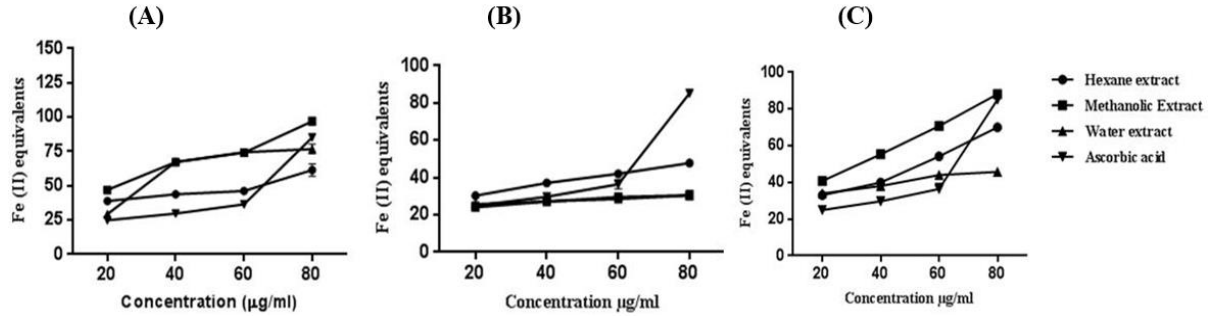

Figure 5: FRAP activity of various extracts of different parts of *R. arboreum.* A: Flower; B: Leaf; C: Bark.

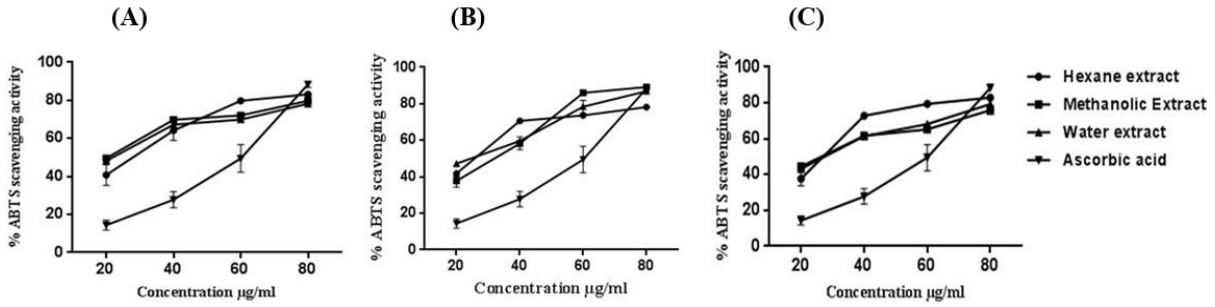

Figure 6: ABTS radical scavenging of various extracts of different parts of *R. arboreum.* A: Flower; B: Leaf; C: Bark.

FRAP assay

FRAP activity also increased with increase in concentration of solvent extracts of different parts of *R. arboreum* as shown in Fig. 5 A-C. Among all extracts, methanolic (22.76±0.39µM) and water extract (33.78±1.39 µM) of flower of *R. arboreum* showed highest FRAP activity as indicated by least value of IC_{50}. Least FRAP activity was shown by methanolic extract of leaf (333.52±30.14µM) and water extract of leaf (168.39±35.73µM). Ascorbic acid showed IC_{50} value of 56.29±1.97 µg/ml with FRAP assay (Table-1).

ABTS radical scavenging activity:

Fig. 6 A-C showed concentration dependent ABTS scavenging activity of different parts of *R. arboreum*. Ascorbic acid was used as standard antioxidant compound. Among all solvents extracts, methanolic (11.49±1.25 µg/ml) and water extract (15.64±1.26 µg/ml) of flower of *R. arboreum* showed highest ABTS radical activity as indicated by least value of IC_{50}. Methanolic extract of leaf (30.39±0.544 µg/ml) and water extract of bark (27.24±2.17 µg/ml). Ascorbic acid showed IC_{50} value of 54.05±2.40 µg/ml (Table-1).

Different part of *R. arboreum* contains high value flavonoids and phenolics which can be used in nutraceuticals and preventive medicines [28]. Ethanolic extract of flower of *R. arboreum* possesses maximum antioxidant and nitric oxide synthase (NOS) activation properties which might be utilized as promising source of therapeutics [30]. However, ethanolic extract of leaves of *R. arboreum* also have in vitro antioxidant activity as evidence by the low IC_{50} values in the scavenging of DPPH (19.91 ± 1.73 µg ml−1) and ABTS (9.50 ± 0.50) hydroxyl radical methods [31]. Methanolic extract of leaves (50%) also show antioxidant activity as it contains high phenol [32]. Antioxidant nature of leaves of *R. arboreum* was also in agreement with study from [33, 34] Antioxidant activity of ethanolic extract of flower was also determined by using DPPH (134.1±2.34 mM TE/g)

and FRAP assay (140.6±2.76 mM TE/g) by [35]. In the present study, flower extracts (methanolic and water) showed high antioxidant activity among all solvent extracts of various parts of *R. arboreum*, which may be due to higher amount of TPC and TFC in methanolic extracts of flower.

Table-1: Half maximal inhibitory concentration (IC_{50}) of different extracts of *R. arboreum* in different antioxidant assays.

Plant part	Type of extract	IC_{50}		
		DPPH*	FRAP**	ABTS*
Ascorbic acid		55.58±1.07	56.29±1.97	54.05±2.40
Flower	Hexane extract	184.01±4.9	57.04±6.58	25.82±7.09
	Methanolic extract	121.55±30.98	22.76±0.39	11.49±1.25
	Water extract	141.98±3.7	33.78±1.39	15.64±1.26
Leaf	Hexane extract	232.07±12.53	87.34±6.7	21.26±2.71
	Methanolic extract	192.51±38.18	168.39±35.73	30.39±0.544
	Water extract	174.02±4.98	333.52±30.14	23.83±0.30
Bark	Hexane extract	147.18±25.39	51.07±1.01	24.32±3.42
	Methanolic extract	178.61±21.53	39.27±4.08	25.79±1.11
	Water extract	229.06±15.44	98.58±16.44	27.24±2.17

Values are expressed as mean ± S.D. of three independent experiments.
*-µg/ml; **-µM Fe (II) equivalents

CONCLUSIONS:

It can be concluded from the present study that extraction yield was highest in methanol and water solvent. The results of present study showed highest antioxidant activity in methanolic and water extract of flower, thereby validating the traditional use of *R. arboreum* as source of natural antioxidants.

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CONFLICT OF INTEREST:

The authors declare no conflict of interest.

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RESEARCH ARTICLE

Serum levels of prostaglandin E2 (PGE2) and interleukin 17 (IL-17) are associated with Angiogenesis and Metastasis in breast cancer patients

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ABSTRACT:

Background: The link between chronic inflammation and tumorogenesis has been noted for a long time. Breast tumor cell-derived prostaglandin E2 (PGE2) induces the production of interleukin 23 (IL-23) that enhances IL-17 secretion. Permanent release of these molecules leads to augmentation of their serum concentrations that may be used as novel clinical markers for diagnosis and prognosis. The aim of this research was to evaluate levels of IL-17 and PGE2 in blood serum of breast cancer patients, and to determine whether these levels correlate with the disease progression **Material and Methods**: Pre-operative serum samples were collected from 41 patients with breast cancer and 28 patients with benign breast tumors. IL-17 and PGE2 levels were measured using ELISA. Furthermore, correlations between levels of these molecules and clinicopathological parameters were determined. **Results**: We demonstrated that serum levels of PGE2, but not IL-17, were significantly increased in breast cancer patients as compared with benign tumor patients. Moreover, both IL-17 and PGE2 levels were increased in cancer patients with angiogenesis and lymph node metastasis. A positive correlation was also identified between IL-17 and PGE2 levels. **Conclusion**: The present study indicates that elevated IL-17 and PGE2 serum concentrations are associated with angiogenesis and metastasis of breast cancer. So, our data suggest that IL-17 and PGE2 could be developed as potential biomarkers of breast cancer diagnosis and prognosis, however more comprehensive results are needed.

KEYWORDS: Breast tumor, prostaglandin E2, IL-17, angiogenesis, metastasis.

INTRODUCTION:

Breast cancer creates a significant public health problem worldwide, and metastasis is considered to be the cause of most deaths in patients[1].

Inflammation appears to be a necessity for both metastasis and elimination of tumor cells. Chronic inflammation has been associated with increased tumor incidence and linked to tumorogenesis[2].

Many human cancers exhibit elevated prostaglandin (PG) levels due to upregulation of cyclooxygenase-2 (COX-2), a key enzyme in eicosanoid biosynthesis[3]. Breast cancer cells secrete PGE2 that plays a role in tumor development and progression, probably through recruitment of leukocytes secreting a variety of cytokines, chemokines and angiogenic factors that promote tumor growth and metastasis[4].

Tumor secreted PGE2 induces IL-23 production in the tumor microenvironment leading to expansion of the newly-defined T helper 17 (Th17) cell subset[4]. IL-17, the hallmark cytokine of Th17, has important roles in protecting the host against extracellular pathogens, but conversely promotes inflammatory pathology in autoimmune disease[5]. In malignancy, IL-17 has a complex role by playing a dual role in the antitumor immunity. On one hand, IL-17 promotes an antitumor

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cytotoxic T cell response leading to tumor regression. On the other hand, studies have demonstrated that IL-17 plays an intricate role in tumorigenesis, proliferation, metastasis, and angiogenesis[6]. IL-17 has been shown to stimulate vascular endothelial cell migration and regulate production of a variety of proangiogenic factors, such as vascular endothelial growth factor (VEGF) and PGE2 (7). Moreover, evidence is now growing which supports the ability of IL-17 to act as a key diagnostic marker, differentiating between benign and malignant pathology. It could potentially be a significant tool in allowing early detection[8].

Therefore, in the present study we assessed the concentrations of IL-17 and PGE2 in blood serum of breast cancer patients and women with benign breast tumors, and their correlation with clinicopathological parameters of breast cancer.

MATERIAL AND METHODS:

The patients were admitted for surgery at Al-Mwasah Teaching Hospital (Damascus), during the period December 2018 – June 2019. Informed written consent was obtained from each patient prior to the specimen collection. 3ml venous blood samples were collected from patients according to the internal review and the Ethics Boards of the Syrian hospitals. Sampling was carried out before any clinical intervention such as chemotherapy, radiotherapy and surgery. Sixty nine female patients recently diagnosed with breast tumors were enrolled in the present study. The age range and the mean age of patients were 22-79 and 45.45±13.01 years respectively. Based on pathologic diagnostic assessment, patients were divided into 2 groups: patients with benign breast tumor (n=28) and patients with breast cancer (n=41). Pathological data [including: tumor grade, lymph node status, angiogenesis] were obtained from medical records of patients, validated by an experienced histopathologist, and summarized in Table 1.

Table 1. Histopathologic Information in Breast Cancer Patients

Factor	Frequency	Percentage
Tumor grade		
I	7	17.1
II	22	53.6
III	12	29.3
Lymph node metastasis		
Metastasic	18	43.9
Non- metastasis	23	56.1
Angiogenesis		
Angiogenic	14	34.1
Non- angiogenesis	27	65.9

Blood specimens were collected in sterile tubes for serum isolation. serum samples were separated from venous blood at room temperature and stored at -30°C until use. Later, serum was assayed by enzyme-linked immunosorbent assay (ELISA) for the presence of IL-17 and PGE2. Concentrations were measured using commercially available ELISA kits (IL-17 and PGE2 ELISA kit, Bender Med Systems, Austria). The procedure was done according to the manufacturers instruction as supplied with kits. The absorbance was read on an ELISA plate reader (Bio Tek, USA) at a wavelength of 450 nm and 405 nm for IL-17 and PGE2, respectively.

Statistical analysis:

All statistical analyses were carried out by SPSS software, version 23.0. Student *t* test was used to assess the statistical significance of difference in median between the 2 study groups. ANOVA test was used for multiple median comparison. For the analysis of correlation, person test was utilized. A P-value of <0.05 was considered to indicate a statistically significant difference.

RESULTS:

The concentration of IL-17 in serum samples of patients with breast cancer and patients with benign tumors was determined. Although comparison of 2 given groups indicated an increase of IL-17 in cancer patients, the increase was of no statistical significance (P >0.05; Table 2). Then, the concentration of PGE2 in serum specimens was measured. Comparison of 2 given groups revealed a significant increase of PGE2 in the serum samples of cancer patients (P = 0.001; Table 2). The serum samples from cancer patients revealed a positive statistical correlation between IL-17 and PGE2 levels (P = 0.011, r = 0.392; Figure 1). However, There was no statistical correlation between IL-17 and PGE2 levels in the serum of patients with benign tumors (P >0.05, r = 0.091; Figure 1).

Table 2. Comparison of IL-17 and PGE2 Concentration in Serum Samples of patients

	Patients	Number	Mean (pg/ml)	SD (pg/ml)	P value
IL-17	**Cancer**	41	1.53	4.16	0.057
	Benign tumor	28	0	0	
PGE2	**Cancer**	41	437.63	381.01	0.001
	Benign tumor	28	73.87	154.83	

There was significantly higher concentration of IL-17 in serum of cancer patients with angiogenesis and those with lymph node metastatis (P<0.05, Table 3). There were similar findings regarding PGE2 Levels. We observed significantly higher serum concentration of PGE2 in patients with angiogenic cancer and those with metastatis to lymph nodes (P<0.05, Table 4). However, neither IL-17 nor PGE2 had statistically significant correlation with the tumor grade of cancer patients (Table 3 and 4).

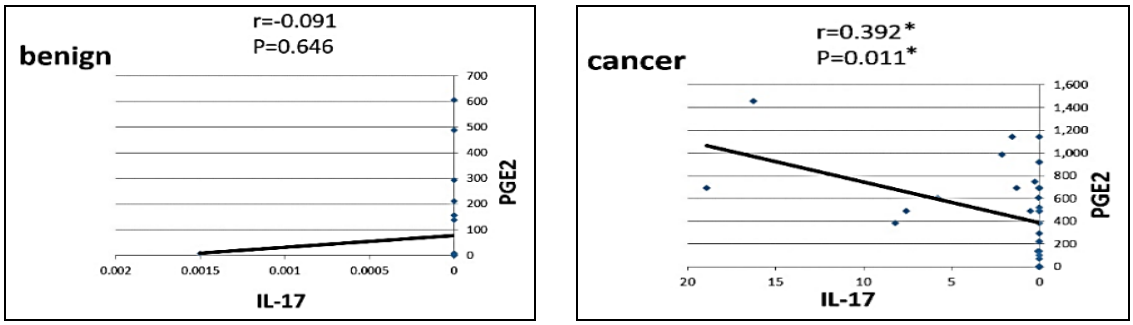

Figure 1. Correlation between serum levels of IL-17 and PGE2 in patients with breast cancer and patients with benign breast tumors. *There was a positive association between the levels of IL-17 and PGE2 in cancer patients. Levels were detected using ELISA and evaluated as pg/ml. IL: Interleukin, PGE2: Prostaglandin E2.

Table 3. The difference in median levels of serum IL-17 (pg/ml) in breast cancer patients according to clinicopathological parameters.

	number	Mean (pg/ml)	SD (pg/ml)	P value
Angiogenic	14	4.46	6.26	0.001[a]
Non- angiogenesis	27	0	0.02	
Metastasic	18	3.04	5.87	0.038[a]
Non- metastasis	23	0.35	1.23	
Grade I	7	0.32	0.64	0.886[b]
Grade II	22	1.94	4.55	
Grade III	12	1.36	4.69	

[a]P-value of student *t* test as appropriate. [b]P-value of ANOVA test as appropriate.

Table 4. The difference in median levels of serum PGE2 (pg/ml) in breast cancer patients according to clinicopathological parameters.

	number	Mean (pg/ml)	SD (pg/ml)	P value
Angiogenic	14	647.64	369.44	0.009[a]
Non- angiogenesis	27	328.74	345.01	
Metastasic	18	591.74	359.74	0.02[a]
Non- metastasis	23	317.02	359.59	
Grade I	7	324.16	289.7	0.761[b]
Grade II	22	404.28	350.25	
Grade III	12	528.09	466.38	

[a]P-value of student *t* test as appropriate. [b]P-value of ANOVA test as appropriate.

DISCUSSION:

In the present study, we assessed the abundance of two major inflammatory factors in serum samples from breast cancer patients and patients with benign breast tumors. We used ELISA and measured the levels of IL-17 and PGE2. We also used data from the patients' records to correlate our findings with clinicopathological results.

In the current study, the serum levels of Th17 cells-related cytokine (IL-17) in patients with breast cancer were higher than those in patients with benign tumors, but there was no significant difference between them. In contrast to these results, Rohani Borj et al. demonstrated significant differences in levels of serum IL-17 in breast cancer patients (n=56) as compared with benign tumor patients (n=60)[9]. Lyon et al. also revealed a significant increase in IL-17 serum concentration in breast cancer using Multiplex bead array assay[10]. However, most cancer patients in the study of Lyon and associates were at least in their 4th week postsurgery, while Mettler et al. reported that the time since surgery could have affected cytokine values in cancer patients[11].

In addition to IL-17, We identified significantly elevated concentration of PGE2 in serum of cancer patients compared with benign tumor patients. Our findings support the demonstrated role of COX/PG signaling in malignancy. Elevated COX-2 protein levels have been detected in approximately 40% of invasive breast carcinomas, with individual studies reporting frequencies ranging from 17% to 84%[3]. Moreover, a work of Mitsuhashi et al. indicated that mammary tumor cells (4T1) secrete many cytokines and lipid molecules, including PGE2[12].

Similarly to our results, an increased plasma levels of PGE2 has also been described in colorectal cancer group compared with benign group[13].

Differentiation of Th17 cells from naïve T cells is dependent on signals from TGF-β, IL-6, IL-21 and IL-23 in mice[14]. Importantly, IL-23 does not act on naïve T cells, but instead acts on T cells that are already committed to the Th17 lineage. IL-23 enhances the production of IL-17 and stabilizes the Th17 phenotype[15]. In humans, it seems that IL-23 enhances IL-17 secretion from Th17 cells[6]. Qian and coworkers demonstrated that breast cancer cell-secreted PGE2 enhanced IL-23 production and tumor-associated Th17 cell development[4]. Importantly, According to our study, the serum levels of PGE2 showed a moderate positive correlation with IL-17 levels in serum of breast cancer patients. These results suggest that PGE2 may play a pivotal role in the regulation of IL-17 secretion in breast cancer. However, we did not find any significant correlation when we examined these two factors in serum of patients with benign tumors.

Angiogenesis has received a lot of attention due to its influence on the tumor grade, metastasis, and therefore patients' prognosis[8]. Numasaki et al. found that Il-17 up-regulates production of a variety of proangiogenic factors, such as vascular endothelial growth factor

(VEGF), prostaglandin E1 (PGE1) and PGE2, by fibroblasts as well as tumor cells. IL-17 also promotes angiogenesis through stimulation of vascular endothelial cell migration and cord formation, resulting in tumor progression[7]. A study of Benevides et al. in 2013 suggested that IL-17 may shift the balance between angiogenic and angiostatic factors toward conditions that promote angiogenesis and tumor development of invasive ductal carcinoma (IDC)[16]. In agreement with these findings, our results revealed that serum levels of IL-17 in breast cancer patients with angiogenesis had been significantly higher than levels in cancer without angiogenesis.

Benevides et al. in 2015 uncovered the mechanism by which the proinflammatory cytokine IL-17 promotes metastatic mammary primary tumor progression; this mechanism is neutrophil dependent. These results support the idea that IL-17 could serve as a biomarker for the prognosis of aggressive breast cancer[17]. According to the present data, our results revealed that serum levels of IL-17 in breast cancer patients with metastasis to lymph nodes had been significantly higher than levels in cancer without metastasis.

Regarding PGE2, we observed significantly higher serum concentration of PGE2 in patients with metastatis to lymph nodes. Our results were in agreement with evidence from studies in vitro that E-series of prostaglandin (EP) receptors might contribute to metastatic behavior of human cancer cells and murine models of cancer[18-20]. An EP receptor expression study by Chang et al. in 2004 indicated that all four subtypes (EP1-4) are expressed in different stages of mammary gland development[21]. Of particular note, It has been shown that breast cancer cells migrate in response to PGE2, and EP4 function was particularly important to migration[18].

In addition, we found a significant increase in serum levels of PGE2 in cancer patients with angiogenesis as compared with those without angiogenesis. A previous data strongly suggested that PGE2 secreted by COX2-expressing mammary tumor cells may promot tumor angiogenesis by inducing many proangiogenic factors[21].

Amano and associates indicated that host stromal PGE2-EP3 signaling appears critical for tumor-associated angiogenesis and tumor growth in a mouse tumor implantation model. They also observed that EP3 signaling pathway is relevant to the induction of a potent proangiogenic growth factor, VEGF[22].

In summary, the present study indicates that preoperative serum PGE2 levels were higher in breast cancer patients as compared to those in benign breast tumor patients. These higher levels could be attributed to tumor-related inflammation and progression. Moreover, PGE2 and IL-17 serum concentrations were associated with breast cancer metastasis and angiogenesis. Thus, these two inflammatory factors may be developed as potential diagnostic and prognostic cancer biomarkers.

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RESEARCH ARTICLE

Awareness of Radiation protection among Dental surgeons in south Chennai -A Questionnaire study

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ABSTRACT:

Aim and Objectives: To assess the attitude and awareness about radiation protection among dental surgeons in south Chennai. **Materials and Methods:** The study participants comprised 150 dental practitioners (general and speciality) in south Chennai. The information was collected from each participant through structured questionnaires regarding attitude and awareness towards radiation protection. Pearson correlation coefficient test was used to assess the validity and reliability of questionnaire, and any $P \leq 0.05$ was considered as statistically significant. **Results:** Of all the 150 dentists enrolled in the study, 69% dental surgeons considered X-ray films as a image receptor. However, majority of the dentists (73%) failed to acquire informed consent of the patient before prescribing dental radiograph. while Majority of dentists were unaware of position and distance rule (54%) and 46% were aware of the rule, it was also found that 59% dental surgeons did not use film holding device and only 41% used film holding device. Majority of them (59%) were aware of AERB certification and 41% were unaware of AERB, 15% of them hold the cone during exposure **Conclusion:** The current study shows the utmost need for further implementation of radiation protection principle among dental surgeons in south Chennai. Majority of them did not practice radiation protection procedures. Emphasis on radiation safety is mandatory for continuing professional education and development of radiographic selection criteria should be recommended.

KEYWORDS: Awareness, radiation protection, dental surgeons, Attitude.

INTRODUCTION:

Radiation hazards evaluation is very important in justifying and ensuring protection. With such evaluation, safe limits of radiation can be evaluated. Radiation exposure limits were introduced by the International Commission on Radiological Protection (ICRP), which was founded in 1928. In India, Atomic Energy Regulatory Board (AERB) is the competent authority. It implements safety provision by Atomic energy Act, 1962. It should be constantly monitored if the radiation protection comes foremost in radiography. The goal of radiation protection procedure is to minimize the exposure of the operator, office personnel, and patient during radiographic examination.

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In dentistry, it is mainly used for diagnostic purposes, and in a dental set-up, usually the practicing dentist exposes, processes, and interprets the radiograph. Even though such exposure is less, it is critical to reduce exposure to the dental personnel and patients to prevent the harmful effects of radiation[(1)].

Biological hazards are classified based on occurrence probability into nonstochastic and stochastic effect [(2).] The radiographic examination carried out in all fields of medical services and dental services contributes the promotion of health, both individually and nationally. In dental practice radiographic examination plays an essential part though certain amount of radiation is delivered inevitably to the patients. It should be as low as reasonably achievable (ALARA). The practicing dentist differs from medical colleagues as he exposes, processes, and interprets the radiograph. Although exposure is minimal it is very important to reduce radiation to avoid the accumulated dose to the dentist in

their lifetime.

MATERIALS AND METHODS:

A questionnaire study was carried out among 150 dental surgeons in south Chennai. A specially prepared structured questionnaire was used to assess the attitude and awareness about radiation protection among dental surgeons in south Chennai. The questionnaires could be returned by mail or in-person. The dentists were informed about the anonymous processing of the questionnaires. A total of 15 questions were included. Statistical analysis was performed using Pearson correlation coefficient test to know the validity of the questionnaire and any $P \leq 0.05$ was considered statistically significant.

RESULTS:

Of all the 150 dentists enrolled in the study, 69% dental surgeons considered X-ray films as a image receptor (figure 1). It was observed that 63% of dental surgeons made less than 5 exposure per day (figure 2). Majority (45%) of them use X -ray to view the periapical region (figure 3). Majority of dental surgeons sent the patient for CT or CBCT to view extension of cyst and tumour (34%), implant planning (30%), for endo and ortho treatment (26%) (figure 4).

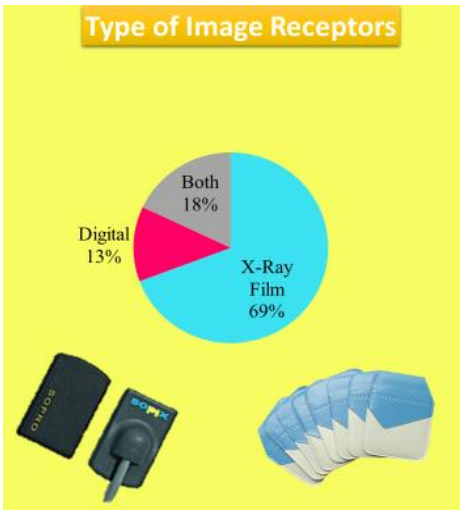
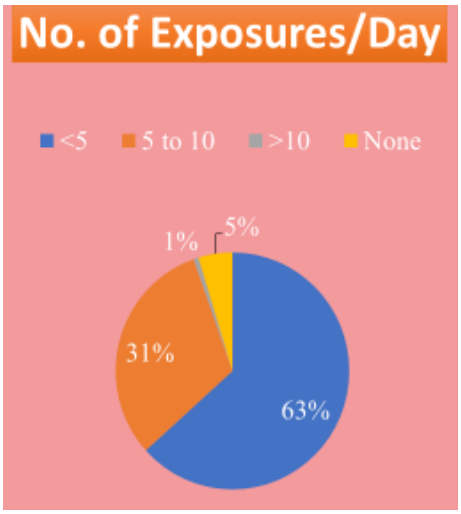


Figure 1



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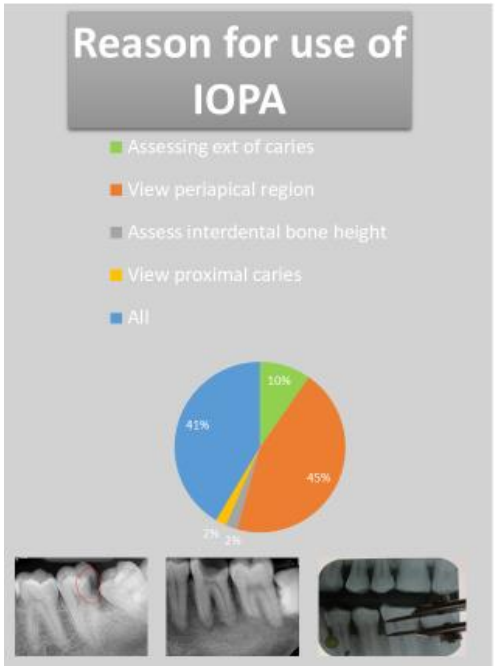

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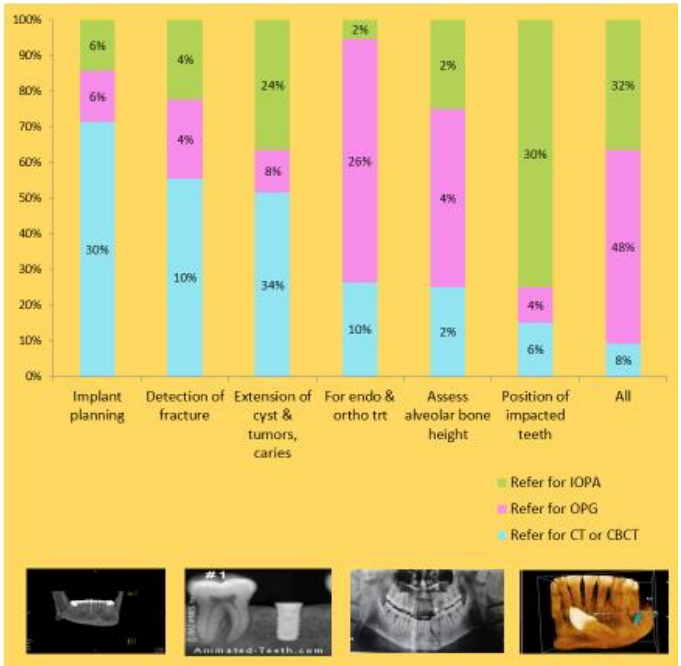

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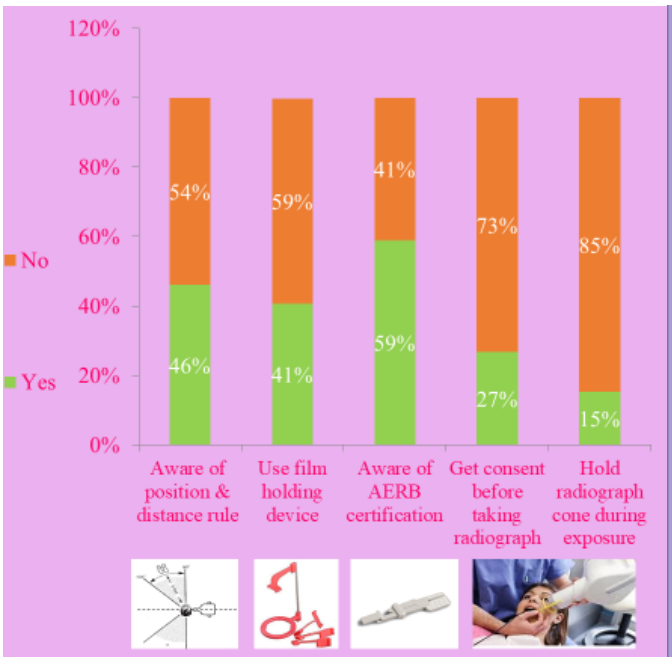

Figure 5

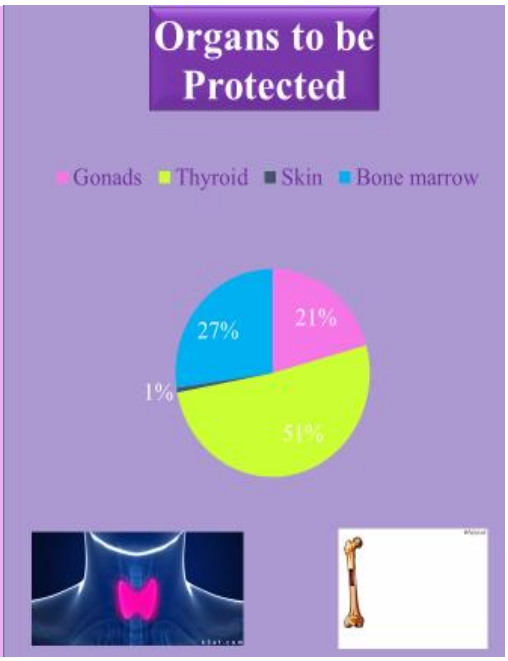

Figure 6

Figure 7

Figure 8

However, majority of the dentists (73%) failed to acquire informed consent from the patient before prescribing dental radiograph. Majority of dentist were unaware of position and distance rule (54%) and 46% were aware of the rule. While 59% dental surgeons did not use film holding device only 41% use film holding device. Many dentists were aware of AERB certification (59%) and 41% were unaware of AERB. 15% of the dentists hold the cone during exposure (Figure 5).

Majority of dental surgeons (51%) were aware of the most commonly protected organ was the thyroid gland during dental X-ray exposure (figure 6). A less percentage of dentists (44%) used lead aprons and thyroid collars to cover their patients during radiographic examination while 50% did not use any protective measure during exposure (figure 7) and also less percentage of dentist (23%) use TLD badge as a personal monitoring device during X ray exposure (figure 8).

DISCUSSION:

The effect of ionizing radiation on living system is well known and well documented. The biologic interaction between ionizing radiation and living organism leads to changes in the electron level immediately within a fraction of seconds of exposure and persist for varied period of time Practitioners who administer ionizing radiation must be familiar with the magnitude of radiation exposure encountered in dentistry, the possible risk that such exposure entails, and the methods used to reduce doses. This information provides the necessary background for explaining the concerned patients the benefits and possible hazards involved with the use of X-rays[(3)].

In our present study majority of dental surgeons were using X-ray film as a image receptor only 13% of them were using digital sensor , which was consistent with the other studies conducted by İlgüy et al., [4] Math SY et al, [5] and Kaviani et al. [6] .73% dental surgeons did not take informed consent before taking radiographs, because majority of them were unaware of the importance of explaining the radiation risk and hazards to the patient.

ADA strongly recommends lead apron and thyroid collar, because lead aprons should be used to minimize patient exposure to radiation, but in our study only 44% dental surgeons use lead apron in their day to day practice. Similar study conducted by Asha et al in coorg, India [7] showed 40% dental surgeons wearing lead apron and in other study conducted by Amanpreet K, et al [8] in Lucknow 38.6% dentist were using lead apron.

Personal monitoring device were used to measure the exposure of operator or associated personal as a protective measure but 76% of dental surgeon did not use any Personal monitoring device which is not in accordance with the study done by Math SY et al.[5] and Kaviani et al.[6]. A strict adherence to what has been termed the position and distance rule is required to reduce the X-ray exposure to the dental surgeons according to which the operator should stand 6 feet from the patient at an angle of 90 to 135 degree to the central x ray of the x ray beam, but 46% of dental surgeon were aware of position distance rule in our study, other similar studies conducted by Asha et al[7] showed 34.4% dental surgeons following this rule.

Film holding device should be used because they improve the alignment of film thereby decreasing the unnecessary exposure and retake of film, but 41% of dental surgeons were only using film holding devices similar to the study conducted in Lucknow and Coorg India[7,8]

CONCLUSION:

The current study shows the utmost need for further implementation of radiation protection principles among dental surgeons in south Chennai. Majority of them did not practice radiation protection procedures. Emphasis on radiation safety is mandatory for continuing professional education, and development of radiographic selection criteria should be recommended.

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RESEARCH ARTICLE

Green synthesis and Characterization of Silver Nanoparticles with Rhizome extract of *Curcuma longa* (AgNPs-RECL) for Antimicrobial activity towards *Xanthomonas* and *Erwinia* species

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ABSTRACT:

Background: Nanotechnology is one the most crucial areas of research with a wide biomedical application including drug delivery. Conventionally physical, and chemical methods have been used for the synthesis of metal-based nanoparticles (NPs) however, they show cytotoxicity and even not better for sustainable environment. Therefore, green synthesis of NPs using plant extract is a better alternative. Herein, we report the synthesis, characterization, and antimicrobial activity of silver nanoparticles with rhizome extract of Curcuma longa (AgNPs-RECL). **Objective:** To perform green synthesis of AgNPs-RECL, followed by their characterization via scanning electron microscopy (SEM), ultraviolet (UV)-visible spectroscopy, X-ray diffraction (XRD), Fourier transform-infrared spectroscopy (FT-IR), and high-performance liquid chromatography (HPLC). Further, antimicrobial activity of AgNPs-RECL was examined using disc diffusion test. **Result:** AgNPs-RECL showed antimicrobial activity towards two plant pathogenic bacteria Xanthomonas anoxopodis and Erwinia amylovora. **Conclusion:** It was concluded that AgNPs-RECL have the potential to inhibit plant pathogenic bacteria like Xanthomonas and Erwinia, which could be useful for nano drug delivery applications.

KEYWORDS: Nanoparticle, *Curcuma longa*, Silver nanoparticle, Antimicrobial activity, *Erwinia*, and *Xanthomonas*.

INTRODUCTION:

In the area of material science, nanotechnology has been explored with a variety of promising approach at molecular as well as cellular level for various biomedical studies including nano drug delivery. However, owing to several limitations of nanoparticles (NPs) based delivery system, including cytotoxicity, there is a demand for an alternative method. In the prevailing scenario, the application of green synthesis method for the synthesis of metal-based NPs has been found to be a boon for various reasons, such as minimal generation of waste, and implementation of sustainable process, leading the production of an ecofriendly NPs.

Metal based green synthesis is more important because of their chemical, optical, photochemical as well as their electronic properties[1]. Among several metals, silver has gained a huge attention for green synthesis of NPs because of its enormous application in various industries including in nonlinear optical, biolabeling, and antibacterial capacity. Silver nanoparticles (AgNPs) are widely applicable in various fields like in drug delivery[2], nanomedicine[3], agriculture, cosmetics and most importantly as antimicrobial agent[4] though many reports revealed that AgNPs cause toxicity[5], but still it plays a major role as disinfectant as well as antimicrobial agent. So, after going through various reports as well as data documented an interest in the field encouraged us to use biological method for the synthesis of AgNPs. Various methods like chemical, physical as well as biological method can be used for synthesizing AgNPs among all three, biological method shows better outcome. Synthesis of AgNPs-with rhizome extract of *Curcuma longa* (RECL) completes in three steps: first the solvent

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medium selection, second reducing agent, and the last non-toxic stabilizers for AgNPs[6]. After the synthesis of AgNPs-RECL, their characterization was accomplished through Fourier-transform infrared spectra (FT-IR), ultraviolet (UV)-visible spectroscopy, X-ray (XRD) diffraction, and scanning electron microscopy (SEM).

In general, medicinal plants are said to be important source owing their pharmacological effects, including as anti-infectious agent[7]. Various plant parts are used to serve the purpose of medicine including root, rhizome, and leaf stem. Huge antibiotic resistance against the available antibiotics due to the evolution of bacteria, antimicrobial compound has been searched. Extract of plant are used as antibacterial, antifungal, and antiviral agents. According to various report 400,000 and above species of flowering plant bears medicinal property in them therefore traditional medicine produced by these medicinal plants are said to be cheaper than the modern medicine[8]. Due to various medicinal properties traditional medicine are investigated for the antimicrobial activity. *Curcuma longa* is the medicinal plant which belongs to *Zingiberaceae* family[9] and is commonly called turmeric. Important component of turmeric is curcuminoids that includes curcumin (diferuloyl methane), demethoxycurcumin, and bisdemethoxycurcumin[10]. Biological activity of turmeric depends upon the most important phytocompound Curcumin. It is said to be soluble in ethanol as well as acetone but insoluble in water[11].

For Antimicrobial activity of *C. longa* oil against different culture such as *Staphylococcus albus*, *S. aureus* and *Bacillus typhosus*, that inhibits the growth of *S. albus* and *S. aureus* were used in different concentrations[12]. Therefore, it is concluded that turmeric has huge antimicrobial property so keeping in mind *C. longa* rhizome was used against various plant pathogenic microorganism like Xanthomonas, *and Erwinia species* on which in-detail studies are yet to be done on the rhizome of *C. longa*.

MATERIAL AND METHODS:

Collection of the Plant root:

Curcuma longa rhizome was collected from Nursery, Patna and its taxonomic identification was done at Botanical Survey of India, Howrah, Kolkata**.** The plant rhizome was washed properly to remove impurities. Later it was dried for a week so that moisture can be removed completely. Further it was powdered and stored in container until use.

Green Synthesis of Silver Nanoparticles with rhizome of *C. longa*:

20ml of distilled water was added to 2gm of *C longa* rhizome powder and was mixed well, then centrifuged at 10,000rpm for about 10 minutes, the supernatant was taken out, from which 10ml of extract was mixed with 90ml of aqueous solution of 5mM Silver nitrate solution ($AgNO_3$) so that silver nitrate can be reduced into Ag+ ions. It was incubated in static condition. The colour change from pale yellow to dark brown suggested the synthesis of AgNPs.[13] Later it was characterized through UV-Visible spectroscopy, FT-IR, XRD, SEM. Further quantitative analysis of Curcumin (which is the most important bio-active compound) was performed through HPLC.

UV-Visible Spectroscopy:

Initial characterization of AgNPs-RECL was done through UV-visible spectroscopy**.** The absorbance was taken between the range of 200-800nm. It was monitored by UV–Vis spectrometer (Systronics, Model 117 available at Central Research Laboratory, Patna Women's College).

FT-IR:

AgNPs binding property which was synthesized by *C longa* rhizome was investigated through Fourier Transform Infrared (FTIR) Spectroscopy. Pellet of dried and powdered Ag NPs was made with potassium bromide (KBr).[14] Further the spectrum was recorded between the wavelength ranging from 600-4000cm^{-1} by (Bruker Alpha, Model No. ECO-ATR available at Central Research Laboratory, Patna Women's College)

XRD:

Measurement of X-Ray Diffraction (XRD) was recorded on (Bruker Alpha, Model No. B8 available at Cytogen Lab, Lucknow, Uttar Pradesh, India). AgNPs were dried first at 60^0C and the dried powder was analyzed on XRD.[15]

SEM:

With the help of Scanning Electron Microscope (SEM), the sample (AgNPs-RECL**)** size and morphology was observed. Before the analysis of structure, the AgNPs-RECL were freeze. Sample thin film was prepared on the grid coated copper further it was dried under mercury lamp for 5 minutes and analysis was done through SEM **(**FEI Quanta 250 available at the Central Drug Research Institute (CDRI), Lucknow, Uttar Pradesh, India).

HPLC:

HPLC analysis of AgNPs-RECL was performed by HPLC system (Shimadzu available at Cytogene Lab, Lucknow). *C longa* rhizome powder was kept away from light and humidity before analysis. The solvent used was Acetonitrile, Methanol, distilled water in the ratio of 70:25:5, the eluent was analyzed at 430 nm wavelength. The column used (Chiralpak IB) was having a size of (4.6mm × 250mm × 5µM) which was maintained at the temperature 25^0C. Injection volume was 20μL having a flow rate 1.5ml/min.

Plant Pathogen bacterial sample:
The bacterial strain was obtained from Microbial Type Culture Collection (MTCC), The Indian Agricultural Research Institute (IARI), New Delhi. The sample procured were *Xanthomonas anoxopodis* (BD0001) and *Erwinia carotovoran* (BL0001).

Assessment of Antibacterial Activity:
For Antibacterial activity of AgNPs two plant pathogenic microorganism were taken, they are *Xanthomonas anoxopodis* and *Erwinia carotovora*. Disc diffusion method was used for antibacterial activity.[16] Whatman No. 1 filter paper was punched uniformly, sterilized and then were impregnated with Crude extract, Antibiotic (Ampecillin), AgNPs and AgNPs along with antibiotic separately.

Preparation of Nutrient Agar:
11.5gm of synthetic nutrient agar was dissolved in 500ml of distilled water. 2 spatulas of agar were added to the solution. The mixture was boiled then autoclaved at 121^0C for 15 minutes.

Preparation of Inoculum:
Serial dilution technique was applied in which master suspension upto 10^{-5} was done. For this six-test tube was taken (for each bacterial strain) in which 9ml of distilled water was poured and sterilized by autoclaving for 15 minutes at 121 C. Further loop of each bacterial strain was inoculated into the first test tube, mixed well with the help of vortex shaker. Then from the first test tube 1ml of diluted suspension was taken out and poured to the next. Same process was repeated upto the dilution suspension 10^{-5}.

Inoculation of the plate:
1ml of diluted suspension (10^{-5}) was taken in the micropipette and dropped on the NA (nutrient agar) plate and evenly swirled with the help of cotton swab.

Loading of Disc:
After swabbing, the plates were kept at the room temperature for 10-15 minutes so that the surface moisture can be absorbed properly before loading the disc impregnated with crude extract, antibiotic (Ampicillin), AgNPs and AgNPs along with antibiotic. After 10-15 minutes it was loaded on plates with the help of sterilized forceps under laminar air flow and then the plates were placed in the incubator for 24 hours at 37^0C.

RESULTS:

Chemical and physical methods are used for synthesis of nanoparticles, but the stability of the product does not exist[17,18]. Therefore biological method for synthesis of NPs became an alternative method for conventional approach, which is more biocompatible and environment friendly as well as cheap.[19,20] According to result aqueous extract of the rhizome of *C longa* pale yellow **(Fig 1B)**, and the solution of $AgNO_3$ was colourless **(Fig 1C)**. After mixing rhizome extract with AgNO3 (1mM) in the ratio of 1:3 **(Fig 1D)** gave result in form of dark brown solution after 24 hr (Fig 1E). The change in colour indicated the evidence of synthesized silver nanoparticle which results due to excitation of Surface Plasmon vibrations which shows formation of AgNPs.[21] At the end synthesized ANPs-RECL were examined through SEM, that showed spherical morphology within the nanometer size range **(Fig 1E)**.

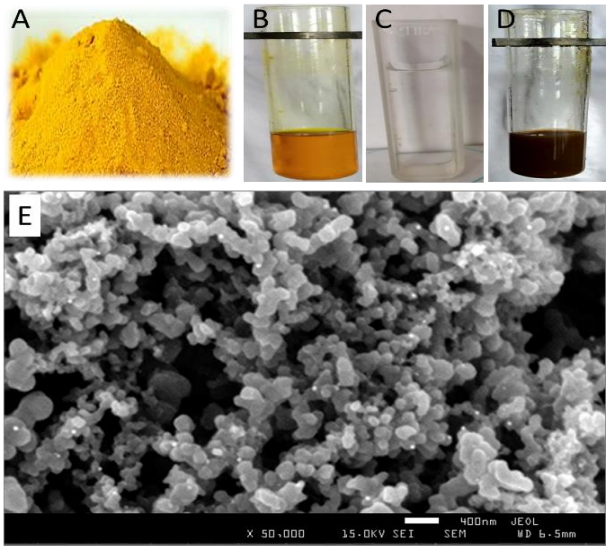

Fig. 1 Green synthesis of AgNPs-RECl.
Representative figure of (A) dried powder of *C longa* rhizome extract. (B) Aqueous extract of *C longa* rhizome (C-E) Respective colour change indicating the synthesis of AgNPs. (C) AgNO3 showing clear solution (D) reaction mixture containing 1 mM AgNO3 and rhizome extract of *C longa* in ratio of 1:3 ratio (E) Change in colour from pale yellow to dark brown after 24 hours showing AgNPs synthesis. (F) Representative SEM image of AgNPs-RECl (Scale bar: 400 nm).

Characterization of AgNPs-RECl by UV-Visible spectroscopy, XRD and FT-IR analysis:
For the identification of synthesized nanoparticle by rhizome extract of *C longa* which was produced through green method, characterization was done via UV-visible spectroscopy, X-ray diffraction (XRD) analysis and FT-IR analysis **(Fig. 2A-C)**. In case of UV-Visible spectrum the peak was observed at 455nm which clearly indicated the particles were polydisperse. (**Fig 2A**). Due to the surface plasmon, the AgNPs-RECL are said to exhibit UV-Visible absorption at its maximum in the range of 400-500nm[22]. XRD analyze both the molecular as well as crystal structure, analyze particle size, and does qualitative identification of different compounds[23].

XRD analysis showed peaks at 2θ value at around 31.7°, 34.3°, 36.2°, 47.4°, 56.5°, 62.7°, 66.2°, 67.9°, 68.9°, 72.5°, 76.8^0 corresponding to interplanar spacing (d) as

0.56 Å, 0.39 Å, 0.59 Å, 0.79 Å, 0.62 Å, 0.71 Å, 1.12 Å, 0.69 Å, 0.83 Å, 0.45 Å, 0.97 Å respectively as per Bragg's law (**Fig. 2B**), this confirms the crystalline nature as the peak intensity shows the crystallinity of high degree for the AgNPS-RECL through green synthesis method. Similar pattern of XRD result is observed by different scientist for AgNPs-RECL through green method[24,25]. AgNPs synthesized by rhizome extract of *C longa* was further characterized by FT-IR that showed peaks at the wavelength 3413.16 cm^{-1}, 1706.47 cm^{-1}, 1647.03 cm^{-1}, 1423.73 cm^{-1}, 1360.50cm^{-1}, 1222.96 cm^{-1} respectively which shows the presence of alcohol, aldehyde, alkene, alcohol, phenol and vinyl-ether functional group. (**Fig 2C**).

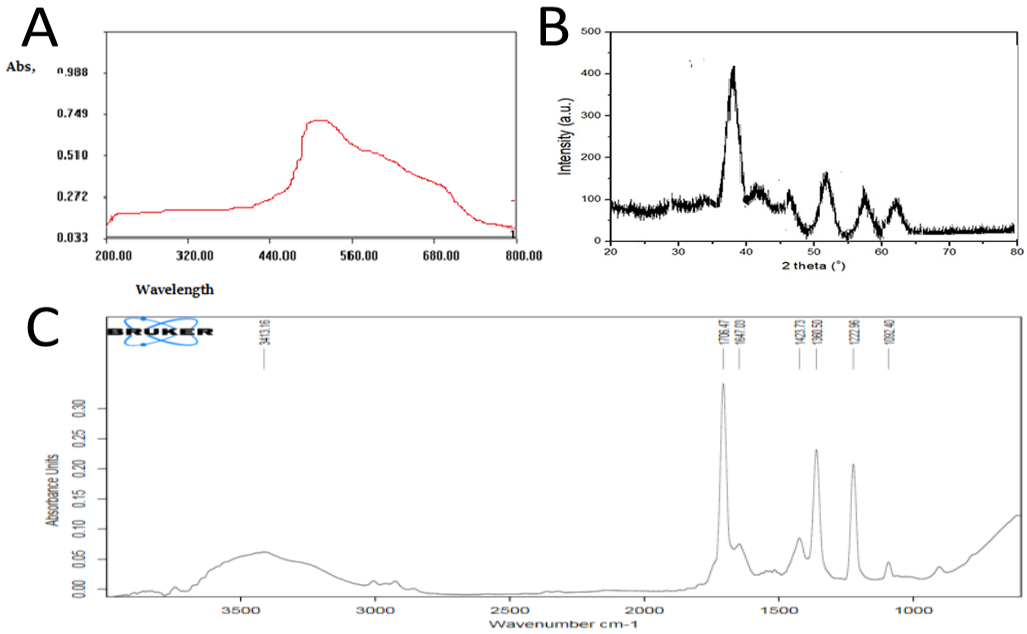

Fig. 2. Characterization of AgNPs-RECl.
(A) UV-visible absorbance spectrum in the range of 200-800 nm of wavelength, (B) XRD spectrum, and (C) FT-IR spectrum, indicating the produced AgNPs-RECl produced by green synthesis.

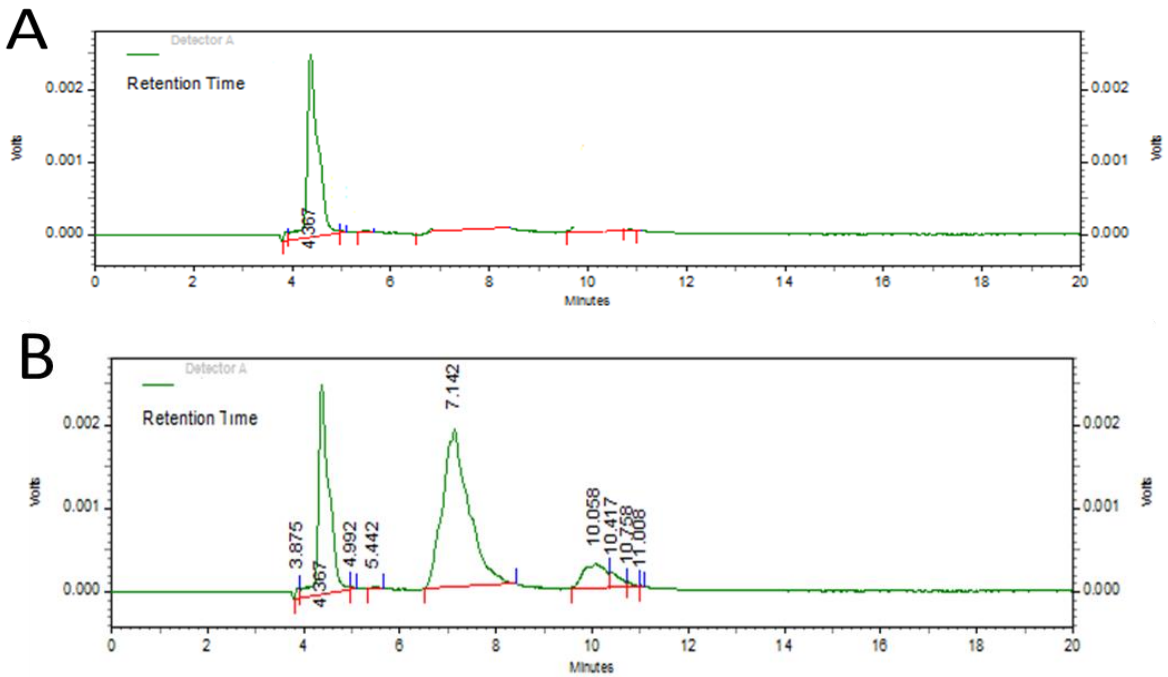

Fig. 3. Quantitative evaluation of Curcumin in the rhizome extract of *C longa* by HPLC analysis.
Representative HPLC chromatogram of (A) standard Curcumin, showing peak at the retention time of 4.367 min, (B) rhizome extract of *C longa*, showing peak at the retention time of 4.367 min.

Rhizome of *C longa* extract was analyzed for its important phytocompound which is responsible for antimicrobial property that is curcumin [26,27,28,29], through HPLC and it was compared with the HPLC chromatogram of standard Curcumin **(Fig 3 A-B).** Symmetrical peak of reference standard curcumin (around 99%) was obtained at the retention time of 4.367min and in the rhizome of *C longa* extract it was estimated to be 49% with same retention time that is 4.367 min. (**Fig 3A-B**)

AgNPs-RECL showed antimicrobial activity towards plant pathogenic bacteria:

In this study antibacterial activity of AgNPs and antibiotic was investigated against two plant pathogenic gram negative bacterias *Xanthomonas anoxopodis* and *Erwinia carotovora*. 12µl of AgNPs, AgNPs along with antibiotic (ampicillin), antibiotic alone and crude extract alone was loaded on sterile disc. The crude extract as well as antibiotic alone was used as control. Further zone of inhibition was recorded **(Fig 4 A, B).** In case of both the pathogenic strain crude extract showed very less zone of inhibition.

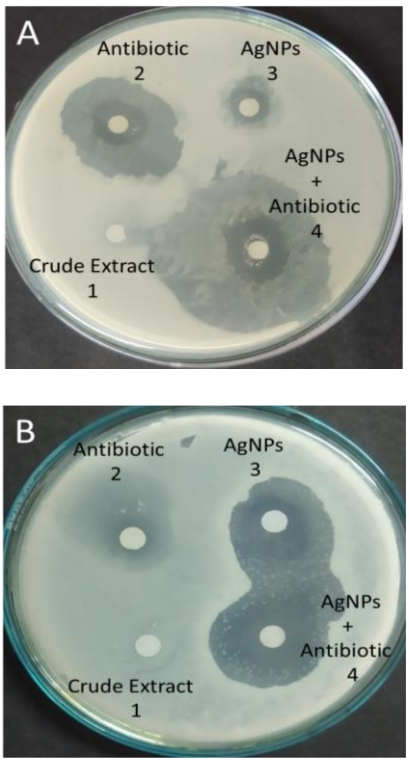

Fig. 4. Antibacterial effect of AgNPs-RECL against plant pathogen (A) *Xanthomonas anoxopodis* (**A1** Crude extract, **A2** Antibiotic, **A3** AgNPs, **A4** AgNPs along with antibiotic) and **(B)** *Erwinia carotovora* (A1 Crude extract, A2 Antibiotic, A3 AgNPs, A4 AgNPs along with antibiotic)**.**

Maximum zone of inhibition in case of antibiotic was 1mm for both *Xanthomonas anoxopodis* and *Erwinia carotovora* respectively. The AgNPs showed maximum zone of inhibition in case of *Xanthomonas anoxopodis* was 0.5mm and in case of *Erwinia carotovora* it was 1mm.

The maximum zone of inhibition for AgNPS in combination with antibiotic was 1.5mm for *Xanthomonas anoxopodis* and 1mm for *Erwinia carotovora.* In case of one selected strain of bacteria that is *Xanthomonas anoxopodis* combination of antibiotic and AgNPs showed the synergistic effect in form of increased zone of inhibition when compaired with AgNPs and antibiotic alone. But in case of *Erwinia carotovora* the zone of inhibition was same for all three that is Antibiotic, AgNPs alone and AgNPs in combination of antibiotic. Therefore, the result reveals that AgNPs when associated with antibiotic shows better antibacterial activity.

DISCUSSION:

Synthesis of nanoparticle with the help of chemical and physical approach has been extensively studied all over the world, however they are toxic to the environment and expensive economically[32], so biological approach came into existence for synthesis of AgNPs with the help of plant, microorganism and enzyme that suggest possible environment friendly alternative[33]. The reduction of $AgNO_3$ is done by the action of reducing agent (extract of plant) which form silver nanoparticle that is further stabilized by the bioactive compound of biological extract which form stable silver nanoparticle. The antibacterial activity of silver nanoparticle is influenced by the particle size which means smaller the particle size is greater will be antimicrobial activity[34]. In the study we took *Curcuma longa* rhizome which has been used traditionally as antimicrobial agent[35]. Different reports reveled that *C longa* has one of the important constituent Curcumin along with other phytocompounds that shows antibacterial, antiviral, antifungal as well as antimalarial activity. Due to their antimicrobial activity they are accessed by clinical trials in human[36,37]. So we for the first time demonstrated antimicrobial activity of green synthesized AgNPs-RECL on two plant pathogenic bacteria *Xanthomonas anoxopodis* and *Erwinia carotovora.*

CONCLUSION:

Overall study developed environment friendly and convenient green approach for synthesizing silver nanoparticle. Confirmation of synthesized nanoparticle was first was done based on colour change from pale yellow to dark brown. Then after further characterization was done through SEM, UV-Visible spectrum that showed the absorption peak at 455 nm, XRD spectrum, FT-IR spectrum. The quantitative analysis of active phytocompound 'Curcumin' was confirmed using HPLC. Most importantly we for the first time performed antimicrobial activity of *C. longa* rhizome extract against

two plant pathogenic microorganism *Xanthomonas anoxopodis* and *Erwinia carotovora* through disc diffusion method.

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CONFLICT OF INTEREST:

Authors declears there is no conflict of interest regarding paper publication.

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RESEARCH ARTICLE

Evaluation of Antibacterial and Antioxidant activities of *Tribulus terrestris* L. Fruits

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ABSTRACT:
The present study comprises screening some phytochemical compounds, antimicrobial activity and antioxidant activity in methanolic and aqueous extracts of *Tribulus terrestris* L fruits. The phytochemical analysis revealed the presence of alkaloids, Tannins, Glycosides, Flavonoids, Saponins, and Phenols. A well-diffusion method determined the antibacterial effect of *T. terrestris* extracts against both Gram-positive (*Staphylococcus auras* and Methicillin-resistant *Staphylococcus aureus* ATCC43300 (MRSA), and Gram-negative bacteria *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Klebsiella pneumoniae*, and *Escherichia coli* ATCC25922. The methanolic extract was the most active extract against tested bacteria with a minimal inhibitory concentration (MIC) value of 6.25mg/ml. An antioxidant activity using DPPH assay showed that IC50 of methanolic and aqueous extracts were 3.94µg/ml and 22.45µg/ml, respectively).

KEYWORDS: *Tribulus terrestris*, Phytochemical, Antibacterial, Antioxidant.

INTRODUCTION:
Presently, the interest in traditional medicine from plant sources has been increasing. Therefore, it's a necessity to carry out a screening of these plant in order to support their use in folk medicine and discover new active components with the possibility of action against multi-resistant bacteria and also to tray decreasing toxicity of commercial antibiotics[1],[2],[3]. Where Pathogenic Microbes are always trying to develop resistance to the different antimicrobial commercial antibiotic drugs[4]. Adverse side effects and high cost of commercial antibiotic are commonly associated with popular synthetic antibiotics (such as allergic reactions, hypersensitivity, immunosuppression, etc.)[5]. Compared to commercial antibiotics, natural phytochemicals are found to be more effective with fewer side effects that is why they are used as an alternative remedy for the treatment of various diseases.

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Bioactive compounds like alkaloids, tannins, flavonoids, phenols, etc., showed antimicrobial activity in the area of food preservation, pharmaceutics, etc.[4],[6]. The strong plant components can compete with human and plant pathogenic bacteria, viruses, and fungi without side effects and environmental dangers[7],[8].

The majority of diseases or disturbances are mainly related to oxidative stress due to free radicals[9],[10]. Free radicals are fundamental to any biochemical process and represent an essential part of metabolism and aerobic life[10]. Phytochemicals occurring antioxidants such as ascorbic acid, vitamin E, flavonoids and phenols compounds possess the ability to reduce the oxidative damage associated with many diseases including cardiovascular disease, atherosclerosis, cancer, cataract, immune deficiency diseases, diabetes, arthritis, and aging[11]. Djeridane *et al*, and Wong *et al*[12],[13], reported that the antioxidant potential of medicinal plants may be correlated to the concentration of their phenolic compounds which include flavonoids, phenolic acids, tannins, and anthocyanin.

Tribulus terrestris L. (the common name in Hindi Gokharu) belongs to the Zygophyllaceae family, an

annual plant and worldwide distribution. Commonly found throughout India, it mostly grows wild especially in Maharashtra, West Rajasthan, Uttar Pradesh, and Gujarat. The genus Tribulus contains about 20 species which increase as bushes around 10-60 cm high.[9][14],[15]. In India, there are three species of common occurrence *Tribulus, Tribulus terrestris*, *Tribulus alatus*, and *Tribulus cistoides*[16]. This plant is used in folk medicine in many countries against various diseases such as eye trouble, edema, skin disorders, urinary troubles, kidney stones, painful micturition and aphrodisiac[17].

Due to the role of plant extracts in the treatment of many infectious diseases, we study in this work, the Antibacterial and Antioxidant activity of methanol and aqueous extracts of *Tribulus terrestris* L. (fruits) on some Pathogenic bacteria in Aurangabad city (India).

MATERIALS AND METHODS:

Plant Materials:

Tribulus terrestris (fruits) were purchased from dealers of a traditional medicinal product at Aurangabad city, India. It was authenticated by Dr. Rafiuddin Nasser, Assistant professor, Department of Botany, Maulana Azad College, Aurangabad.

Preparation of the extracts:

Fruits of *T. terrestris* were crushed to powder form. Methanol and Aqueous extracts were accomplished according to established protocols[18].

Methanol extract:

50g of plant fruits powder were extracted with 500ml methanol (50–55°C) using a Soxhlet apparatus. After extraction, the solvent was evaporated by a rotary evaporator, then leaving it in an incubator at a temperature of 40°C for 24 h to get a dried form. It was weighed and kept in a refrigerator.

Aqueous extract:

50g of dry plant powder was taken in a beaker, 500ml of distilled water was added and the mixture was stirred by a magnetic stirrer for 24 h. After that, it's filtered by Whatman paper No. 1 and filtrate was centrifuged at speed of 3000rpm for 10 minutes. The supernatant extract was evaporated by leaving it in an incubator at a temperature of 60°C for 24 h. Finally, the extract was weighed and kept in a refrigerator in sterilized and dark glass containers.

Phytochemical tests:

Extracts were subjected to phytochemical tests to investigate the presence of the following phytochemicals: alkaloids, tannins, glycosides, saponins, flavonoids, sterols, and Phenolic[18],[19].

Bacterial Strains:

The bacterial strains used for this study were Gram-positive, *Staphylococcus aureus* (clinical sample) and Methicillin-resistant *Staphylococcus aureus* MRSA (ATCC 43300) and Gram-negative, *Pseudomonas aeruginosa*, *Proteus mirabilis, Klebsiella pneumoniae* (clinical samples), *and Escherichia coli*, (ATCC 25922). All bacterial strains were obtained from Government Medical College, Aurangabad. India.

Antibacterial assay:

Bacterial suspensions were prepared and adjusted to 0.5 McFarland turbidity stander to get a final density of 1.5 x 106 CFM/ml. Agar well diffusion method is used to evaluate the antibacterial activity of plant extracts according to[20]. Methanol and water extracts were dissolved in 10% dimethyl sulphoxide (DMSO) and in distilled water to final concentrations of (100, 200, 300, 400mg/ml). Nutrient agar plates were prepared, allowed to solidify and 50µl of bacterial suspensions were seeded on the surfaces of these plates using cotton swabs. Wells of 8mm in diameter were bored on the agar using a sterile cork borer. 100µl of extracts were then introduced into the wells (10, 20, 30, 40mg/well). Antibiotic Streptomycin (100µg/well) was used as a positive control and 10% DMSO or distilled water as a negative control. Then the plates were incubated at 37℃ for 18-24 h. The experiment was performed in triplicate and the mean of the diameter of the inhibition zones was measured.

Minimum Inhibitory Concentration (MIC):

MIC was evaluated by the classical double dilution method in Nutrient broth as described by[21]. A series of different concentrations for methanol and water extracts were prepared (ranging from 100mg/ml to 3.125mg/ml). The MIC knows as the lowest concentration of extract that resulted in no apparent growth after 24 h incubation at 37°C.

DPPH free radical scavenging assay:

Antioxidant activity of *T. terrestris* methanolic and aqueous extracts were determined using 1, 1-Diphenyl-2-picryl hydrazyl radical method with minor modification[22].

0.05mL of the extracts dissolved in methanol were diluted to 1.0ml using (ethanol) to attain the concentrations 1-200µg/ ml, and were added to DPPH (Final concentration 200µM, in 95% ethanol). The absorbance of the resulting solution was read at 515 nm after 20 min using a spectrophotometer. The tests were performed in triplicate and the percentage inhibition was calculated by using the following formula:

$$\% \text{ Inhibition} = [(Abs_{control} - Abs_{sample}) / Abs_{control}] \times 100$$

Where $Abs_{control}$ is the absorbance of DPPH radical solution without extract, Abs_{sample} is the absorbance of DPPH radical solution mixed with the extract.

IC_{50} values were calculated using Linear regression by plotting scavenging activity against sample concentrations using Microsoft Excel software. Ascorbic acid was Stander.

STATISTICAL ANALYSIS:

Experimental data are expressed as means ± Stander error. Statistical analyses were performed by one-way ANOVA and t-value. The difference was considered to be statistically significant when $p < 0.05$. This analysis was done using SPSS ver. 20.0 software.

RESULTS AND DISCUSSION:

The present study showed phytochemical analysis, antibacterial and antioxidant activity of the methanolic and aqueous extracts of *T. terrestris* fruits. The weight and percent (%) of methanol and distilled water extracts were 4.64 g (9.28%) and 7g (14%), respectively.

Phytochemical analysis:

Phytochemical screening of aqueous and methanol extracts of the *Tribulus terrestris* (fruits) plants revealed the presence of alkaloids, tannins, glycosides, flavonoids, Phenols, are found in both extracts and are shown in (Table1). Glycosides and saponin were also found in methanol extract and absent in aqueous extract. Steroids was absent in each extract. Phytochemicals such as alkaloids, tannins, flavonoids and several other which are naturally happening in most plants, are known to have medicinal importance, which can be used in realizing a defense mechanism against many microorganisms[23]. The presence of secondary metabolite compounds in the plants is affected by several environmental factors such as light intensity, drought stress, temperature, salinity[24].

Table 1: Phytochemical screening of methanolic and aqueous extracts of *Tribulus terrestris* L. fruits.

		Extracts	
Phytochemical	Test	**Methanol**	**Aqueous**
Alkaloids	-Mayer -Wagner	+ +	+ -
Tannins	-Lead acetate -5% $Fecl_3$	+ +	+ +
Glycosides	-Keller-killianit	+	-
Saponin	-Foam test	+	-
Flavonoids	- Lead acetate -Sulphuric acid -5%FeCl3	+ - +	+ - +
Steroids	-Salkowski	-	-
Phenolic	-5%$FeCl_3$	+	+

Note: (+) Presence and (-) Absence.

Antibacterial activity:

In this study methanol and aqueous extracts of the fruit of *Tribulus terrestris* plant were tested for their antibacterial activity. As the results showed a positive effect in inhibiting the growth of bacterial strains, the results were different depending on the extract used, the type of bacteria, and concentrations. (Table 1 and Fig 1 and 2).

Methanolic extract, with a concentration of 40mg/Well, showed the best inhibitory effect against *S. aureus*, *S. aureus* (MRSA), *K. pneumonia*, *P. aeruginosa* and *P. mirabilis* with inhibition zones of 26mm, 24mm, 22.2 mm, 16.7mm and 15.7mm respectively. No activity was observed at concentrations of 10 and 20mg/well for *P. mirabilis* (Table 2 and Fig 1). Methanolic extract showed no inhibitory effect on *E. coli.*

Aqueous extract at a concentration of (40mg/well) showed more potent activity against *K. pneumonia* and *P. mirabilis* in which the diameter of inhibition zone were 18.3mm and 16mm, respectively. No activity was observed at concentrations of 10mg/well for *K. pneumonia.* The aqueous extract has no activity against *S. aureus,* MRSA, *P. aeruginosa* and *E. coli* (Table 2 and Fig 2).

MIC was applied only on bacteria that was sensitive to extracts at concentration 10mg and had an inhibition zone of 10mm and more. MIC value of the methanolic extract of *T. terrestris* fruits against *S. aureus*, MRSA, *K. pneumonia* and *P. aerunginosa* was 6.25mg/ml. MIC value of the aqueous extract of *T. terrestris* fruits against *P. mirabilis* was 6.25mg/ml (Table 3).

Methanolic extract showed higher activity than aqueous extract (Table 2). According to Sonam Pandey[25], ethanolic extract showed a better inhibition than aqueous extract and this is maybe attributed to the phytochemical compounds less solubility in water. Methanolic extract of *T. terrestris* fruit in this study revealed close results to the methanolic extract of *T. terrestris* leave against most of the bacteria under study with MIC value 6.25 in Nigeria[26]. Similar results also were reported by Kianbakht, and Jahaniani[27], in which MIC value was 2 mg/mL for methanolic extracts of Iranian *T. terrestris* fruits, leaves and stems against all bacteria. Another study revealed that all extracts from different parts of The Iraqi *T. terrestris* showed antimicrobial activity against most tested bacteria[28]. Also, in one study, the Indian *T. terrestris* were active exclusively against, *E. coli*, *K. pneumonia*, and *P. mirabilis*; among ten bacterial were studied[29]. In another finding, ethanolic extract of Yemeni *T. terrestris* had no detectable antibacterial activity against any bacterial used[30]. Different results concerning the antibacterial activity of *T. terrestris* might be due to different types of strains used and different geographic sources of the plant used.

Table 2: Antibacterial activity of methanol and aqueous extracts of *T. terrestris* (Fruit) against bacterial strains.

Extracts / Organism	Methanol					Aqueous				
	Conc. Of the extract (mg/well)									
	10	20	30	40	St	10	20	30	40	St
	Zone of inhibition (mm) (Mean ± SE)									
Gram-positive:										
S. aureus	20.1±1.3	22.0 ±0.57	24.3 ±0.37	26.0 ±1.2	34.7 ±0.60	-	-	-	-	-
S. aureus (ATCC)	18.0 ±1.2	18.0 ±0.67	23.0 ±0.58	24.0 ±0.58	29.2 ±0.44	-	-	-	-	-
Gram-negative:										
P. aerunginosa	10.0 ±0.29	13.0 ±0.58	14.8 ±0.44	16.7 ±0.88	21.5 ±0.76	-	-	-	-	-
P. mirabilis	-	-	12.3 ±0.64	15.7 ±0.33	28.0 ±0.0	10.3 ±0.88	12.0 ±1.15	13.0 ±0.0	16.0 ±1.0	27.3±0.33
K. pneumonia	15.2 ±0.44	16.3 ±0.88	20.3 ±0.33	22.2 ±0.44	29.0 ±0.58	-	10.7 ±0.33	16.3 ±0.88	18.3 ± 0.33	28.3 ±0.60
E. coli (ATCC)	-	-	-	-	-	-	-	-	-	-

St: Streptomycin (positive control 100µg /well), - : No zone of inhibition. All values are expressed as Mean ± SE (n=3).

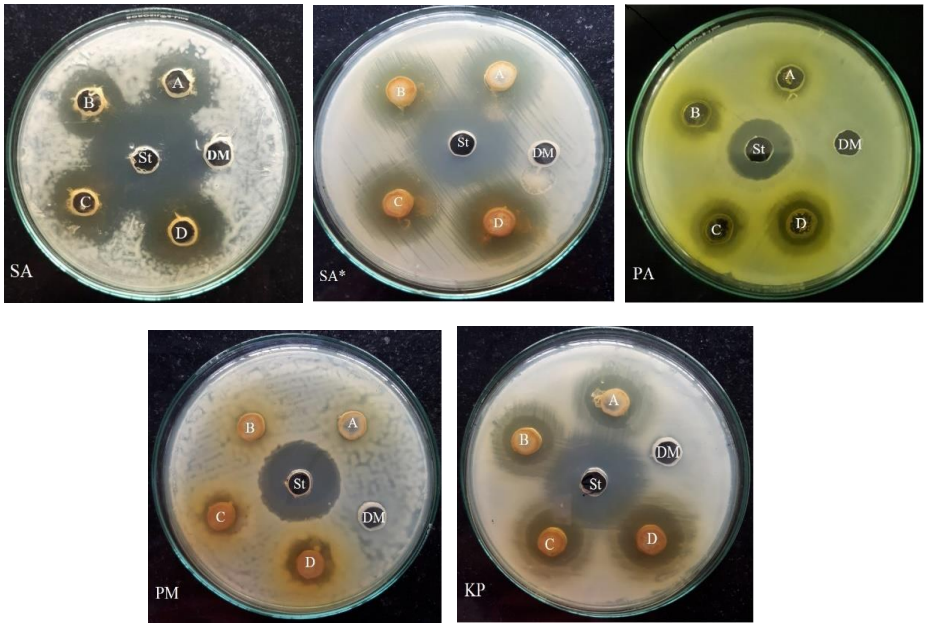

Figure 1: Growth inhibition activity of methanolic extract of *T. terrestris* against: *S. aureus* (SA), MRSA (SA*), *P. aeruginosa* (PA), *P. mirabilis* (PM), and *K. pneumonia* (KP) at concentrations 10 (A), 20 (B), 30 (C), and 40 (D) mg/well, negative control 10% v/v DMSO (DM) and the positive control 100 µg /well Streptomycin (St).

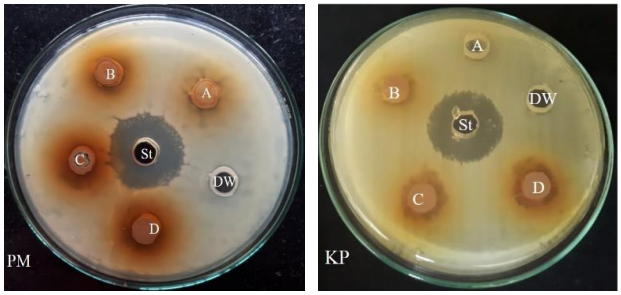

Figure 2: Growth inhibition activity of aqueous extract of *T. terrestris* against *P. mirabilis* (PM), and *K. pneumonia* at concentrations 10(A), 20(B), 30(C), and 40(D) mg/well, negative control distilled water (DW) and the positive control 100 µg /well Streptomycin (St).

Table 3. MIC values of *T. terrestris* fruits extracts (mg/mL).

Bacteria / Extracts	*S. aureus*	*MRSA*	*P. aerunginosa*	*P. mirabilis*	*K .pneumonia*	*E. coli*
Methanolic	6.25	6.25	6.25	NT	6.25	NT
Aqueous	NT	NT	NT	6.25	NT	NT

NT: Not tested

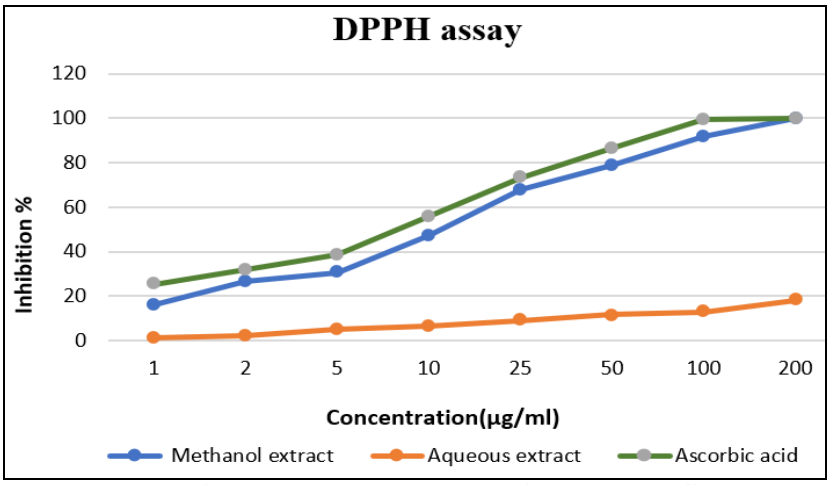

Figure 3: DPPH radical scavenging activity of Methanolic and aqueous extract of *T. terrestris* at different concentrations

DPPH free radical scavenging activity:

Antioxidant activity of methanolic and aqueous extracts of *T. terrestris* fruits were determined by DPPH radical scavenging assay and both extracts showed antioxidant activity in a concentration-dependent manner. Methanolic extract showed a higher scavenging ability with IC_{50} value of 3.94µg/ml and aqueous showed medium scavenging ability with IC_{50} of 22.45µg/ml. The IC_{50} value for the standard (ascorbic acid) was 3.35 µg/ml.

A study conducted by Lokhande, *et al*,[31] using *T. terrestris* leaves showed that aqueous and alcoholic extracts have a strong antioxidant activity in which IC_{50} were 43.96 and 41.66µg/ml, respectively.

In another study, among a total alcoholic, ethyl acetate fractions, rutin, and quercetin extracts of *T. terrestris* fruits, the maximum scavenging ability was recorded in rutin and quercetin (IC_{50}=12.1 and 11.2µg/ml respectively), when compared with ascorbic acid (10.2 µg/ml)[32]. On the other hand, antioxidant activity from acetone, hexane, isopropanol, and aqueous extracts of *T. terrestris* fruits, the strongest free radical scavenging activity was showed in hexane extract[33]. Similar results highlighting the scavenging ability of ethanolic extracts of *T. terrestris* fruits, with IC_{50} 142.27µg/ml[34].

According to Hetty Manurung,[24] there are potent relationships between secondary metabolite compounds such as phenolic, alkaloids, and flavonoids and antioxidants. And it has been shown that the scavenging effects of the DPPH radical is a dose-dependent in both samples and standards[33],[34]. *T. terrestris* is a possible new powerful natural source of antioxidants[15], [31], [32].

CONCLUSION:

Methanolic extract of *Tribulus terrestri* fruits have more phytochemical compounds, higher antibacterial and a strong antioxidant activity than aqueous extract. Further studies are recommended to isolate and identify antioxidant components in *T. terrestris.*

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RESEARCH ARTICLE

Microbiological Evaluation of Clean Rooms in Pharmaceutical Industries and Molecular Characterization of Microbial Isolates

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ABSTRACT:
Environmental monitoring in pharmaceutical industry was studied by using settle plate, air sampling, surface monitoring and personnel monitoring. These methods are commonly employed to collect the samples in pharmaceutical industry. The collected samples were incubated and predominant bacteria were isolated based on the colony morphology and identified by molecular characterisation. The PCR sequenced bi-directionally for seven isolates i.e, *Micrococcus luteus, Bacillus sp, Bacillus Sp. Strain S3Sr84, Kocuria sp. LWYT2000(2016), Staphylococcus sp. Bca53(2016), Brevibacterium sanguinis strain CF52, Staphylococcus hominis strain HB14*. Environmental monitoring statistical trend data will provide the process control and environmental isolate must be identified to understand their disinfectant susceptibility.

KEYWORDS: Environmental monitoring, Molecular Characterization, 16S rDNA, Phylogenetic tree, Disinfectant Validation.

INTRODUCTION:
The development of systems to control the numbers, survival and proliferation of microorganisms during manufacturing of pharmaceutical products is most vital in pharmaceutical industry. The facility where pharmaceutical products are manufactured is in closed environment, where men and materials movement will be frequent for carrying out different processes. The result from environmental monitoring provide the information on physical construction of the room, the performance of heating, ventilation and air conditioning (HVAC) system, gowning practices, personnel cleanliness, equipment, and cleaning operation[1]. Which will ensure concentration of airborne particles including viable and non-viable is controlled and classified, and which is designed, constructed and operated in a manner to control the introduction, generation and retention of particles inside the room (international standard ISO-14644-2015)[2,3].

The design and construction of clean rooms and controlled environments are demonstrated in USFDA GMP (United States food and drug association, Good manufacturing practice) guideline, adequately separating areas of operation is an important for contamination prevention in the pharmaceutical clean rooms and should maintain at least 10-15 Pascal's of positive pressure differential between adjacent clean room[4,5,6]. USFDA recommends and classified aseptic processing area to meet minimum, Class 10,000 (ISO 7) standards under dynamic conditions. Manufacturers can also classify this area as Class 1,000 (ISO 6) or maintain the entire aseptic filling room at Class 100 (ISO 5). An area classified at a Class 100,000 (ISO 8) air cleanliness level is appropriate for less critical activities (e.g., equipment cleaning)[4].

Aseptic processing facility should be appropriately controlled to achieve different degrees of air quality depending on the nature of the operation[7,8]. As the level of airborne contaminants in the environment may have impact on the level of product quality, hence microbiological assessment of aseptic processes is crucial[7,8,9,10].

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The presence of high numbers of microorganisms and pathogens represents a serious health threat to consumers as the products are consumed by humans. The microbiological quality is necessary for their efficacy and patient safety, because microbial contamination of drug cause immediate adverse effects on patient's health in morbidity and mortality[4]. The disease can be based upon microbial infection or metabolic disorders. Therefore, minimizing the numbers or preventing the introduction of significant numbers of microorganism into pharmaceutical clean rooms is necessary[11].

Hence to have a controlled environment pharmaceutical industry should have an effective cleaning and sanitization programme[12]. Pharmaceutical facilities must be kept clean and microbial count under control in order to protect the quality of the products and ultimately the safety of the patients[10,13].

Microbiological testing alone does not provide completed or absolute assurance of absence of microbial contamination. However, such testing combined with robust environmental monitoring program and the use of validated manufacturing processes provides a high degree of assurance of the microbial safety of drugs[14]. The suitability, efficacy, and limitations of disinfecting agents and procedures should be assessed[4]. A comprehensive cleaning and sanitization program is needed for controlled environments used in the manufacture of Pharmacopeial articles to prevent the microbial contamination[15].

The development of recent technologies to enumerate microbial population have greater resolution and sensitivity to describe the presence of microbial distribution on Earth. All microbes in nature do not grow on plate media. Similar results have been observed in pharmaceutical environments. The available information from environmental monitoring programme support on optimizing process control and controlling microbial contamination[16].

Environmental monitoring programs for sterile and non-sterile pharmaceutical facilities comprise the analysis of personnel, processes, raw materials, and finished products. Critical areas during pharmaceutical manufacturing must always be in control to minimize the distribution, viability, and proliferation of microorganisms. When an environmental monitoring program is in place, environmental monitoring data are evaluated to determine whether or not the series of environmental controls continue to operate as intended. Statistical analysis is used to evaluate an environmental monitoring program. A gradual increase or decrease in microbial counts over time, or a change in Microbial flora or counts on several plates of a particular area on a given day, would constitute a trend. Environmental fluctuations are intrinsic of an environmental monitoring system. This is because clean rooms and controlled environments are not supposed to be sterile, and constant intervention by personnel and materials represents continuous challenge to process control and cGMP. Optimization of pharmaceutical manufacturing relies on the integration of different systems and processes to minimize microbial insult resulting in safe and efficacious products[11,16].

The presence of microorganisms in air can impact the quality of the processes and products manufactured in pharmaceutical environments. Although Quantitation of the air borne microbial flora depends upon the sensitivity and accuracy of the methods used, several methods are recommended for air monitoring[2,3]. The most common viable monitoring methods used in pharmaceutical industry such as Settle plate, active air sampling, surface monitoring and personal monitoring[16].

The purpose of the study was to identify the predominant bacteria from pharmaceutical clean rooms by molecular characterization and to minimize and control the microbial proliferation in the clean room by using appropriate disinfectant[17,18,19]. This is important in order to understand if certain species are being recovered pose a product or environmental risk and to check if the cleaning and sanitization practices are effective [6,20].

MATERIALS AND METHODS:

Chemicals and reagents:

Pre-incubated Soyabean casein digest agar Catalogue Number M290-500G, Tryptone soya agar with polysorbate 80 and soya lecithin Catalogue Number M449-500G, Dey-Engley Neutralizing Agar Catalogue Number M186-500G, Grams staining – crystal violet, grams iodine, decolorizer and safranin obtained from Himedia. 0.45µ Membrane filter Catalogue Number HVWP047S6 obtained from Merck Millipore, Microbial culture *Bacillus subtilis* ATCC-6633, *Staphylococcus aureus* ATCC-6538, *Pseudomonas aeruginosa* ATCC-9027, *Escherichia coli* ATCC-8739, *Aspergillus brasilliensis* ATCC-16404, *Candida albicans* ATCC-10231 obtained from American type culture collection (ATCC) Chemistry: Big Dye Terminator version 3.1", Cycle sequencing Kit. Polymer and Capillary Array: POP_7 polymer 50cm Capillary Array, Data Analysis: Seq Scape_ v 5.2 Software.

Instrumentation:

Air sampler Make: VWR PBI Model: SAS super ISO, Incubators Make: Thermolab: 800L, Autoclave make machine fabric Model: 180, Laminar airflow unit Make: Esco, Microscope Make: Zeiss Mode; Primo star. Sequencing Machine: ABI 3500 Genetic Analyzer.

Passive Air Sampling (Settle Plate):
Pre-incubated Soyabean casein digest agar plates were exposed at predetermined sampling location at working level from the floor on petri plate stand, lifted and slide open the lid of the media plate and kept aside. Exposed individual media plates for 4 hours[21,22]. After the exposure period, closed the media plate with the lid. Collected the exposed media plates in Petri plate's carrier and brought back to the microbiology department. Incubated the media plates at 20 - 25ºC for 72 hours; recorded the observations after 72 hours for fungal count. Incubated the same Media plates, further at 30 - 35ºC for 48 hours and recorded the observation after 48 hours for bacterial count.

Active air sampling:
Air sampling was performed by using 90mm Pre-incubated Soyabean casein digest agar by using active air sampler SAS PBI. Collected 1000L of air hours[21]. After air sampling, collected the plates in petriplates carrier and brought back to the microbiology department. Incubated the media plates at 20º to 25ºC for 72 hours; recorded the observations after 72 hours for fungal count. Incubated the same Media plates, further at 30º - 35ºC for 48 hours and recorded the observation after 48 hours for bacterial count.

Surface monitoring:
Surface monitoring was performed by using contact plate method. The plates contain Tryptic Soy Agar with Lecithin and Polysorbate 80 added to inactivate residual disinfectants and are used for enumeration of microorganisms on environmental[3,15]. and personnel gowning surfaces.

Identification of microbial isolate from environmental monitoring:
Primary Screening:
The predominant bacterial colonies identified were (named EI-121, EI-122, EI-129, EI-130, EI-133, EI-134 and EI-135) isolated and performed grams staining by using microscope. Grams staining will provide information's i.e cell arrangement (single cell, cluster and clumps), shape (rod or cocci) and grams-Staining characteristics[23]. Transferred a loopful of the liquid culture to the surface of a clean glass slide and allowed to spread over a small area, set aside the film to air dry. Dried film fixed by passing it briefly through the Bunsen flame two or three times without exposing the dried film directly to the flame. The slide should not be so hot as to be uncomfortable to the touch. Deluged slide with crystal violet solution for up to one minute. Wash off briefly with tap water (not over 5 seconds) and drain. Flooded slide with Gram's Iodine solution, and allowed to act (as a mordant) for about one minute. Wash off with tap water and drain. Removed excess water from slide and blot, so that alcohol used for decolorization is not diluted. Flood slide with 95% alcohol for 10 seconds and wash off with tap water. (Smears that are excessively thick may require longer decolonization. This is the most sensitive and variable step of the procedure, and requires experience to know just how much to decolorize) and drained the slide. Flooded slide with safranin solution and allowed to counterstain for 30 seconds. Wash off with tap water. Drain and blot dry with bibulous paper. The slides of bacteria were examined under the oil immersion lens.

Molecular Characterization:
Genomic DNA was extracted from 7 isolates following DNAzol-based cell lysis protocol and the lysates were purified on DNA-binding columns. Polymerase chain reaction (PCR) amplification performed in ABI-2720 thermal cyclers using 341F and 907R as primers (2). The PCR amplification combination include 0.2mmol/L (each) dNTP, 400nmol/L (each) primer, 5mmol/L MgCl, and 1 U Taq polymerase in a final volume of 50µl. After an initial denaturation step at 94°C for 3 min, 30 cycles of PCR reaction were run as follow: denaturation at 94 °C for 1 min, annealing at 55°C for 45 s, and extension at 72°C for 1 min. In addition, a final extension at 72°C for 10 min was added. The resulting products were analysed by electrophoresis in 1.0% agarose gel and purified with PCR Clean-up Kit Refer Fig. 1 and 2. Sequences were determined in an ABI-3500 XL Genetic Analyser using 341F as a sequencing primer, and their closest matches were found by blasting against the short and nearly exact matches from NCBI (National Center for Biotechnology Information) databases (http://www.ncbi.nlm.nih.gov). Sequences were aligned and the phylogenetic tree was generated using DNAMAN package (Lynnon Biosoft, Canada) with evolutionary distances method (boot-strapping 100-times)[24] Refer Fig.3.

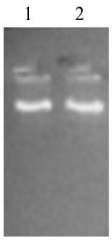

Figure 1: Genomic DNA from Bacterial sample using the Bacterial Genomic DNA Isolation Kit.

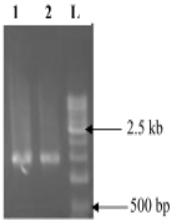

Figure 2: PCR amplification of 16s rDNA fragment from Bacterial sample loaded on 1% Agarose Gel.

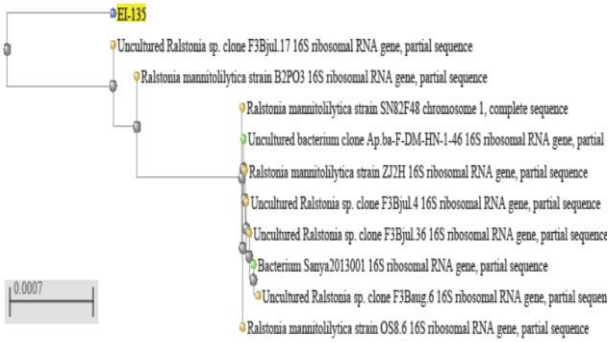

Figure -3: Represented phylogenetic tree of Sample – EI-135.

Disinfectant Efficacy Validation:

Prepared the desired volume of Dey-Engley agar medium, Normal saline, prepared inoculum for the test *Bacillus subtilis* ATCC-6633, *Staphylococcus aureus* ATCC-6538, *Pseudomonas aeruginosa* ATCC-9027, *Escherichia coli* ATCC-8739, *Aspergillus brasilliensis* ATCC-16404, *Candida albicans* ATCC-10231, and isolate obtained from environment i.e., *Micrococcus luteus* strain CL10 16S (EI-121), *Bacillus sp*. H1-115 (EI-122), *Kocuria sp*. LWYT2000 (EI-129), *Staphylococcus sp*. Bca53 (EI-130), *Brevibacterium sanguinis* (EI-133), *Staphylococcus hominis* strain HB14 (EI-134), *Bacterium Sanya* 2013001(EI-135). Culture suspension prepared for the above mentioned cultures & environmental isolates in predetermined the inoculum count to achieve 10^{6}-10^{7} cells/ml bacteria and 10^{5}-10^{6} fungi cells/ml by dilution method. 10-100 cfu culture suspension is prepared by diluting the stock culture appropriately.

Selection of disinfectants:

Selected based on the chemical composition and characteristics of isolates i.e. broad spectrum antimicrobial activity i.e., Vesphene IISe (2-phenylphenol- 9.09%, p-tertiary amylphenol- 7.66%, Potassium hydroxide-5.00%, Sodium hydroxide - <2.00%) LpH Se (7.7%.), -7.7%, Isopropanol- ~10%, Phorsphoric acid- ~14.0%, Dodecylbenzene sulfonic acid-5.0%), Bacillocid extra (Dimethanol-14.0%, Glutraldehyde-5.0%), Microbac Forte (Benzyl-C12-18-alkyldimethylammonium chlorides 199mg/g, N-3aminopropyl)-N-dodecylpropane-1,3-diamine 50mg/g). Collected the required disinfectant and prepared the disinfectants concentrations in a sterilized container by using sterile water. Above disinfectant solution was filtered by using 0.2micron, 47mm diameter membrane filter. Collected the filtered disinfectant solution in a sterilized container and labeled the container which shall represent the name of disinfectant, concentration and date of preparation.

Verification of Filtration technique:

10mL of selected higher concentration of diluted disinfectant was filtered through 0.45µ membrane filter. Rinsed the membrane with 100mL Normal saline solution three times and added the 10-100cfu culture in the final rinse and placed the membrane in to pre-incubated Dey-Engley agar. Incubated at respective temperature to verify the suitability of the test method.

Disinfectant Efficacy Test:

Transferred 10ml of prepared disinfectant solution into different sterilized test tubes and marked with different time intervals, Inoculated 0.1ml (10^{6}-10^{7} cells/ml bacteria and 10^{5}-10^{6} fungi cells/ml) of any one of the challenge microorganisms in to each test tube, inoculum should not exceed 1% of the test solution volume. Mixed well and allowed it to the pre-determined contact time such as 0 minutes, 15 minutes, 30 minutes for Vesphene IISe, LpH Se, Bacillocid extra, Bacillocid extra, Pre-sterile for each organism. At the end of contact time, serially diluted the solution and filtered the contents of each tube through sterilized 0.45 micron, 47mm diameter membrane filter. Rinsed the membrane filter with 100ml of sterilized normal saline three times. Aseptically transferred the membrane filter on to the surface of pre- incubated Dey-Engley agar plate repeated the steps for the other disinfectant solution with all microorganisms. Taken separately, 10ml of disinfectant used in the study for all the disinfectants and filter through a sterilized 0.45-micron 47mm diameter membrane filter. Rinsed the membrane filter with 100ml of normal saline three times, inoculated with 10-100 cells of one of the microorganisms. Aseptically transferred the membrane filter on to the surface of pre-incubated Dey-Engley agar plate for positive control. Filtered 10ml of disinfectant solution through a sterilized 0.45micron, 47mm diameter membrane filter. Rinsed the membrane filter with 100ml quantity of Normal saline. Aseptically transferred the membrane filter on to the surface of pre-incubated Dey-Engley agar plate for negative control. Incubated the bacterial challenged plates at 30-35°C for 72 hours and fungal challenged plates at 20-20ºC for 5 days. Observed the plate at the end of incubation period and recorded. Required achieve the acceptance criteria of Not less than 3 log reduction for vegetative bacteria and 2 log reductions for spores and Fungi[3].

Surface Test:

Took sterilized 2 x 2 inches Epoxy template and added 0.1mL of test culture suspension containing 10^{6} to10^{7} bacterial cells and for fungi 10^{5} to10^{6} cells using micropipette and spread using a sterile spreader. Allowed to dry all the culture inoculated Epoxy templates inside the laminar air flow cabinet. Flooded 1.0mL selected disinfectant listed in table - 5 of test concentration and allowed the surface as it is with

disinfectant until contact time 10 minutes. After the specified contact time, discarded the flooded disinfectant by tilting the template and swabbed the total area with the help of sterile swab Collected swab sample from the entire template surface adequately and immediately inoculated the swab into 10mL of normal saline vortexed the test solution vigorously and serially diluted using 10mL normal saline to get 10-100 CFU/mL for bacteria and fungi after dilution, content filtered through sterile 0.45micron, 47mm diameter membrane filter. Rinsed the membrane filter with 100ml of sterilized normal saline three times. Aseptically transferred the membrane filter on the surface of pre-incubated Dey-Engley agar plate and incubated. Performed the test for all the organisms listed in Table -5 and repeated the procedure for all other disinfectant solution.

RESULTS:

The samples were collected from different location of pharmaceutical industry by using settle plate, air sampling, surface monitoring and personnel monitoring. Total numbers of samples by settle plate was 53663 while 44004 samples (82%) were showed positive and 9659 (18%) samples showed no growth, by air sampling 39312 while 34987 samples (89%) were showed positive and 4325 (11%) samples showed no growth, by surface monitoring 25584 were tested among that 23793 samples (93%) were showed positive and 1791 (7%) samples showed no growth and personnel monitoring 14976 samples among which 898 samples (6%) showed positive growth while 14077 samples (94%) showed no growth Refer Table No 1,2,3, and 4.

Settle plate (Passive Air Sampling):

Samples were taken from microbiology laboratory and production area in facility using settle plate technique. Results were shown in table-1.

Table 1: Environmental Monitoring by Settle Plate in pharmaceutical industry

No. samples	Positive		Negative	
	No.	%	No.	%
53663	44004	82	9659	18%

Active air sampling:

Samples were taken from microbiology laboratory and production area in facility using air sampling apparatus (SAS PBI Air sampler) for quantitative determination of microorganisms**.** Results were shown in Table-2.

Table 2: Environmental Monitoring by Air sampling in Pharmaceutical Industry

No. samples	Positive		Negative	
	No.	%	No.	%
39312	34987	89	4325	11%

Surface Monitoring:

Samples were taken using contact plates from production area and microbiology laboratory in facility results were shown in Table-3.

Table 3: Environmental Monitoring by Surface Monitoring in Pharmaceutical Industry

No. samples	Positive		Negative	
	No.	%	No.	%
25584	23793	93	1791	7%

Personnel Monitoring:

Samples were taken using contact plates from production area and microbiology laboratory in facility Results were shown in Table-4

Table 4: Environmental Monitoring by Personnel Monitoring in Pharmaceutical Industry

No. samples	Positive		Negative	
	No.	%	No.	%
14976	898	6	14077	94%

Molecular characterization:

Seven bacterial species were isolated from different locations in the pharmaceutical facility. Primary screened microbial isolates were further molecular characterization performed for the microbial isolates. Genomic DNA was isolated from the sample and further ~1.3 kb/1.5kb, 16s-rDNA fragment was amplified using high–fidelity PCR polymerase. The PCR product was sequenced bi-directionally. Represented Gel photo of Sample EI- 133 and EI-134. Refer Figure – 1 and 2.

The results of molecular characterization of bacterial isolate are as below:

Sample: EI-121:

The Microbe was found to be most similar to *Micrococcus luteus* strain CL10 16S. The next closest homologue was found to be *Micrococcus sp.* SK22 gene for 16S rRNA, partial sequence ribosomal RNA gene, partial sequence.

Sample: EI-122:

The Microbe was found to be most similar to *Bacillus sp*. H1-115 16S ribosomal RNA gene, partial sequence. The next closest homologue was found to be *Geobacillus stearothermophilus* strain NB3-8 16S ribosomal RNA gene, partial sequence.

Sample: EI-129:

The Microbe was found to be most similar to *Kocuria sp*. LWYT2000 16S ribosomal

RNA gene, partial sequence. The next closest homologue was found to be *Kocuria palustris* partial 16S rRNA gene, strain Marseille-P699.

Sample: EI-130:
The Microbe was found to be most similar to *Staphylococcus sp*. Bca53 16S ribosomal RNA gene, partial sequence. The next closest homologue was found to be *Staphylococcus cohnii* strain PC-05 16S ribosomal RNA gene, partial sequence.

Sample: EI-133:
The Microbe was found to be most similar to *Brevibacterium sanguinis* partial 16S rRNA gene, strain CF 52. The next closest homologue was found to be *Brevibacterium sanguinis* strain T124 16S ribosomal RNA gene, partial sequence.

Sample: EI-134:
The Microbe was found to be most similar to *Staphylococcus hominis* strain HB14 16S ribosomal RNA gene, partial sequence. The next closest homologue was found to be *Staphylococcus hominis* strain 77 (BC26) 16S ribosomal RNA gene, partial sequence.

Sample: EI-135:
The Microbe was found to be most similar *Bacterium Sanya* 2013001 16S ribosomal RNA gene, partial sequence. The next closest homologue was found to be *Ralstonia mannitolilytica* strain OS8.6 16S ribosomal RNA gene, partial sequence.

Disinfectant validation:
Every manufacturing facility is different and hence each qualification study is different. Considering the various combinations of surfaces, organisms, disinfectant products and disinfection procedures, the study were conducted using disinfectant i.e., Vesphene IISe 0.8%, LpH Se 0.4%, Bacillocid Extra 1%, Microbac Forte 2% by dilution method. Surface test carried out using Epoxy. More than 3 log reduction obtained for vegetative bacteria and more than 2 log reduction obtained for spore former and fungi refer Table-5. Validity of method and controls are meeting the acceptance criteria.

Table-5: Disinfectant Efficacy test and surface test Log Reduction

Disinfectant	Microorganism	Efficacy test			Surface test
		0 min	15 min	30 min	Epoxy
Vesphene IISe 0.8%	*Escherichia coli* ATCC-8739	6.69	6.69	6.69	6.94
	Pseudomonas aeruginosa ATCC-9027	6.67	6.67	6.67	6.67
	Staphylococcus aureus ATCC-6538	6.69	6.86	6.86	6.82
	Bacillus subtilis ATCC-6633	2.69	4.63	5.15	4.59
	Candida albicans ATCC-10231	6.06	6.06	6.06	6.00
	Aspergillus brasiliensis ATCC-16404	3.18	4.43	5.43	5.43
	Micrococcus luteus strain CL10 16S (EI-121)	5.60	7.55	7.55	5.04
	Bacillus sp. H1-115 (EI-122)	4.04	5.48	7.60	5.43
	Kocuria sp. LWYT2000 (EI-129)	5.83	6.83	6.83	6.46
	Staphylococcus sp. Bca53 (EI-130)	3.76	5.06	5.67	6.67
	Brevibacterium sanguinis (EI133)	4.76	7.60	7.60	6.51
	Staphylococcus hominis strain HB14 (EI-134)	2.84	7.61	7.61	6.46
	Bacterium Sanya 2013001(EI-135)	4.64	6.02	7.54	5.37
LpH Se 0.4%	*Escherichia coli* ATCC-8739	6.66	6.66	6.66	6.92
	Pseudomonas aeruginosa ATCC-9027	6.74	6.74	6.74	6.69
	Staphylococcus aureus ATCC-6538	7.01	7.01	7.01	6.86
	Bacillus subtilis ATCC-6633	2.69	7.02	7.02	6.85
	Candida albicans ATCC-10231	6.08	6.08	6.08	5.99
	Aspergillus brasiliensis ATCC-16404	2.10	3.47	4.47	5.38
	Micrococcus luteus strain CL10 16S (EI-121)	4.64	7.54	7.54	6.82
	Bacillus sp. H1-115 (EI-122)	4.69	6.72	7.59	6.94
	Kocuria sp. LWYT2000 (EI-129)	6.87	6.87	6.87	6.43
	Staphylococcus sp. Bca53 (EI-130)	4.87	7.56	7.56	6.56
	Brevibacterium sanguinis (EI133)	7.00	7.00	7.00	6.67
	Staphylococcus hominis strain HB14 (EI-134)	4.73	7.57	7.57	6.79
	Bacterium Sanya 2013001(EI-135)	4.43	7.73	7.73	6.51
Bacillocid extra 1%	*Escherichia coli* ATCC-8739	6.69	6.69	6.69	6.94
	Pseudomonas aeruginosa ATCC-9027	6.67	6.67	6.67	6.67
	Staphylococcus aureus ATCC-6538	6.86	6.86	6.86	6.82
	Bacillus subtilis ATCC-6633	4.74	5.09	6.99	5.19
	Candida albicans ATCC-10231	6.06	6.06	6.06	6.00
	Aspergillus brasiliensis ATCC-16404	3.26	4.43	5.43	5.43
	Micrococcus luteus strain CL10 16S (EI-121)	6.97	6.97	6.97	6.51
	Bacillus sp. H1-115 (EI-122)	4.25	7.84	7.84	5.38
	Kocuria sp. LWYT2000 (EI-129)	6.83	6.83	6.83	6.46
	Staphylococcus sp. Bca53 (EI-130)	4.32	7.55	7.55	6.60
	Brevibacterium sanguinis (EI133)	3.39	7.71	7.71	6.07
	Staphylococcus hominis strain HB14 (EI-134)	2.20	7.43	7.43	5.83

	Bacterium Sanya 2013001(EI-135)	4.64	7.54	7.54	6.77
Microbac Forte 2%	*Escherichia coli* ATCC-8739	6.66	6.66	6.66	6.94
	Pseudomonas aeruginosa ATCC-9027	6.74	6.74	6.74	6.67
	Staphylococcus aureus ATCC-6538	5.71	7.01	7.01	6.82
	Bacillus subtilis ATCC-6633	3.44	4.34	7.02	4.28
	Candida albicans ATCC-10231	6.08	6.08	6.08	6.00
	Aspergillus brasiliensis ATCC-16404	2.15	5.47	5.47	5.43
	Micrococcus luteus strain CL10 16S (EI-121)	7.00	7.00	7.00	6.51
	Bacillus sp. H1-115 (EI-122)	3.56	5.22	6.82	5.44
	Kocuria sp. LWYT2000 (EI-129)	7.02	7.02	7.02	6.46
	Staphylococcus sp. Bca53 (EI-130)	6.87	6.87	6.87	6.82
	Brevibacterium sanguinis (EI133)	5.88	6.84	6.84	5.68
	Staphylococcus hominis strain HB14 (EI-134)	6.84	7.28	7.28	6.04
	Bacterium Sanya 2013001(EI-135)	7.42	7.42	7.42	5.66

DISCUSSION:

Predominant bacterial isolates from the environmental monitoring were identified by molecular characterization. The identified isolates were submitted in NCBI are *Micrococcus luteus* (EI-121) Accession No. KX082816, *Bacillus sp* (EI-122) Accession No KX082817, *Bacillus sp. strain S3Sr84 (*EI-135) Accession No KY678781, *Kocuria sp. LWYT2000 (2016)(* EI-129) Accession No. KX845571, *Staphylococcus sp. Bca53 (2016)* (EI-130) Accession No KX845572, *Brevibacterium sanguinis strain CF 52 (*EI-133) Accession No KX953856, *Staphylococcus hominis strain HB14 (*EI-134) Accession No KX953857. Statistical trend data will provide the process control and further available isolate must be identified to understand the existing flora in the clean room and must be challenged with disinfectant periodically to confirm their susceptibility.

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CONFLICT OF INTEREST:

The authors declare no conflict of interest.

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RESEARCH ARTICLE

Exploration of a new Fluoride resistance gene (*FRAM* gene) in oral *Streptococcus mutans MAW*

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ABSTRACT:

In the present investigations pretended one highly fluoride ion (F) tolerant bacterial strains like *Streptococcus mutans MAW* was isolated from Iraqi dental patients. The study focuses on a new bacterial fluoride resistance gene that contribute to each of the main virulence defenses to surviving in the oral cavity. The study showed same bacteria was developed fluoride resistance ways using model from hypothetic genes over lapping kind, it confirmed as SNARE -associated domain family genes of cell wall. **Material and methods**: It was detected by the potential function resistant at 2000 ppm/ml sodium fluoride, which appeared highest level of resistance among four gradient levels of concentrations. The amplification was identified the hypothetic gene of fluoride-resistant using specific primer. **Results**: The *FRAM* gene was sequenced with a length 400 bp comparing with NCBI database. The qRT- PCR results had been appeared high expression of *FRAM* gene in extra level was 5.25-fold compared with control. The sequencing analysis led to detect a new mutant gene for resistance has identity 49% in NCBI database. The result submitted a new *Streptococcus mutans MAW* (Gen Bank Acc. LC431527) was correlated in genetic distance neighborhood with other fluoride resistance genes of NCBI strains. **Conclusion**: It was concluded that fluoride exporter confers fluoride resistance to oral *S. mutans MAW*. The sequencing analysis was able to explain genetic converting in chromosome lead to a fluoride-resistant strain. These explorations can providea new insights into a new mechanism of microbial F^+ resistance mutations.

KEYWORDS: *Streptococcus mutans MAW*, Fluoride Resistance Genes, SNARE -associated domain family genes, qRT-PCR.

1. INTRODUCTION:

A long term use of fluoride can result in emergence of fluoride-resistant oral *Streptococcus sp*. are including *Streptococcus mutans*, which strongly implicated as a basically etiological factor in dental caries. One of the important virulence defenses of these organisms is their ability to form as dental plaque on tooth surfaces. [1] The studies focus on bacterial products that contribute to each of main virulence properties, one of the consequences of a patent survey how *S. mutans* has adapted to surviving the oral environment [2], to defense against host factors included application of fluoride is acute of egression of fluoride resistance in microorganisms.

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Fluoride-resistant strains have been discovered in several clinical studies [3]. Presently, the strains have fluoride resistant show clear phenotypic like difference sing growth, adherence, and metabolic activity compared to the fluoride sensitive strains. It was believed that these phenotypic converting was led to stable genotypic changes in the fluoride-resistant strains. the reports have explored these genotypic changes lead to many differences in fluoride resistance machines included a new mutation genes with different sequence [4]. The distinctness of fluoride-resistant in oral *Streptococcus sp*. may not only decrease the anti-caries effects of fluoride, but also, disrupt aggregation of oral streptococci in the oral cavity may be not only dispose fluoride effects like anti-caries, on other hand disrupt aggregation of oral streptococci in the oral cavity to attain understanding of resistance mechanisms involved fluoride resistance may be shed insight for the antimicrobial fluoride effects [5]. The family genes of fluoride exporters have reported in microorganisms such as subclass of family of SNARE-

associated domain; a small membrane protein that able to camphor resistance mutants. [6][7] Genome sequencing of S. mutansUA159 strain has in charged of fluoride exporters has directly interrelated [8] In this study, we attempted to detect and explore fluoride-resistance-related hypothetical gene like mutant gene in oral *Streptococcus mutans*. The involvement of the gene in fluoride resistance has been confirmed by sequencing analysis, this gene participated in expression of fluoride resistance proteins belong to microflora like *S. mutans* isolate from oral cavity. The hypothetical gene confirmed to do fluoride exporter machines in bacterial cell membrane for fluoride resistance [9].

2. MATERIALS AND METHODS:

2.1-Bacterial identification and fluoride resistance level determination:

Streptococcus mutans MAW was isolated by using nutrient agar media, a DNA extraction set was employed for identification was confirmed through genotypic diagnosis of microorganisms species by specific *16S rRNA* gene primer (*16S rRNAF*. (5'GCGACGATACATAGCCGACC'3), *16S rRNAR*. (5'CTCGGTCAGACTTTCGTCC'3) [10][53]. The PCR reaction consist of (25µl) PCR reaction mixture, it was following an initial 4-min hold at 94°C, a *16S rRNA* gene fragment was amplified by 30 cycles of denaturing at 94°C (30 s), annealing at 55°C (30 s), and extension at 72°C (45 s). [11] (Fig.1).

The fluoride resistant of *Streptococcus mutans MAW* was examined by culturing to an optical density at (OD_{600}) (2 * 10^7 cells/ml) of the bacteria by streaking the nutrient agar plates containing gradient concentrations of sodium fluoride (500, 1000, 1500, 2000) ppm of sodium fluoride per ml) in order to show two different levels of fluoride resistance. Plates were incubated at 37°C for 45hr., growth colonies were indicated to positive result. The higher resistance colonies for fluoride was selected and inoculated in broth culture with concentration of sodium fluoride equal to 2000ppm/ml, then bacteria were incubated until early exponential phase at 37C˚. To evaluate bacterial proliferation at (OD_{600}) (4* 10^5 cells/ml), more specifically, bacteria was reducing five more times, corresponding to fluoride resistance generation. The hypothesis was that mutants carrying *FRAM* gene caused diminished *FR* expression [12][52].

2.2- Identification of *Streptococcus mutans* strain and *FRAM* gene sequencing for phylogenic analysis:

The resistance *Streptococcus mutans MAW* colonies were selected from higher fluoride resistance concentration plates have 1500, 2000ppm/ml NaF, a DNA extraction was employed to amplify *FRAM* gene by specific-gene primer at conventional PCR with *FRAM* gene primers RF.(5'-CATATGCTTAATCCCCATCTAATGCT- '3) *R*. (5'-GCATGCACTGATATTACTGGCTATTTA- '3). Using of Exo SAP-IT reagent as per manufacturer's instructions to PCR product were purified and sequenced when used specific forward and reverse primers. Sequencing reactions were performed with Big Dye Terminator cycle sequencing ready reaction kit reagents on an ABI Prism DNA sequencer. Sequences were appeared, edited, and identified using (Clone manager demo $_{9.2}$) software program to comparing alignments with conference sequences database in NCBI Gene Bank reference sequences. [12][13]. The sequence was analyzed in the nucleotide databases led to submitted to Gen Bank. Related sequences of *Streptococcus mutansUA159* were obtained from the NCBI's nucleotide database (www.ncbi.nlm.gov/nucleotide) and included in the multiple alignment employed (clone manager demo$_{9.2}$) software program [14]. The evolutionary history was inferred using the Neighbor-Joining method among ten FR genes in NCBI database [15] via (clone manager demo $_{9.2}$) software with 1000 bootstrap replicates [16]. The evolutionary distances were computed using the Jukes-Cantor method and run at MEGA6 model.

2.3- Genes expression assay:

The RNA was extracted from *Streptococcus mutans MAW* which was fed with higher NaF concentrations (control without NaF.). Isolated RNA integrity an average optical density (OD) OD_{260}/OD_{280} nm absorption ratio of range 2.12. Therefore, concentrations of 25 ng cDNA (= reverse transcribed total RNA) per µl were achieved [17][18]. To Real-time PCR expression highly purified salt-free primer for especial gene (*FRAM gene*) and housekeeping gene (*16SrRNA* gene) RT-PCR was performed using a standard TaqMan® PCR kit protocol. The 10µl PCR included 0.67µl RT product, 1× TaqMan® Universal PCR Master Mix (Applied Biosystems), 0.2 µM probe, 1.5µM forward primer and 0.7µM reverse primer. The reactions were incubated at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The threshold cycle (C_T) is known as the fractional cycle number when the fluorescence passes the fixed threshold [19].

For explicated mathematical model is necessary to determine the CPs for each transcript using Light Cycler Software 3.5 (Lin Reg PCR" to "Dynamic) [20].

3. RESULTS:

3.1- Bacterial *16S rRNA* identification:

Streptococcus mutans MAW of oral isolate from Iraqi dental patients was performed to confirm their identification species by amplifying of the *16S rRNA* gene. Primers of conserved region of *16S rRNA* were designed and used for amplification of DNA, then PCR

products were separated on 1% agarose gel, the electrophoresis results were documented in (Figure 1), they were demonstrated DNA of *S. mutans MAW* species given*16S rRNA* gene band with 125 bp [10].

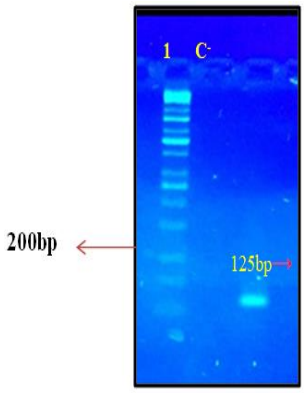


Figure (1): Agarose gel electrophoresis for amplification of *16SrRNA* gene of *S. mutans MAW* on (1%) agarose gel, 70 voltage for 90 minutes. Lane (1): DNA marker Ladder (100bp). Lane (C^-): Amplicon of *16S rRNA* primer with *S.salivarious* . Lane (2): Amplicon of *16S rRNA* gene (125bp) of *S.mutansMAW*.

3.2- Higher level of fluoride resistance of *Streptococcus mutans MAW*

Streptococcus mutans MAW bacteria was showed the highest Fluoride tolerance (NaF) at 2000ppm/ml, it was selected for further studies. The results detected the specificity of fluoride resistance in bacterial phenotype that given quantitative determination of turbidity for *S. mutans MAW* through bacterial proliferation at NaF culture as shown in (table 1). The results appeared using NaF was caused releasing in bacterial growth to understand practically roles of the confirmed variant in the *S.mutans MAW* to fluoride-resistant until early exponential phase according to fluoride-resistant genes activity of mutated intergenic region may be let to appear mutant strain [21][32].

(Table 1): - Sodium Fluoride effects on *S. mutans MAW* growth

Growth type	Growth value ($O.D_{600}$) (cell /ml)	Incubation period (hr.)
S. mutans MAW culture without NaF	2×10^7	18
S. mutans MAW with 2000 (µg/ml) NaF	4×10^4	48

3.3- Identification of fluoride-resistance-related genes in *S. mutans MAW*

In the present study the fluoride resistance gene was used in bacterial diagnosis through amplification of conserved region of SNARE family genes by using specific Primers were designed and used for amplification of DNA by PCR, the products were separated on 1% agarose gel (Figure 2), the result demonstrated that *S. mutans MAW* has hypothetical gene band with 400bp.

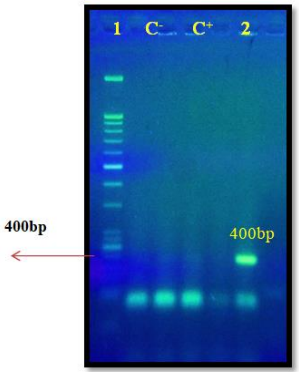


Figure (2): Agarose gel electrophoresis for amplification of *FRAM* geneof *S. mutans MAW* on (1%) agarose gel, 70 voltage for 90 minutes. Lane (1): DNA marker Ladder (100bp). Lane (C^-): Amplicon *FRAM* geneprimer with *S.salivarious*. Lane (C^+): Amplicon *FRAM* geneprimer with sensetive *S. mutans MAW*. Lane (2): Amplicon of *FRAM* gene(400bp) with resistance *S. mutans MAW*.

3.4- The sequencing analysis of fluoride resistance genes

The sequencing results appeared a new *FRAM* gene in *S. mutans MAW* have 400 bp and 44% long similarity with blast gene bank of *S. mutans UA159*(ID: CP007016.1) as shown in (Figure 3 A) [22][13][14]. Sequence similarity analysis was run by (ClonemanagerDemo$_{9.2}$program) the revealed genes which named (hypothetical gene) provisional gene was included overlapping gene belonged to SNARE-associated domain family genes, SNARE-like motif present coded to bacterial proteins responsible to inhibitory function for NaF toxicity, it considerate one of bacterial cell wall machines for bacterial resistance were promoting from SNARE - associated domain family genes [23]. *FRAM* geneof *S. mutans MAW* ishypothetical genes are responsible for catalyzes the conversion of charismatic to preheated in the pathway of tyrosine and phenylalanine biosynthesis [24][25][26][54] which responsible about fluoride resistance by unknown enzymes [26], mostly it are negatively regulated by tyrosine, tryptophan and phenylalanine and belonged to SNARE family genes (Gene bank PRK07248 is a number of the super family cl00693) (Figure3 B).

Differentiation modeling can produce homologous structures for many of the remaining sequences having unknown structures [27][3], but still in many cases is unable to identify good template structures because of inadequacies in sequence matching that mutant *FRAM* gene has identify 49% to *FR* gene of *S. mutans UA159* (ID: CP007016.1) came to low similarity 44%. Sequence matching is really important because it is a highly efficient key to compare proteins (genes),[28] identify protein (gene) functions and permits rapid comparisons and analyses of whole genomes. Therefore, the predicating *FRAM* protein matrix has 50 amino acid got similarly 80% alignment with *S. mutans UA159* FR

proteins using BLOSUM 62 matrices alignment (http///G:/today/SIM/Alignment/Tool/Protein/Sequences.html) [28]. These proteins pairing had high structural similarity but low genes sequence similarity, matching sequence can be sufficiently informative about the effects of mutations. [29][30][31] Many studies would have observed a higher percentage of overlaps genes called "hypothetical." Coding to unknown proteins [32], to investigate about hypothetical genes account for 53% of all annotated genes and are somewhat less likely to overlap than are remaining genes, the information explain *FRAM* gene has high verity sequence was correlated with mutation occurrence . The main peculiarities of the overlaps, such as quantity and quality are analogous between hypothetical and non-hypothetical groups [33][34][52] · Overlapping genes are appeared abnormal within a genome concluded (84%) of overlapping genes occur on the same strand in shape (tandem overlaps,→→)with some remaining (16%) overlaps occurring on opposite DNA strands in term (antiparallel overlaps,→←or←→)*[9][35].

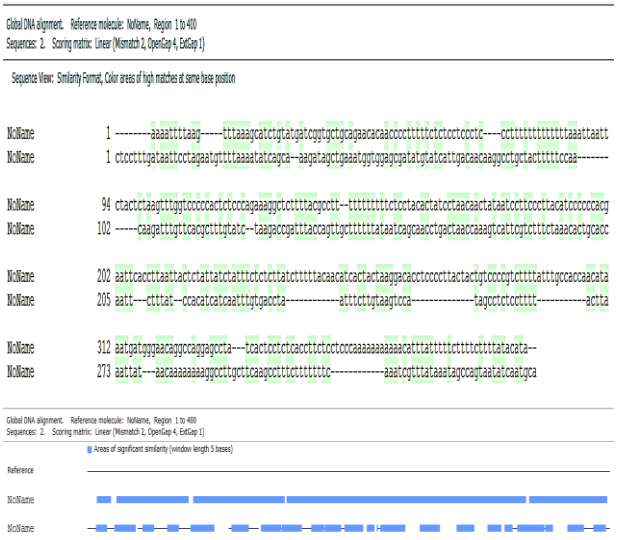


Figure (4): A- Amplification and sequencing of (*FRAM* gene) in *S. mutans MAW* bacteria,B - The similarity of *FRAM* gene sequence length is (44%) and identity is (49%) between *S. mutans MAW*and *S. mutans UA159* (ID: CP007016.1) .

3.5-FRAM gene submission in NCBI and phylogenetic tree:

The bacterial strain was identified up to other NCBI fluoride resistance species level by *FRAM* gene sequence analysis .The sequenced data obtained were edited a new data , aligned, and submitted to the NCBI GenBank for accession number became *S. mutans MAW* (ID: LC431527), that the detection show this gene belonged to unknown hypothetical proteins, generally they are negatively regulated by tyrosine, tryptophan and phenylalanine and belonged to SNARE family genes (Gene bank PRK07248 is a number of the superfamily cl00693) [36][23][25] (figure 4). Notably, homology searches with the *FRAM gene* sequence have returned only low-similarity hits was 44% [37][38], the closest matches (which all were putative FR genes from NCBI strains) shared 49% sequence identity were conducted using (clone manager demo $_{9.2}$) model .

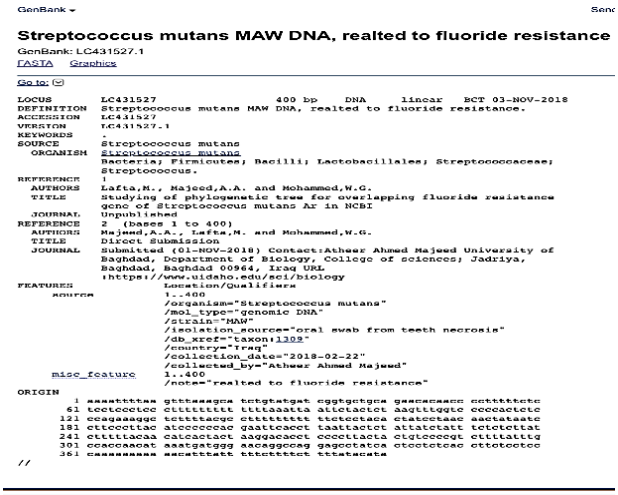


Figure (4): -The information page of *FRAM* gene 400bp submission of *S. mutans MAW* and the authors affinity in NCBI.

Specifically, all cases always attitude where protein manifestation alignment match but sequences gene do not necessary match well are resolved. Phylogenetic analyses and multiple-sequence alignment were conducted using MEGA6 model, the phylogenic tree structure was mainly appeared nearest to *S. mutans UA159* (ID: CP007016.1)than other groups that it take branched cluster of tree were closely related (ranging 20 nucleotide substitutions per site [nss^{-1}], respectively. Neighborhood graduation came from the comparison between *S. mutans* isolates have been recorded in the National Center Biotechnology Information (NCBI) to appear they have under sequence respectively with source of isolation and showed compatibility(figure5).

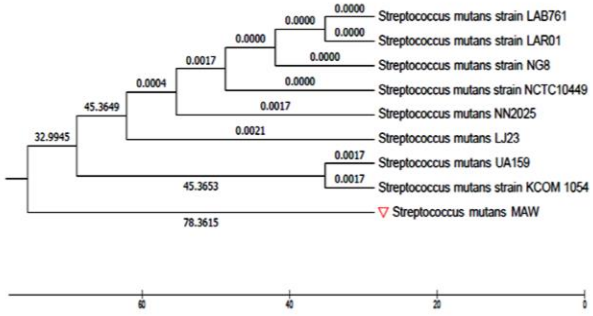
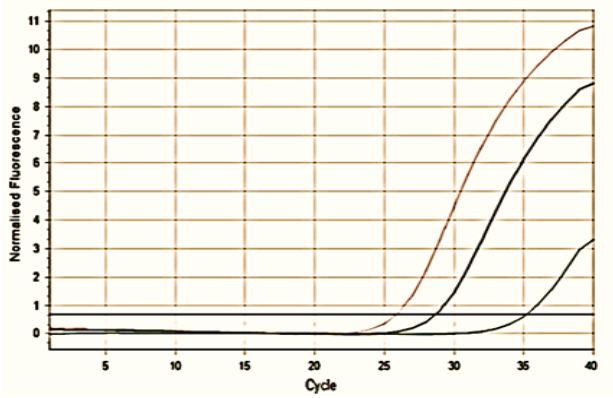


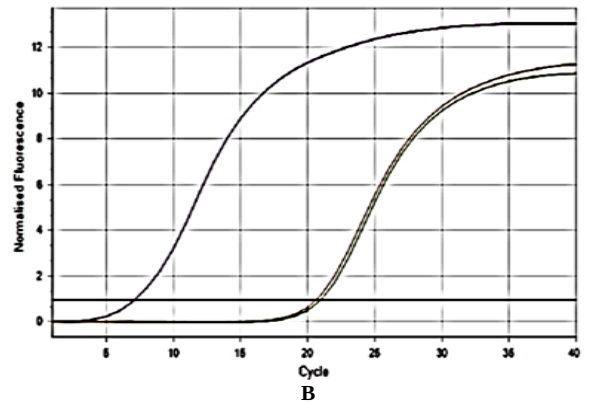
Figure (5): Genogram showing phylogenetic relations of the promising oral *Streptococcus mutans MAW* has closely related type and genetic distance with other fluoride resistance strains retrieved from NCBI GenBank. The GenBank accession numbers of the reference strains are explained in parentheses. Horizontal bars in the genogram represent branch length. Similarity of the neighboring full length of *FRAM* gene sequences have been indicated by bootstrap values. Distance matrix was calculated using Mega6 model. The scale bar indicates 20 substitutions per nucleotide position [nss^{-1}].

3.6- *FRAM* gene expression:

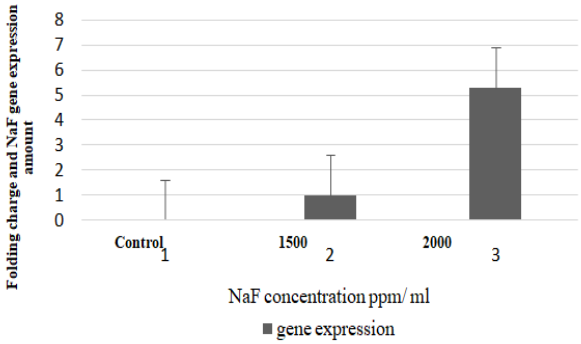
The study takes care to demonstrate mRNAs for *FRAM* gene such as SNARE family genes (hypothetical gene) was upregulated by fluoride resistance presetting in bacterial media. When *S. mutans MAW* was grown without NaF (control) and with 1500, 2000ppm NaF founding was compared, the bacterial resistance expression pattern when fluoride absenting didn't appear and it just showed expression increasing relevant to NaF presenting, when it acts as a genes stimulator in growth culture. Real-time RT-PCR data demonstrated *FRAM* gene expression was quantified and described the errors related to common practice of single control [39] normalization strategy based on single housekeeping gene (*16SrRNA*gene) leads to erroneous normalization up to 5.25 fold in this analysis. In conclusion, the *S. mutans MAW* cells at early, late exponential, and stationary phases were collected and subjected to whole RNA extraction, DNase treatment, cDNA synthesis [40] (Figure 6 A, B), real-time PCR has shown $2^{\Delta\Delta Ct}$ software method was used to calculate relative gene expression [41][42][36], the expression compered between NaF concentration (2000ppm) was grown up 5.25-fold higher than bacterial growing in (1500ppm), according to control when fluoride was absent. The results confirmed that *S.mutans MAW* acquired fluoride resistance through higher expression of overlapping fluoride resistance gene, constitutively higher activity of hypothetical gene (Figure 6 C).



A



B



C

Figure (6): A- melting curves of *FRMA* gene and housekeeping gene *16SrRNA* during the amplification program melts the unspecific Light Cycler PCR products. B- The effect of fluorescence acquisition in samples segment at elevated temperatures. SYBR Green I acquisition at 75°C in the 3rd segment and one "negative control ".C- The Folding change in *FRAM* gene expression in *S. mutans MAW* when it has grown with NaF (1500,2000) ppm/ml and the gene expression compared with control when bacteria has grown without NaF concentration.

4. DISCUSSION:

Many mechanisms may play roles in the antimicrobial activity of fluoride are included inexpensive physical and chemical procedures have been introduced for F removal, such as electro-chemical methods, adsorption operations, and ion exchange ways [3][5]. This study has been applied to identify and study one Fluoride-resistant gene responsible about one evidenced mechanism to support this hypothesis in dental *S. mutans MAW*, which is detected contrast to transient fluoride resistance is believed to be due to chromosomal mutations. [14][16]

Last a few years, multiple gene mutations have explored in a fluoride-resistant strains [32]. These recent studies brought a new concepts or candidates for the mechanism of fluoride resistance is not only the antimicrobial target sites of fluoride, but also particular sites are involved fluoride resistance.[32,14] The dental *S. mutans MAW* are classified according to its *16SrRNA* gene sequences, The present fluoride resistance phenotype when the bacteria was cultured at 1500, 2000ppm NaF, it showed The differences in the fluoride-resistance level replaced to genes type responsible to mechanism differentiated and examined in order to detection the relationship between the type of this gene and the fluoride resistance genes founding (table 1).The study suggests that oral *S. mutans MAW* possesses SANAR-family genes contain hypothetical gene like over-lapping genes kind which are associated with fluoride resistance observed through of PCR amplified to gene fragment and separated in 1% gel agarose appeared length 400bp. The sequence analysis for 400bp fragment and similarity analysis (clone manager demo $_{9.2}$) was leaving to 44% strand length similarity after cooperated with *Streptococcus mutans UA159* and other NCBI data base (figure 4), its lead to

discover the type of gene is for SNARE family genes responsible fluoride exporter machines such as one of bacterial cell wall changing genes.

The work investigation studies detected that, *FRAM* gene is annotated as 'hypothetical gene, of bacterial cell wall genes were included SNAR –family genes, It could assume enclose to the remaining genes are either homologous to genes of unknown function, therefore, it has identity 49% compared with NCBI database. It is often unclear whether they encode actual proteins, the confront genes are commonly coded to as `hypothetical', `uncharacterized', proteins. A general prediction of its function can be often made based on a conserved sequence modifying and mutation, subtle sequence similarity to a previously characterized protein or the presence of diagnostic structural manifestations [43][23][36]. Most reports have supported the hypothesis of protein - protein interactions data reported a high rate of positive interactions [44][45]. As well as, protein - protein interactions alignment typically offer the exact structure of the protein in BLOSUM62 take up matrices alignment 80% amino acid similarity compared with *Streptococcus mutans UA159* amino acid hypothetical proteins matrices in whole genome [45][46][47][52], in essence, functional characterizations can be obtained from genome wide protein interaction data through a new matrices alignments. [46] At least, the gene function could come from genome context analysis, phyletic patterns, of similarity lengths interaction NCBI data using PSI-BLAST after gene submission, [46][48][23] led to two more general functional investigations that were not made from sequence analysis alone but also we have taken a close sight on *FRAM* gene expression was confidently detected at the mRNA levels. these findings support a novel role for the *FRAM* gene products in *S. mutans MAW* accumulation and suggest that *FRAM* expression in this oral pathogen is subject to complex mechanisms of control imposed by growth phase, dietary F, and other factors present in the plaque environment [49][53][54].

Computational methods, including sequence analysis, phyletic patterns, domain fusions, structural threading of the branch lengths Similarity and gene neighborhoods can and should be used for prediction [50][51] of the likely mutation properties of this gene through direct experimentation because the furthest genetic distance between *FRAM* gene of *S. mutans MAW* and other NCBI data was 20 [nss^{-1}] [52][53][54].

5. CONCLUSION:

We conclude that fluoride exporter confers fluoride resistance to oral *S. mutans MAW*, in addition, using sequencing analysis was able to cover genetic changes in the genome of a fluoride-resistant strain that does not belong to the other fluoride resistance class. These findings can provide new sights into mechanisms of microbial fluoride resistance, its will be important for developing F^- removing engineered bacteria in the future. To the best of our knowledge, it is the first report on NaF absorption by the bacterial strains.

6. KNOWLEDGE:

The results described in this article was supported by Department of Biology, College of Science, University of Baghdad, like any rating research for instructors as annual achievements in their scientific field.

7. CONFLICTS OF INTEREST:

The authors declare that there are no conflicts of interest.

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RESEARCH ARTICLE

In-vitro Analysis of Phytochemical, Anti-oxidant capacity of seed Ethanolic extracts of *Sapindus saponaria* Vahl and Anti-bacterial activity on Common Dental Pathogens

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ABSTRACT:

In the present study, seed kernel ethanolic extract of *Sapindus saponaria* Vahl was analysed for its phytochemical, anti-oxidant capacity and anti-bacterial activity on Common Dental Pathogens. The extractive value was found to be 2.8 (%w/w). The phytochemical analysis revealed the presence of alkaloids, flavonoids, steroids, terpenoids, tannins and saponins. The dose response curve of DPPH radical scavenging activity of ethanolic extract of *S.sapindus* Vahl was compared with BHT and ascorbic acid showed nearly equal free radical scavenging activity (80%) at 0.1mg/ml. The scavenging activity of controls (BHT and ascorbic acid) was found to be 78.68 and 95% respectively. The IC_{50} values obtained was 0.04± 0.40mg/ml for seed kernel extract and for BHT (0.014±0.70 mg/ml) and ascorbic acid (0.027± 0.35 mg/ml) respectively. In ABTS radical scavenging method the inhibition was nearly 98% at 0.1 mg/ml for BHT. The IC_{50} values obtained was 0.012± 0.32 mg/ml for seed kernel extract and for BHT (0.018±0.60 mg/ml). The anti-bacterial activity of extracts was examined by ager well diffusion method at 200 mg mL^{-1}sample concentration. The results of anti-bacterial activity were found that ethanolic extract of S.saponaria was most effective against all tested bacterial pathogens (*Staphylococcus aureus, Streptococcus mutans, Streptococcus salivarius, Streptococcus sanguinis* and *Lactobacillus acidophilus*).. Maximum anti-bacterial activity was observed against S.salivarius (23 mm) and lowest activity against S. sanguinis (9 mm). The results validate the traditional uses of *S.saponaria* Vahl in treatment of dental diseases and as a potent anti-oxidant.

KEYWORDS: Anti-oxidant, Anti-bacterial, Seed Kernel, ethanolic Extract, *Sapindus saponaria*.

INTRODUCTION:

Medicinal plants occupy a very prominent place in folk medicine and synthesis of various biologically active secondary metabolites, many of which have been out come to have numerous roles in human ailments[1]. In the recent era the urge and priority for the plant secondary metabolites have raised to full extent to decipher various diseases and disorders all over the world. Now-a-days they became the key compounds for various therapeutic purposes or as precursor's models for the synthesis of useful drugs[2].

Till recent times, much of the investigation in the pharmacological field was going on to develop new novel compound with greater stability and can act on wide range of health issues. As a part of it analysis on phytochemicals, anti-inflammatory and anti-microbial activity of these medicinal plants have gained much attention[3]. The novel science research communities already confirmed the use of natural anti-oxidants and anti-microbial compound will become a key to unlock various oxidative stress-related and pathological aspects. Microbial resistance to almost all antibacterial agents has been reported[4]. This resistance is largely due to indiscriminate use of antibacterial drugs commonly used in treatment of these diseases. A parts from resistance, some antibiotics has side effects which limit their usage. So there is a urgent need to discover new spectrum of antibacterial agents with will minimize the side effects. The plants are considered as potential source to discover

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novel anti-bacterial and anti-microbial prototypes[5,6]. The present study describes the evaluation and phytochemical screening of antibacterial potential plant species *Sapindus saponaria* against four common human pathogens. *Sapindus saponaria (*family: Sapindaceae) with alternative Botanical Names *Sapindus thurstoniiI* and commonly known as Manele, A'e, Hawaiian Soapberry was a known medicinal plant with various potential medicinal values. As a part of search for anti-bacterial compounds from this plant we extracted and screened the seed (kernel) ethanolic solvent for anti-bacterial activity on dental pathogens. Dental diseases are due to pathogenic stimulus have became one of the most common health issue in the human community[7] and are mainly of considered to be three types (dental plaques, periodontal diseases and dental caries)[8]. Dental caries are common oral bacterial pathological strains causing a biofilm and causing a great damage to enamel, dentin or cement of teeth due to bacterial activities[9,10]. Periodontal diseases are bacterial infections that affect the opinionated structure of the teeth. A large number of resident's bacterial strains like *Streptococcus, Actinomycetes*, *Lactobacillus* and *Porphyromonas* were considered to be responsible for this type of disease in humans[11].

MATERIALS AND METHODS:

Chemical:

All the solvents used in this study were purchased from Merck Chemicals, India and are of analytical grade.

Seed Collection:

The fresh matured healthy fruits were collected from the plant species *Sapindus saponari Vahl* which was observed and located in the local village area of Narasaraopet, Guntur district Andhra Pradesh (India). The plant was identified based on its vegetative and floral description given in the botanical literature. The fruits were air dried under shade and after complete drying the seeds were separated. The seed coat of dried seeds was manually separated and the kernels alone were used for preparation of extracts.

Preparation of Ethanolic Extracts:

The dried seeds kernels were finely powder for 5 min using a sterilized mixer grinder and stored in air-tight bottles. The kernel powder (50g) was added to 200ml of ethanol and kept overnight undisturbed at 10°C. Then next day the solution was allowed to stand for 1-2 hrs in boiling water bath with occasional shaking, and kept undisturbed for 24h. The preparation was filtered through a sterilized Whatman No.1 filter paper and the filtered extract was concentrated under vacuum below 40ºC using rotaevaporator[12,13]. The dried extract thus obtained was exposed to UV rays for 24h and checked for sterility on nutrient agar plates and stored in labelled sterile bottles in a freezer at 4°C until further use[14].

Determination of extractive value:

The filtrate was concentrated on a rotary evaporator under reduced pressure. The ethanolic extracts obtained were weighed and the percentage yield of the extract was determined by using the method adopted by Banso & Adeyemo, (2007)[15] with minor changes.

In Vitro Phytochemical Screening:

In order to analyse various secondary metabolites (phytochemical) constituents present in the organic solvent (ethanol) extract, various standard protocols were applied[16,17,18].

In-vitro antioxidant analysis:

1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay:

The effect of ethanolic seed kernel extract on DPPH radicals was estimated according to the method of Blois[19] with minor changes. The seed kernel ethanolic extract was lyophilised under vacuum and dilutions were prepared with concentrations of 0.02mg/ml to 0.1mg/ml. 1ml (i.e) (0.135mM) of DPPH solution was mixed with 1.0ml of extract (in ethanol). The reaction mixture was thoroughly mixed and kept in the dark for 30 min under 25°C±2°C. Reduction in the absorbance of the mixture was recorded at 517nm filter using ascorbic acid as control. Scavenging of DPPH radicals by the extract was calculated using the following formula:

Percentage Inhibition = [(Abs of control – Abs of the sample)]/ *(*Abs of control*)*] × 100

Where Abs of control is the absorbance of DPPH and Abs of the sample is the absorbance of the DPPH radical + sample ethanolic extract. The half maximal inhibitory concentration (IC_{50}) values denoted the concentration of sample required to scavenge 50% of DPPH free radicals.

2, 2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical scavenging assay:

The ABTS assay method was also applied to have a comparative analysis for the extract by using the method followed by Re *et al* [20]. ABTS solution was prepared by mixing equal volume of 7 mM ABTS in water and 2.45 mM potassium persulfate (PPS) solution and left for 12-16hrs indark. To prepare a working solution 1ml of diluted working solution of ABTS-PPS was mixed with 1ml of seed kernel ethanolic extract, and the absorbance read at 734 nm after incubation for 10 minutes. ABTS.+ the scavenging capacity of the extract were compared with standard butylated hydroxytoluene (BHT). The percentage inhibition of the formation of

ABTS.+ was calculated using the following formula:

Inhibition (%) = [(Abs of control – Abs of the sample)]/ *(*Abs of control*)*] × 100

Where Abs of control is the absorbance of ABTS radical + methanol and Abs sample is the absorbance of the ABTS radical + ethanolic extract.

Dental pathogens tested:

The strains of dental infection related bacteria used in this study were *Staphylococcus aureus*, *Streptococcus mutans*, *Streptococcus salivarius*, *Streptococcus sanguinis* and *Lactobacillus acidophilus* respectively. All the bacterial strains were grown and maintained on nutrient agar slants at 4ºC. All the strains were procured from the Microbial Type Culture and collection, Chandigarh, India.

Inoculums preparation:

Stock cultures were maintained at 4ºC on slopes of nutrient agar. Active cultures for experiments were prepared by transferring a loopful of cells from stock cultures to test tubes of Mueller-Hinton Broth for bacteria that were incubated without agitation for 24 h at 37ºC.

Screening for anti-bacterial activity:

The seed kernel methanolic extract of *S.saponaria Vahl* was used for the screening. Anti-bacterial activity of various extracts was determined by the agar well diffusion method[21]. The plates were incubated at 37ºC for 24 h during which activity was evidenced by the presence of a zone of inhibition surrounding the well. Each test was repeated three times and the antimicrobial activity was expressed as mean of diameter of inhibition zones (mm) produced by the extracts when compared to controls.

RESULTS AND DISCUSSION:

In the present study the phytochemical, anti-oxidant capacity of Seed (kernel)Ethanolic extracts of *Sapindus saponaria* Vahl and anti-bacterial activity on Common Dental Pathogens was analysed *in vitro* as the data regarding was very scanty. The extractive value of the seed kernel ethanolic extract was found to be 2.8 (% w/w) **(Table 1)**. This may be due to the availability of various secondary soluble metabolites in the organic solvent mixture. The preliminary phytochemical analysis of the ethanolic extract revealed the presence of alkaloids, flavonoids, steroids, saponins, tannins and terpenoids these results are in full agreement with the date published by Prabhaker *et al*., 2012[22] and percentage of flavonoids, saponins and volatile oils were more when compared to others **(Table 2)**. Various plants are known to have beneficial therapeutic effects as noted in the conventional Indian system of medicine, *Ayurveda*. The effect of many medicinal plants organic extracts on bacterial and microbes have been investigated by large group of researchers in different locations of the worlds. It has been recommended that aqueous and ethanolic extracts from diverse medicinal plant species was used in allopathic medicine are potential sources of anti-viral, antitumoral and anti-microbial agents due to the presences of secondary metabolites[23].

Table 1: Extractive Value of Seed Kernel ethanolic extract.

Name of the Plant Source	EV (% w/w)
Sapindus saponaria Vahl (Seed Kernel)	2.8

Table 2: Qualitative Analysis of Phytochemical from the seed kernel ethanolic extract of *Sapindus saponaria* Vahl.

S. No	Phytochemicals	Methanolic Extarct
1	ALKALOIDS	+
2	FLAVONOIDS	+++
3	PHENOLS	+
4	SAPONINS	+++
5	VOLATILE OILS	+++
6	TANNINS	+
7	TERPENOIDS	++

+ Present; - Absent: + Less; + + Moderate; + + + High; + + + + Very high

The *In vitro* antioxidant potentiality of seed kernel ethanolic extract was studied by the inhibition of DPPH radical assay[1]. The dose response curve of DPPH radical scavenging activity of ethanolic extract of *S.sapindus* Vahl was compared with BHT and ascorbic acid **(Fig 1).** The extract almost showed nearly equal free radical scavenging activity (80%) at 0.1mg/ml. The scavenging activity of controls (BHT and ascorbic acid) was found to be 78.68 and 95% respectively. The IC_{50} values obtained was 0.04±0.40mg/ml for seed kernel extract and for BHT (0.014±0.70mg/ml) and ascorbic acid (0.027±0.35mg/ml) respectively. Another method of radical scavenging was also adopted to have a comparative analysis of the study, by using ABTS assay [5]. The ABTS free radicals are generated by the oxidation of ABTS + cations radicals were produced by the reaction between 7mM ABTS in water and potassium persulfate. The kernel ethanolic extract of *S.saponari* showed nearly 80% (0.1mg/ml) of rapid and effective scavengers of ABTS radicals **(Fig 2)** and this activity was compared with BHT. The inhibition was nearly 98% at 0.1mg/ml for BHT. The IC_{50} values obtained was 0.012±0.32mg/ml for seed kernel extract and for BHT (0.018±0.60mg/ml). The results showed that the relative antioxidant activity of the ethanolic extract was good when compared to the standard controls. Proton-radical scavenging is also an important trait of antioxidant activity of the extract[24]. This implies that the plant seed kernel extract was found to be significant and useful for treating pathological damage at higher concentration when used.

The anti-bacterial activity of seed kernel ethanolic extracts of *S.saponaria* was assayed under *in vitro* conditions by agar well diffusion method against the selected dental pathogens. The inhibition of microbial

growth by ethanolic solvents extract was shown in **(Table 3)**. The extract, chosen for the study and extraction process showed significant anti-bacterial activity. The organic solvent extract showed highest zones of inhibition on the selected dental pathogens. The highest zone of inhibition was recorded with *Lactobacillus acidophilus* (27mm) and lowest zone was noticed against *Streptococcus mutans* (17mm). The results are in agreement with the previous work done by [25-28]. This suggests that the seed kernel ethanolic extract can be used as an anti-bacterial agent for various dental pathogens and this may be due to the presence of secondary metabolites[29,30,31].

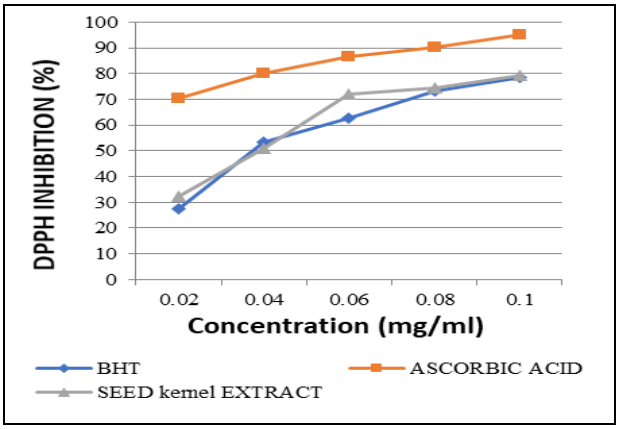

Fig1: DPPH scavenging activity of ethanolic extract of Seed kernel of *Sapindus saponaria* Vahl.

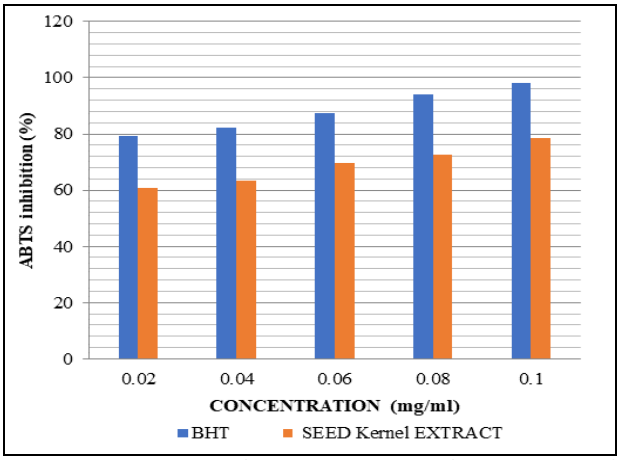

Fig 2: ABTS activity of ethanolic extract of Seed kernel of *Sapindus saponaria* Vahl

Table 3: Anti-bacterial activity of *S.saponaria* Vahl seed kernel ethanolic extract against dental pathogens

Dental pathogens	Diameter of zones of inhibition (mm)
	See kernel Methanolic extract
Staphylococcus aureus,	24
Streptococcus mutans,	17
Streptococcus salivarius,	25
Streptococcus sanguinis	22
Lactobacillus acidophilus	27

*Values are means of three replicates, Bore diameter is 6 mm

CONCLUSION:

Since the tested ethanolic extract of *S.saponaria* was very effective against the tested dental pathogens, purification and toxicological studies on these plant seed (kernel) extracts and *in vivo* trials should be carried out so that it may be used as a probable source for the development of a phytomedicine to act against dental pathogens. The enhancement in the anti-bacterial activity of these extracts can be done by purification of phytoactive compounds and administration of appropriate dosages. As the global scenario is now changing towards the use of nontoxic and non-synthetic products, these plants metabolites can be used for traditional medicinal and development of modern drugs.

CONFLICT OF INTEREST:

The authors have no conflict of Interest.

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RESEARCH ARTICLE

A Crystal Engineering design to enhance the Solubility, Dissolution, Stability and Micrometric properties of Omeprazole via Co-crystallization Techniques

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ABSTRACT:

Introduction: Omeprazole, a proton-pump inhibitor used in peptic ulcers, gastro-oesophageal-reflux disorder, Zollinger-Ellison syndrome and in H.pylori infections. The omeprazole is unstable at acidic pH, undergoes degradation in stomach. To prevent the degradation in stomach, dosage forms are supplied as enteric-coated tablets or granules encapsulated in gelatin capsules. The efficiency of such dosage forms depends upon the extent of coating, solubility of coating material, type of dosage forms; in addition this it is insoluble in water, having poor bioavailability. To overcome these major drawbacks of omeprazole, an novel technique: co-crystallization was attempted, to produce a stable, enhancement in solubility and improved micromeritic properties of omeprazole. Co-crystals consists of API and a stoichiometric amount of a pharmaceutically acceptable co-crystal former. Pharmaceutical Co-crystals are non-ionic supramolecular complexes and can be used to address physical property issues such as solubility, stability and bioavailability in pharmaceutical development without changing the chemical composition of the API. Co-crystal is a crystalline entity formed by two different or more molecular entities where the intermolecular interactions are weak forces like hydrogen bonding and π- π stacking. **Materials and methods**: As omeprazole is unstable in acidic pH, very hygroscopic character, photosensitive and thermolebility in nature, Anti solvent addition method was used for preparation of crystals. Poly Vinyl Pyrrolidone is taken as co-former, magnesium chloride and sodium bicarbonate as basic substances. Further prepared crystals were characterized by Powder X-ray Diffractometer (PXRD), Differential scanning colorimeter (DSC), Fourier Transformation Infra-red Spectroscopy (FTIR).Moreover they were studied for melting point determination, flow property studies (micromeritic properties),dissolution studies, solubility test and stability tests. **Results and discussion:** FTIR revealed that there is no formation of hydrogen bonding between drug and co-former; hence it states that co-crystals are not formed, but XRD and DSC states that there is a formation of some physical interaction, partial crystalline and amorphous form which is a new crystal lattice. **Conclusion:** Formed crystals produce moderate improvement in micromeritic properties, solubility, stability and dissolution behaviour of drug. Thus physicochemical properties of omeprazole were improved via crystal engineering technique – co-crystallization.

KEYWORDS: co-crystallization, dissolution behaviour, micromeritic properties.

INTRODUCTION:

Omeprazole, a proton-pump inhibitor is widely prescribed for the treatment of peptic ulcer, Zollinger-Ellison syndrome, Gastro-oesophageal reflux disease (GERD), H.pylori infection and NSAID associated ulcers. Its oral bioavailability (40-50%) in humans is poor, due to acid sensitivity and first pass metabolism. Attempts were made earlier to improve the

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bioavailability by formulating it as enteric-coated granules encapsulated in gelatin shell and enteric-coated tablets etc. The efficiency of such dosage forms depends upon the number of parameters such as extent of coating, solubility of coating material and type of dosage form [1]

The maximum development and interest area is being diverted to co-crystallization. Co-crystals are homogeneous solid phases containing two or more neutral molecular components in a crystal lattice with defined stoichiometric proportions, which are solids at room temperature and are held together by weak interactions, mainly hydrogen bonding. Pharmaceutical co-crystals can enhance the essential properties of the APIs such as flowability, chemical stability, compressibility and hygroscopicity. Co-crystallization can be achieved only when the physiochemical properties like Hygroscopicity, solubility, micromeritic properties and compaction behaviour of the formulation is to be improved at one step. Co-crystals basically consists of two components that are the API and the co-former, the co-former can be any other excipient or API which when given in combination reduces the dose and also the side effects. Changing the co-former will also changes the pharmaceutical properties, chemical stability, bioavailability, solubility, melting point, moisture uptake, dissolution.

As mentioned earlier co-crystallization is the most dynamically developing group of solid pharmaceutical substances, it is a very vast area. Hence, they can be divided into co-crystal anhydrates, co-crystal hydrates (solvates), anhydrates of co-crystals of salts and hydrates (solvates) of co-crystals of salts [2, 3].

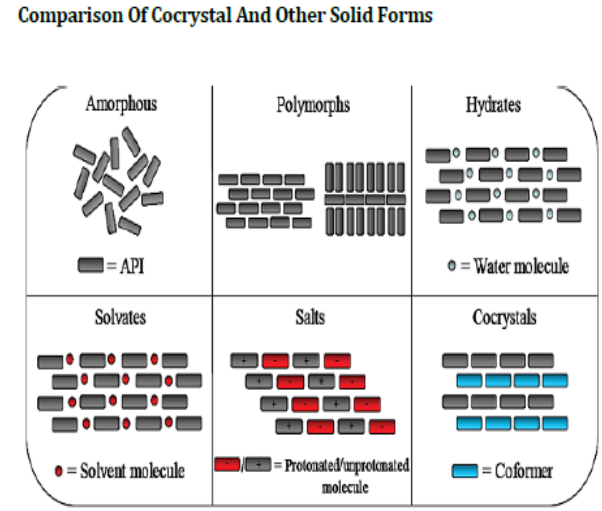

Figure.1 Comparison of co-crystals with other solid forms

The according to the BCS classification the API belonging to class II and IV have always posed a challenged in case of enhancing the solubility. Hence, one such option is crystallization. Thus the knowledge of crystal engineering along with the molecular properties of active pharmaceutical ingredients can pose a great option. Co-crystals consists of two or more molecules with a hydrogen bonding. The most appropriate co-crystal can be selected using various analytical techniques and rational physicochemical studies that include investigations of solubility and stability.

A more refined definition of a co-crystal can be "multicomponent crystal that is formed between two compounds that are solids under ambient conditions, where at least one component is an acceptable ion or molecule. Co-crystallization with pharmaceutically acceptable compounds does not affect pharmacological activity of API but can improve physical properties, such as solubility, hygroscopicity, compaction behaviour [4, 5].

Co-crystals often contain self-assembly units based on supramolecular synthon that are derived from motifs that are commonly found in crystal structures. As co-crystallization basically involves the conversion of molecule to a neutral form it does not matter whether the API is ionic, non-ionic and acidic or basic. The co-former or the counter molecule used in co-crystallization may be considered non-toxic, thus increasing the scope of co-crystallization over salt formation. The counter molecule may be an excipient, food additive, preservatives, vitamins, minerals, amino acids and other biomolecules or another API [6].

The first co-crystal synthesized was quinhydrone which is a 1:1 co-crystal between benzoquinone and hydroquinone. To design a co-crystallization experiment, two aspects should be kept in mind the first one being the evaluation of the robustness of the potential intermolecular interactions and considering the hydrogen bonding rules. Robustness can be checked by analyzing the trends within the Cambridge Structural Database (CSD) or by retrospective data [7,8]. In a co-crystal there exists hydrogen bond which imparts a robust and directional nature to it. Hence in case where hydrogen bonding plays an importance this rule should be considered which says that string hydrogen bond donor tends to interact with the best hydrogen bond acceptor in a given crystal structure. This 'best-donor–best-acceptor' rule can be of great utility in the design of specific hydrogen bonding interactions [9]. The methods of preparation were carried by different techniques.

Table .1 Methods used for co-crystal preparation [10]

Solid based methods	Solution based methods	Supercritical fluid methods	Electrically assisted methods
Co-grinding/dry grinding	Isothermal slurry conversion	Supercritical slurry crystallization	Sono-crystallization
Solvent drop grinding	Slow evaporation	Supercritical anti-solvent (SAS) and gas anti-solvent (GAS)	Microwave assistance
Extrusion	Assisted evaporative co-crystallization	Supercritical assisted spray drying	
Hot melt extrusion	Spray drying		
High shear wet granulation	Reactive co-crystallization		
	Cooling co-crystallization		
	Anti solvent addition		

MATERIALS AND METHODS:

Materials:

Omeprazole (USP) was obtained as a gift sample from Rawchem Laboratories Pvt. Ltd. (Hyderabad, India). PVP, magnesium chloride, sodium bicarbonate, Methanol, Ethanol, Acetone, 2-propanol, Sodium dihydrogen orthophosphate dihydrate, Disodium hydrogen orthophosphate dihydrate (AR grade) were obtained from different commercial suppliers.

Methods:

Experimental attempts to Prepare omeprazole Co-crystals:

To prepare omeprazole co-crystal, a best co-former should be selected first with hydrogen bonding donor and acceptor ability. All the dicarboxylic acids, esters, ethers, some drugs and excipients can have good co-former character. But omeprazole is a basic drug, it is unstable in acidic medium, hence co-former must be a basic compound with donor, acceptor ability. Co-grinding method, solvent drop grinding method, slurry crystallization method, slow evaporation method were failed due to instability, hygroscopicity, photosensitivity and thermolebility of omeprazole. Then basic compounds were selected and preparation method was optimized and finalized to anti solvent addition method.

Table. 2 Experiments attempted to make co-crystals of omeprazole

Compound	Co-formers attempted	Method of preparation	Inference
OMEPRAZOLE	Di carboxylic acids	Co-grinding	Unstable preparation
	PVP	**Anti solvent addition***	**Stable crystalline substance obtained.**
	PVP	Co-grinding	Unstable preparation
	PVP	Solvent drop grinding	Unstable preparation
	Calcium carbonate	Slow evaporation	Unstable preparation
	Magnesium carbonate	Co-grinding	No crystal formed
	PEG	Co-grinding	No crystal formed
	Sodium carbonate	Slurry crystallization	Unstable preparation
	PVA	Co-grinding	Unstable preparation

Method of crystals preparation:

Anti–solvent addition method:

Pharmaceutical crystals of omeprazole were prepared with PVP as co-former, magnesium chloride and sodium bicarbonate as basic substances for maintaining stability in basic pH with different solvents like acetone, methanol, ethanol, 2-propanol by anti solvent addition method. crystal was prepared by dissolving 1:1 molar ratio of omeprazole and PVP in 10ml of solvents separately followed by addition of both solutions to magnetic stirrer, allow to mix thoroughly for 20 min, 20 ml of 2% magnesium chloride solution (cool water at 10^{O}C -20^{O}C) severs as anti-solvent is added slowly to the preparation, a turbidity is formed initially gradually it produces precipitate allow to stir continuously for about 2 hrs. Then filtered and dried at 40˚C and stored in vials at cool and dark place.

Characterization studies of prepared crystals:

Melting point:

Melting point of pure omeprazole, co-formers and co-crystals were obtained by capillary method using liquid paraffin. The capillary filled with drug powder was placed in melting point apparatus and then liquid paraffin was heated, then drug is melt the melting point of drug powder was noted.

Differential Scanning Calorimetry (DSC):

Thermal analysis of omeprazole, co-former and prepared crystal were recorded individually on DSC (Q200), Waters. The samples were scanned at 10^{O}C/min over a temperature range of 50^{O}- 400^{O}C with nitrogen purging in aluminium pan.

Fourier Transform Infrared (FT-IR) Studies:

FT-IR of pure omeprazole and prepared crystals spectra were recorded individually by a Spectrum RXI, Perkin Elmer FTIR spectrophotometer by mixing them with potassium bromide. Scans were recorded in the range of 400-4000 cm-1 at spectral resolution of 4 cm-1.

Powder X-Ray Diffraction:

The X-ray diffractogram were generated using a Bruker diffractometer D8 Advance. Multiscans over 10-60 minutes were employed over the 2θ- range 10-80^{O}, with a scan speed 4^{O}/min.

Drug Solubility study:
The solubility studies of omeprazole and prepared crystal were performed by shake flask method. The omeprazole was added in excess to the distilled water in vials. The vials were sealed and kept in the rotary shaker (Orbitek- Scigenics Biotech, India) at 25ºC ± 0.5 and 100 rpm. After equilibrium the solutions were filtered using whatmann filter paper, diluted suitably with distilled water and subjected for quantification of omeprazole by UV spectrophotometrically at the wavelength of 301nm [8].

Drug Dissolution study:
The dissolution study for pure omeprazole and prepared crystal was carried out using USP type I (basket type). Dissolution apparatus with 900 ml of 6.8 pH phosphate buffer as dissolution medium at 37^{O}C±0.5. The samples were withdrawn at definite time intervals (10 min) and replaced by fresh dissolution medium for one hour and were quantified using UV spectrophotometer (Perkin Elmer lambda 25) at 301nm [11,12].

Micromeritic properties [13]
Angle of repose
The angle of repose for pure drug and preparations were determined by fixed funnel method (Liberman et al., 1990). The accurately weighed pure drug and preparations was taken in a funnel. The height of the funnel was adjusted in such a way that the tip of the funnel just touches the apex of the heap of the powder. The powder was allowed to flow through the funnel freely into the surface. The height and diameter of the powder cone was measured and angle of repose was calculated.

Tan θ= h/r. Where, θ is the angle of repose, h is the height in cm; r is the radius in cm

Values for angle of repose ≤ 300 usually indicate a free flowing material and angles ≥ 400 suggest a poorly flowing material. 25- 30 showing excellent flow properties, 31-35 showing good flow properties, 36-40 showing fair flow properties, 41-45 showing passable flow properties.

Bulk Density:
The loose bulk density and tapped density were determined by using bulk density apparatus. Apparent bulk density was determined by pouring the blend into a graduated cylinder. The bulk volume (Vb) and weight of the powder (M) was determined. The bulk density was calculated using the formula,
Db =M/Vb
Where, M is the mass of powder, Vb is bulk volume of powder

Tapped Density:
The measuring cylinder containing a known mass of blend was tapped for a fixed time. The minimum volume (Vt) occupied in the cylinder and the weight (M) of the blend was measured. The tapped density was calculated using the formula,

Dt = M/Vt

Where, M is the mass of powder, Vt is tapped volume of powder

Carr's Index (%)
The compressibility index has be proposed as an indirect measure of bulk density, size and shape, surface area, moisture content and cohesiveness of material because all of these can influence the observed compressibility index.

The simplest way for measurement of free flow of powder is Carr's Index, a indication of the ease with which a material can be induced to flow is given by Carr's index (I) which is calculated as follows:

CI (%) = [(Tapped density – Bulk density)/Tapped density] x100

ρt tapped density and ρb is bulk density. The value below 15% indicates a powder with usually gives rise to good flow characteristics, where as above 25% indicates poor flowability. 1-10 showing excellent flow properties, 11-15 showing good flow properties,16-20 showing fair to passable, 21-25 showing passable.

Hausner's ratio:
Hausner's ratio is an indirect index of ease of powder flow. It is calculated by the following formula.

Hausner's Ratio = Tapped density (ρt)/ Bulk density (ρb)
Where ρt tapped density and ρb is bulk density. Lower Hausner's ratio (<1.25) indicates better flow properties than higher ones, between 1.25 to 1.5 showing moderate flow properties and more than 1.5 poor flow.

Stability studies:
Stability study of omeprazole crystals was carried out for 90 days at specified condition. They are stored at temperature and humidity conditions of 25ºC ±2C/60% RH ± 5% RH and 40 ± 2ºC/ 75% RH ± 5% RH. [15], then optimized crystals were analysised for melting point, micromeritic properties, solubility test and dissolution test.

RESULTS AND DISCUSSION:

Table 3. physical characterizations studies of preparations with different basic compounds and solvents.

S. NO	CODE	CO-CRYSTAL COMPONENTS	2% BASIC COMPOUND	SOLVENT	MELTING POINT (°C)	SOLUBILITY mg/ml
1.	CO 1	Omeprazole + PVP	$NaHCO_3$	Ethanol	142	0.794
2.	CO 2*	Omeprazole + PVP	$MgCl_2$	Ethanol	135	1.0*
3.	CO 3	Omeprazole + PVP	$NaHCO_3$	Acetone	137	0.822
4.	CO 4	Omeprazole + PVP	$MgCl_2$	Acetone	135	0.799
5.	CO 5	Omeprazole + PVP	$NaHCO_3$	Methanol	145	0.810
6.	CO 6	Omeprazole + PVP	$MgCl_2$	Methanol	134	0.832
7.	CO 7	Omeprazole + PVP	$NaHCO_3$	2-Propranol	144	0.816
8.	CO 8	Omeprazole + PVP	$MgCl_2$	2-Propranol	136	0.438
9.	-------	Omeprazole	----------	----------	146	0.31

*Mark is denoted for the significant in the solubility and selected for preparation of crystals.

DISCUSSION:

From the table 2, solubility and melting point data of different preparations were reported. CO 2 code preparation is having enhanced in solubility when compared with pure omeprazole. Thus it is chosen for further characterizations studies and dissolution studies.

Melting Point:

Discussion

Melting point is amongst the physicochemical properties of co-crystals. It is the temperature of solid and liquid phase equilibrium. This test was used as a preliminary test for confirmation of co-crystal formation. When the co-crystals are formed the melting point changes and comes in between the melting point of two individual molecules. If such results are obtained it can be confirmed that the co-crystals are formed.[9] It was found that the melting point of the crystals 135 ^{O}C showed a significant deviation with respect to the melting point of pure drug omeprazole 146^{O}C and the individual co-former PVP 174^{O}C indicating there must be some interactions between pure and co-former. [3]

Characterization by DSC

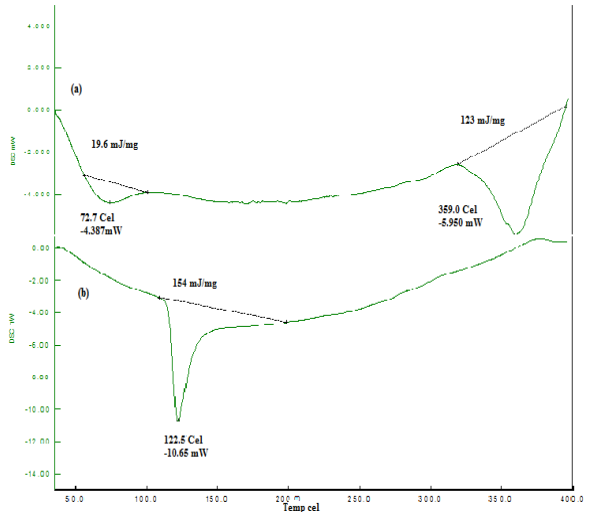

Figure 2. DSC of omeprazole-PVP crystals (a) pure omeprazole (b)

DISCUSSION:

From the fig 2 DSC thermograms, it was observed that the prepared crystals were different in pattern and intensity, as compared to pure omeprazole, which indicates their interaction. This shift in the melting point is due to the change in crystal lattice of the omeprazole in presence of co-former, forming a relatively different crystal lattice. The DSC thermogram of pure omeprazole(b) shows sharp melting peak at 122.5°C while DSC scan of their prepared crystals (a)shows a large broad peak at 72.7°C followed by another quite broad peak at 359°C depicting that melting peak of both omeprazole have shifted to lower and PVP have shifted to higher temperatures. These changes occurred as a consequence of interaction induced by thermal energy between the drug and the co-former, during the DSC scan of sample. The DSC thermogram for omeprazole-PVP crystal shows a broad peak at 72.7°C followed by a sharp melting peak at 359°C this DSC scan of crystals suggesting the formation of a new phase.

Fourier Transform Infrared (FT-IR) Studies:

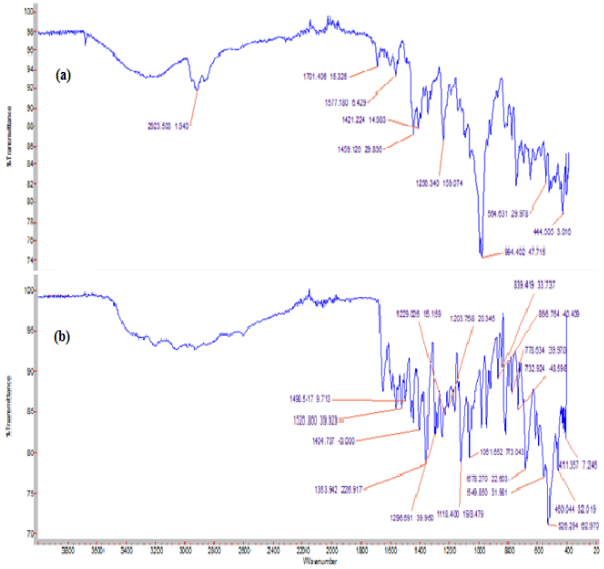

Figure 3. FTIR spectrum of omeprazole –PVP crystals (a) and pure omeprazole (b)

DISCUSSION:

From the fig 3 FTIR patterns of pure omeprazole(b) shows characteristic absorption at 1456 C-H stretching of methyl groups, 1353 of S=O stretching of sulfinyl group, 1296 of C-N stretching for aromatic amine. But there is a change in the functional groups present in the prepared crystals (a) compared to that of pure omeprazole drug, absorption at 2923 of C-H stretching of alkanes, 1701 of C=O stretching of conjugated acids, 1421 O-H stretching of alcoholic group, 1459 C-H stretching of methyl groups, 1256 stretching of C-O aromatic esters showing presence of new bond formation in the optimized crystals. But it confirms no formation of hydrogen bonding between drug and the co-former. Thus it formed a new form of crystal lattice.

Powder X-Ray Diffraction

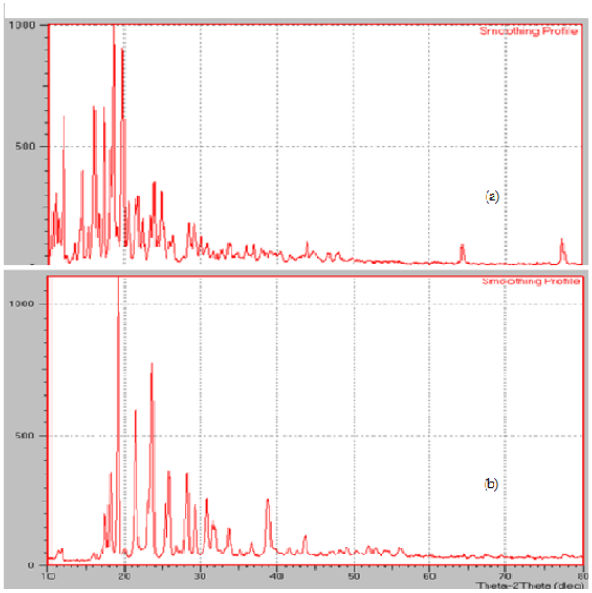

Figure. 4. XRD pattern of omeprazole-PVP crystals (a) and pure omeprazole (b)

DISCUSSION:

XRD is a useful method for fast identification of the new phases. A different XRD pattern for the crystals from those of the individual components confirms the formation of a new crystal phase [6].

The crystallogram pattern of prepared crystals (a) shows a very high variation in peak intensities as compared to that of the pure omeprazole (b) as shown in Fig.4. Hence it was concluded that, some physical or chemical bonding incurred between pure omeprazole and PVP resulting in the formation of respective new crystals habitant. The intensity of 2θ values of pure omeprazole and prepared crystals were as followed.

Omeprazole values (2θ- Intensity) were 17.4- 112, 18.1-213, 19.1- 730, 23.3- 505, 25.9-222

Optimized omeprazole crystals values were (2θ-Intensity) 12- 403, 14.4- 249, 16- 428, 17.35- 415, 18.5-649, 19.7- 572, 23.8-205

Drug Solubility study:

Discussion:

Increased in saturation solubility for prepared crystals (1mg/ml) when compared to pure omeprazole (0.3mg/ml). Solubility is increased by 3.33 times than pure omeprazole.

Comparative Drug Dissolution study:

Discussion:

From Fig.5 it was observed that prepared crystals of omeprazole-PVP showed enhanced drug release as compared to pure omeprazole alone.

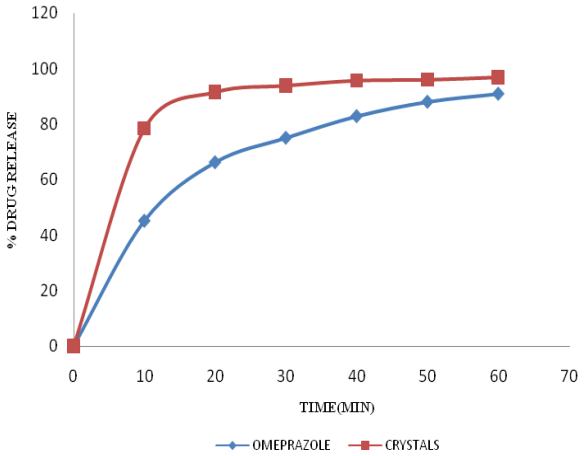

Figure 5. Comparative dissolution profile of pure omeprazole and crystal

Micromeritic properties:

Table 4. Micromeritic properties

S. No	Compound	Bulk density (mg/ml³)	Tapped density (mg/ml³)	Angle of repose (°)	Carr`s index (%)	Hausner`s ratio
1.	Pure omeprazole	0.184±0.01	0.236±0.02	32 ± 1	17.2 ±1.02	1.28 ± 0.01
2.	Omeprazole crystals	0.188 ±0.02	0.223 ±0.01	29 ± 0.5	15.69 ±1.01	1.19 ± 0.02

n = 3 , results was expressed in mean ± SD

DISCUSSION:

From the table 4 obtained values, prepared crystals shows slightly improvement in the micromeritic properties when compared to pure omeprazole

Stability studies of omeprazole optimized crystals:

Table 5 comparative stability studies of omeprazole optimized crystals

S. No	Evaluation parameters	25^{O}C with 60%RH	40^{O}C with 75%RH
1.	Physical appearance	No change in colour	Colour is darken
2.	Melting point (^{O}C)	135	133
3.	Bulk density(mg/ml^{a})	0.188±0.03	0.184±0.02
4.	Tapped density(mg/ml^{a})	0.223±0.02	0.237±0.04
5.	Angle of repose(θ)	29±1	33±1
6.	Carr`s index (%)	15.69±1.04	17.3±0.05
7.	Hausner`s ratio	1.19±0.02	1.29±0.03
8.	Solubility(mg/ml)	1	0
9.	% drug release for 90 days	95.5%	0 %

DISCUSSION:

Stability study table 5 omeprazole optimized crystals was carried out for 90 days at specified condition. From the stability studies it was revealed that there is no significant changes in the physical parameters, micromeritic properties, solubility and % drug release at 60 min in phosphate buffer pH 6.8 when stored at temperature and humidity conditions of 25 ºC ±2C / 60%. But at condition 40 ± 2 ºC/ 75% RH ± 5% RH shows slightly decreased in percentage drug release and crystals under gone darken in colour. So, It can be concluded that at 25 ºC ±2C / 60% condition have good stability than the 40 ± 2 ºC/ 75% RH ± 5% RH [15].

CONCLUSION:

The omeprazole-PVP optimized crystals were prepared successfully using co-former PVP, magnesium chloride as basic compound by anti- solvent addition method. These crystals were analytically characterized by melting point, FTIR, DSC and XRD. Results of studies reveals that formation of new crystal phases due to physical and chemical interactions between API and co-former, but by the FTIR results it was confirmed that there is no formation of co-crystals. Prepared crystals showed better solubility, dissolution and micromeritic properties as compared to pure omeprazole. By the stability studies it can be concluded that at 25 ºC ±2C / 60% condition have good stability than the 40 ± 2 ºC/ 75% RH ± 5% RH. Many attempts were made to produce a stable, novel crystalline form of omeprazole to overcome all the major drawbacks of drug, but it failed in preparation of co-crystals by a novel particle engineering technique: co-crystallization. Further investigations were on progress to produce at most best formulation.

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RESEARCH ARTICLE

UHPLC Method development, Validation and Forced degradation study for simultaneous estimation of Vaborbactam and Meropenem in bulk drug

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ABSTRACT:

The accurate, precise, sensitive and economical spectrophotometric method was developed, validated and force degraded studies for simultaneous estimation of Meropenem and Vaborbactam in bulk drug. The UV method employed was simultaneous equation method. Chromatographic conditions involved in UHPLC C18 column with the mobile phase consisting of methanol and water. The method employs 261nm as λ1 and 273nm as λ2 for formation of equations. Vaborbactam and Meropenem obeys vandeemter equation in the concentration range 20-50µg/mL^{-1} (0.999) and 20-50µg/mL^{-1} (0.999). The calibration graph were plotted linear. The mean recovery for vaborbactam and meropenem was found to be 99.6% and 99.4% respectively. The limit of detection and quantification are determined and were found to be 0.025µg/ml and 0.045µg/ml. The developed method were validated according to ICH guidelines and values of accuracy, precision, LOD and LOQ, robustness and ruggedness. were found to be in good accordance with the prescribed values. Upon validation, the developed method effectively detected the drug as a pure compound. The drug was subjected to stress condition of peroxide, photolytic, acid, alkaline and thermal degradation, considerable degradation was found in thermal degradation studies. Thus the proposed methods were successfully applied for simultaneous determination of vaborbactam and meropenem in routine industry work.

KEYWORDS: UHPLC, Vaborbactam, Meropenem, System suitability, Precision, and Degradation studies.

INTRODUCTION:

UHPLC (Ultra High-Performance Liquid Chromatography) is a relatively new technique. UHPLC brings dramatic improvements in sensitivity, resolution and speed of analysis can be calculated[1,2]. It has instrumentation that operates at high pressure but it has no negative impact on column and uses fine particles (<2.5µm) and mobile phases at high linear velocities decreases the length of column, reduces solvent consumption and saves time. The UHPLC is based on the principle on vandeemter equation.

The binary solvent manager uses two individual serial flow pumps to deliver a parallel binary gradient. The binary solvent manager is a high-pressure pump that moves solvent through the system. Vaborbactam (VBB) and Meropenem (MPN) standard drugs are used. Vaborbactam is a β-lactamase inhibitor based on a cyclic boronic acid pharmacophore[3,4]. It is a combination of antibacterial therapy. It is potent inhibition of Klebsiella pneumoniae carbapenemase enzymes and other ambler class A and C enzymes. Meropenem is broad spectrum carbapenem antibiotic. It is active against gram positive and gram-negative bacteria. It was approved for treatment of adult patients with complicated urinary tract infections[3,4]. From the various literature findings it has been seen that vaborbactam in combination with meropenem were analytically validated by different spectroscopic and chromatographic techniques. The major objective of this research is to develop convenient UHPLC for determination of Vaborbactam and Meropenem in bulk drug, to maintain low retention time,

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better resolution and only two solvents are used in our study when compare to previous other spectroscopic methods.

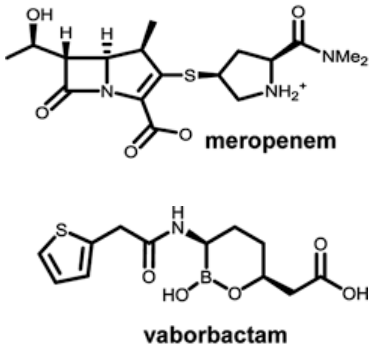


Fig. 1: Chemical structure of Vaborbactam and Meropenem

MATERIALS AND METHODS:

Chemicals and reagents:

Meropenem and Vaborbactam were procured from Madras Pharmaceuticals. Ammonium hydrogen phosphate, Acetonitrile, Water, Sodium hydroxide used were of analytical grade (Rankem or Merck chemicals) 0.45µm nylon filter (Madras pharmaceuticals, Chennai, India) was used. All other chemicals and reagents used were analytical grade unless otherwise indicated.

Selection of solvents:

On the basis of solubility study methanol was selected as the solvent for dissolving Meropenem and Vaborbactam.

Preparation of standard stock solution:

Vaborbactam Stock Solution:

An accurately weighed quantity of vaborbactam (1mg/ml) was taken in 50ml volumetric flask and dissolved in methanol (50ml) with the help of ultrasonication for about 10 min. Then the volume was made up to the mark using methanol to get vaborbactam standard stock solution (1mg/mL).

Meropenem Stock Solution:

An accurately weighed quantity of Meropenem (1mg/ml) was taken in 50ml volumetric flask and dissolved in methanol (50ml) with the help of ultrasonication for about 10min. Then the volume was made up to the mark using methanol to Meropenem.

Validation Method:

Vaborbactam and Meropenem were validated by various parameters like system suitability, precision, linearity, accuracy, limit of detection, quantification, robustness and ruggedness[5].

Forced degradation study was carried out in alkaline, peroxide, acid and thermal methods according to ICH guidelines[5].

METHOD DEVELOPMENT:

Determination of λ max of individual component:

Appropriate necessary dilutions are made from standard stock solutions to get the concentration range of 10µg/ml of vaborbactam and 10µg/ml of meropenem. Drug solutions were scanned separately between 200-400nm. Vaborbactam shows at 261nm while meropenem shows at 273nm respectively.

Overlay spectra of Vaborbactam and Meropenem:

The overlain spectrum of both drugs was recorded (Fig:2) and two wavelengths 261nm(λ max of vaborbactam) and 273nm(λ max of meropenem) were selected for further study.

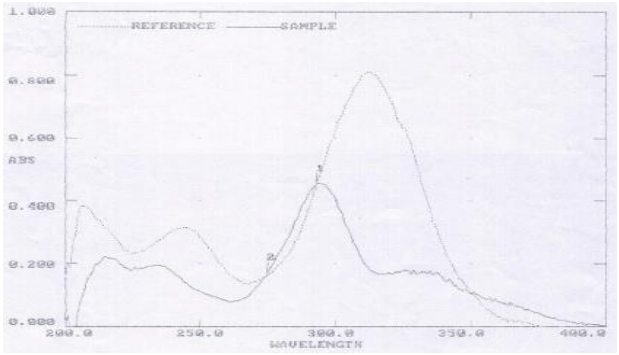


Fig. 2: Isobestic point of Vaborbactam and Meropenem

OPTIMIZATION METHOD:

Preparation of mobile phase solution:

Mix 700ml of methanol and add 300ml of water and it is filtered through 0.45µm membrane filter. The solution was sonicated up to 10min and again the solution was filtered through 0.22µm membrane filter and again sonicated for degassing and proper mixing purpose. The column temperature has to be maintained at 50°C with gradient composition pressure.

Chromatographic condition:

By acquity UHPLC (Zodiac, C18(150x4.6x5µm) with isocratic elution with flow rate 1ml/min. The column temperature was maintained at 40°C. The prepared standard solutions automatically injected through column.

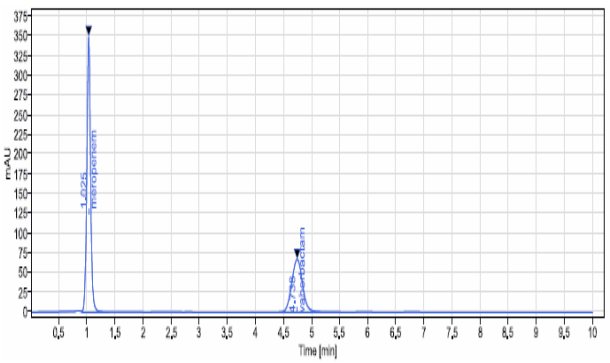


Fig. 3: Optimized method of Vaborbactam and Meropenem

Observation:
In this trail, observed that, good resolution and good peak shape. So, this trail is considered as optimized method.

RESULTS AND DISCUSSION:

System suitability:
System suitability tests are an integral part of gas and liquid chromatographic methods. They are used to verify that the resolution and reproducibility of the chromatographic system are adequate for the analysis to be done. The tests are based on the concept that the equipment, electronics, analytical operations and samples to be analyzed constitute an integral system that can be evaluated as a whole[1,2]. System suitability is the checking of a system to ensure system performance before or drying the analysis unknowns. To verify that the analytical system is working properly and can give accurate and precise results were evaluated by 100µg/ml of MPN and 100µg/ml of VBB were injected six times and the chromatograms were recorded for the same. Various parameters such as theoretical plates tailing factor, retention time and resolution factor were reported as per the ICH and USP guidelines[5,6]. The relative standard deviation and tailing factor not more than 2.0 within the acceptance criteria.

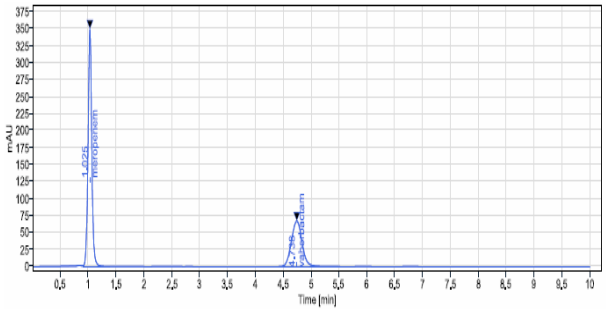


Fig. 4:System suitability chromatogram

Precision:
Precision can be defined as "the degree of agreement among individual test results when the procedure is applied repeatedly to multiple samplings of a homogenous sample". A more comprehensive definition proposed by the International Conference on Harmonization (ICH) divides precision into three types[2,3,4]:

- Repeatability
- Intermediate precision and
- Reproducibility

Repeatability is the precision of a method under the same operating conditions over a short period of time. Intermediate Precision is the agreement of complete measurements (including standards) when the same method is applied many times within the same laboratory. Reproducibil*ity* examines the Precision between laboratories and is often determined in collaborative studies or method transfer experiments[7,8,9].

Procedure:
Method precision was determined by injecting six different solutions of sample solutions MPN (100µg/mL) and VBB (100µg/mL) for six times are prepared separately. The relative standard deviation and tailing are 2.0 within the limits. The results of precision were shown in table 1.

Table 1: Precision readings of Vaborbactam and Meropenem

	VABORBACTAM		MEROPENEM	
	Area	**%Assay**	**Area**	**%Assay**
1	535328	100.0	2877968	100.4
2	561133	100.5	271334	99.8
3	565523	99.9	262343	100.4
4	544261	99.0	273760	98.6
5	571227	99.0	295910	99.8
6	622800	98.5	295173	99.5
Average	566712	99.5	279414.7	99.7
SD	27951.72	0.8	12323.61	0.6
%RSD	**0.049322**	0.8	**0.441036**	0.6

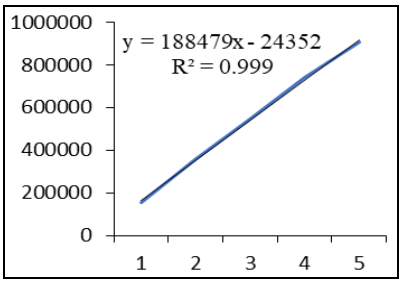
Linearity:
The Linearity of a method is a measure of how well a Calibration plot of response against concentration approximates a straight line. Linearity can be assessed by performing single measurements at several analyte concentrations. The data are then processed using a linear least-squares regression. In the resulting plot slope, intercept and Correlation coefficient provide the desired information on Linearity[10].

Procedure:
An accurately measured aliquot portion of working standard solution of Vaborbactam and Meropenem were transferred into a five separate 10ml volumetric flasks. The volume was made up to the mark using methanol to obtain concentrations (20-50µg/ml). Absorbance of these solutions was measured at 270nm. Calibration curve was plotted, absorbance vs concentration as shown in fig 5. The results of linearity data were given in table 2.

Table 2: Linearity data of Vaborbactam and Meropenem

S. No	Parameter	Vaborbactam	Meropenem
1	Correlation coefficient	0.999	0.999
2	Slope	9069	20547
3	Intercept	101920	26213

Specificity:
Specificity is the ability to assess accurately the analyte in the presence of components which may be expected to be present in the sample matrix. Typically these might include impurities, degradants, matrix etc. It is a measure of the degree of interference from such other things such as other active ingredients, excipients, impurities, and degradation products, ensuring that a peak response is due to a single component only[11,12].



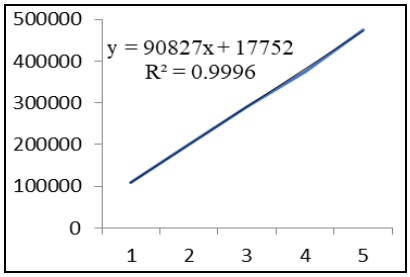


Fig. 5: Linearity graph of Vaborbactam and Meropenem

Specificity is divided into two separate categories like identification and assay and impurity tests. For identification purpose, specificity is demonstrated by the ability to discriminate between compounds of closely related structures or comparison to a known reference standard[1]. For assay/impurity tests, specificity is demonstrated by the resolution of the two closely eluting compounds. These compounds are usually the major component or the active ingredient and an impurity. Blank solution was injected and the chromatogram was recorded for the same as Placebo solution was prepared and it was injected and the chromatogram was recorded. Interference with the peak and mobile phase is does not interfere with the two drugs. The results are specified within the limits[13,14].

Accuracy:

The accuracy of a measurement is defined as the closeness of the measured value to the true value. In a method with high accuracy, a sample (whose "true value" is known) is analyzed and the measured value is identical to the true value. Typically, Accuracy is represented and determined by recovery studies, but there are three ways to determine accuracy.

Procedure:

Accuracy of the method was determined by recovery studies. To the formulation (preanalysed sample), the reference standards of the drugs were added at the level of 50%, 100%, 150%. The recovery studies were carried out three times and the percentage recovery and percentage mean recovery were calculated for each drug. The results of accuracy were given in table 3.

Limit of detection and limit of quantification:

These limits are normally applied to related substances in the drug substance or drug product. Specifications on these limits are submitted with the regulatory impurities method relating to release and stability of both drug substance and drug product[13,14].

Limit of detection is the lowest concentration of analyte in a sample that can be detected, but not necessarily Quantified, under the stated experimental conditions. With UV detectors, it is difficult to assure the detection precision of low-level compounds due to potential gradual loss of sensitivity of detector lamps with age or noise level variation by detector manufacturer.

$$LOD = 3.3\ \sigma / S$$

Limit of quantitation is the lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy under the stated experimental conditions. Several approaches for determining the quantitation limit are possible, depending on whether the procedure is a non-instrumental or instrumental[15,16].

$$LOQ = 10\ \sigma / S$$

Observation:

The lower amount of sample was quantified and detected. The vaborbactam was detected at 0.025µg/ml and meropenem was detected at 0.045µg/ml.

Robustness:

The concept of robustness of an analytical procedure has been defined by the ICH as "a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters." A good practice is to vary, important parameters in the method, systematically and measure their effect on separation. The Robustness of the method was determined. The results obtained by deliberate variation in method parameters are summarized[17]. The results of robustness were summarized in table 4.

Table 3: Accuracy data of Vaborbactam and Meropenem

Vaborbactam					Meropenem				
% Recovery	Amount present (µg/mL)	Amount Found (µg/mL)*	Percent Recovery *	%Mean Recovery	% Recovery	Amount present (µg/mL)	Amount found (µg/mL) *	Percent Recovery *	% Mean Recovery
50%	50	49.64	99.3	99.2	50%	50	49.86	99.7	100.4
100%	100	99.17	99.2		100%	100	99.98	100.0	
150%	150	148.63	99.1		150%	150	152.08	101.4	

Table 4: Robustness data for Vaborbactam and Meropenem

Chromatographic changes		Theoretical Plates		Tailing factor		Resolution
		VBB	MPN	VBB	MPN	Between VBB & MPN
Flow rate (mL/min)	0.8	11114	16033	1.27	1.27	3.08
	1.2	11284	16140	1.25	1.43	2.85
	1.5	11096	15870	1.25	1.43	2.64
Wavelength(nm)	268	10985	15759	1.25	1.45	2.96
	272	11372	16183	1.26	1.43	2.93
	273	10927	15727	1.24	1.44	3.06

Ruggedness:

Degree of reproducibility of test results obtained by the analysis of the same samples under a variety of condition such as different laboratories, different analysts, different instruments etc, normally expressed as the lack of influence on test results of operational and environmental variable of the analytical method[18,19]. Ruggedness is a measurement of reproducibility of test results under the variation in condition normally expected from laboratory to laboratory and from analyst to analyst[20]. The ruggedness of the method was studied by the determining the analyst to analyst variation by performing the Assay by two different analysts.

Forced degradation studies:

Forced degradation or stress studies are undertaken to deliberately degrade the sample. These studies are used to evaluate an analytical method ability to measure an active ingredient and its degradation products, without interference by generating potential degradation product. During validation of the method, drug substance are exposed to acid, base, heat, light and oxidizing agent to produce approximately 10% to 30% degradation of active substance[21,22]. The studies can also provide information about the degradation pathways and degradation products that could from during storage. These studies may also help in the formulation development, manufacturing and packaging to improve a drug product. Reasons for carrying out forced degradation studies include development and validation of stability-indicating methodology, determination of degradation pathways of drug substances and products, discernment of degradation products in formulations that are related to drug substances versus those that are related to non-drug substances[23,24]. The results of forced degradation studies were summarized in table 5.

Peroxide degradation:

Sample solution of Vaborbactam and Meropenem (10µg/ml) and 1 ml of 20% hydrogen peroxide (H_2O_2) was mixed. For UHPLC study, 10µl were injected into the system and the chromatogram was recorded to assess the stability of sample.

Photolytic degradation:

The photochemical stability of the drug was studied by exposing the 100µg/ml solution to UV light by keeping the beaker in UV chamber for 7 days. For UHPLC study, the resultant solution 10µl was injected into the system and the chromatogram were recorded to assess the stability of sample.

Acid degradation:

Take 1 tablet, powdered and place in a 50ml volumetric flask and dissolve in mobile phase up to 75% then sonicate it for 10 minutes then add 1 ml of 0.1N HCl then kept in oven at 60^0c for 1 hour then cool and add 1 ml of 0.1N NaOH it then make up the volume up to 50ml with mobile phase, then place the sample in the vial and measure the chromatogram.

Alkaline degradation:

Take 1 tablet, powdered and place in a 50ml volumetric flask and dissolve in mobile phase up to 75% then sonicate it for 10 minutes then add 1 ml of 0.1N NaOH then kept in oven at 600°C for 1 hour then cool it and add 1ml of 0.1N HCl then make up the volume up to 50ml with mobile phase, then place the sample in the vial and measure the chromatogram.

Thermal degradation:

Sample solution of Vaborbactam and Meropenem (10µg/ml) was placed in oven at 105°C for 6hr to study dry heat degradation. for UHPLC study, the resultant solution was injected into the system and the chromatograms were recorded to assess the stability of the sample.

Table 5: Degradation data for Vaborbactam and Meropenem

	Vaborbactam				Meropenem			
Method	**std area**	**Degradation area**	**% Obtained**	**% Degraded**	**std area**	**Degradation area**	**% Obtained**	**% Degraded**
Peroxide	559559	1041210	101.488	0.412	282306	2046738	101.493	0.407
Photolytic	178620	1041237	101.491	0.409	863520	2046725	101.492	0.408
Acidic	561133	1041221	101.489	0.411	271334	2046742	101.493	0.407
Alkaline	178620	1041238	101.491	0.409	863520	2046731	101.492	0.408
Thermal	267582	1041241	101.491	0.409	267582	2046729	101.492	0.408

Acceptance criteria:
The % Degraded for Vaborbactam and Meropenam from these stability methods should be not more than 1.0 %.

Observation:
Degradation studies were carried out with acid, base, peroxide, thermal. It was observed that the response of peak area and retention time of Vaborbactam and Meropenem were nearly same as obtained in control samples of Vaborbactam and Meropenem. Degradation was found in acid, base, peroxide, thermal and UV conditions because extra peaks were observed.

CONCLUSION:

A new precise, accurate, rapid method has been developed for the estimation of Meropenam and Vaborbactam pharmaceutical drug by UHPLC. From the above experimental results and parameters it was concluded that, this newly developed method for the estimation Meropenem and Vaborbactam was found to be simple, precise, accurate and high resolution and shorter retention time makes this method more acceptable. The present recovery was found to be 98.0-101.5% was linear and precise over the same range. Both system and method precision was found to be accurate and within range. Detection and quantification limit was found to be 0.025 Vaborbactam and 0.045 Meropenem. The analytical method was found linearity over the range of 20-60ppm of the target concentration for both the drugs. The analytical method passed both robustness and ruggedness tests. On both cases, relative standard deviation was well satisfactory.

CONFICT OF INTEREST:

No conflict of interest.

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RESEARCH ARTICLE

In vitro Antilithiatic study of Ethanolic extract of roots of *Ipomoea digitata* Linn

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ABSTRACT:
The main objective of this study was to evaluate in vitro antilithiatic activity of ethanolic extract of roots of *Ipomoea digitata* Linn. Inhibitory effect of extract on in vitro crystallization was analysed by nucleation assay. Cystone tablet a marketed drug used as a standard drug which is dispersed into 100ml buffer solution for compare of various concentration of ethanolic root extract of *Ipomoea digitata* Linn. The inhibitory activity of root extract on the nucleation of calcium oxalate crystals and the rate of aggregation in calcium oxalate crystals was determined by spectrophotometric assay. Investigation revealed that ethanolic extract of roots of *Ipomoea digitata* Linn is positive effect on inhibition for formatting precipitation of calcium and potassium in vitro.

KEYWORDS: Antilithiatic, *Ipomoea digitata* Linn, urolithiasis, Calcium oxalate, aggregation.

INTRODUCTION:
Stone formation is the oldest and serious painful urologic disease with significant prevalence in the population due to change in lifestyle and dietary factors. The risk of developing urolithiasis have been reported in adults appears as in western countries which are 5 - 9% in Europe, 12% in Canada, and 13-15% in the USA and also in some Asian countries such as Saudi Arabia 20.1% with lifetime recurrence rates of up to 50%.[1-4] According to an estimate every year 600000 Americans suffer from urinary stone. In India about 12% of Indian suffers out of which 50% from urinary stone.[5] Urolithiasis is a complex process which is created from imbalance between inhibitors and promoters in the kidney. Stone formation or lithiasis is characterized by calculi formation. It has two types such as nephrolithiasis and urolithiasis. Calculi formation in urinary bladder, ureter or any part of urinary tract rather than kidney is known as urolithiasis while nephrolithiasis is characterized calculi formation in kidney. It is the solid non –metallic minerals found in the urinary track. It is a stone which is formed by an aggregation of solute materials from urine such as calcium, oxalate, phosphate and uric acid. The formation of lithiasis involves several physiochemical events which is beginning with super saturation, crystal nucleation, crystal growth, aggregation and end with retention within the urinary tract. The most common calcium oxalate stones represent up to 80% of the analysed stones.

Ipomoea digitata (Convolvulaceae) is perennial medicinal herb which is known as Bedari kand and Teliakand in Chhatisgarh. Its roots used as tonic and aphrodisiac. It is used in treatment of uterine pain, infertility, lactation, gastric ulcer, blood dysentery, high blood pressure and heart disease.[6] Pharmacological studies showed that this plant have properties of antioxidant, antibacterial, antidiabetic, antihypertensive and antihyperlipidaemic. The present study was to evaluate in vitro antilithiatic activity of ethanolic extract of roots of *Ipomoea digitata* Linn by nucleation assay.

MATERIAL AND METHODS:
Identification, collection and extraction of plant material:
The roots of *Ipomoea digitata* Linn were collected from the Chhatisgarh, India and were identified by Botanist, Government of India, Ministry of environment and forests, Botanical survey of India, Central national herbarium Howrah-7111103. The rhizomes of *Ipomoea digitata* Linn were powdered with a mechanical grinder

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to obtain a coarse powder. Powdered material was passed through 40 mesh sieve to get a coarse powder of desired particle size. The powdered material was subjected to successive extraction with ethanol in a soxhlet apparatus. The extract was concentrated under vacuum pressure. It is dried and stored in a refrigerator throughout the study.

Screening of phytochemicals:

Extracts were subjected to qualitative analysis for identification of different phytochemicals present in the plant with standard method.[7-8] Phytochemical test revealed that the presence of fixed oil, carbohydrate, tannins, phenolic compound, alkaloids saponins, sterols and flavonoids in extract of plant tuber.

Nucleation assay method:

In this test method rate of nucleation of calcium oxalate was estimated by comparing the induction time in the presence of ethanolic extract of *Ipomoea digitata* Linn. Crystallization of calcium oxalate was started by adding of solution of Calcium chloride and Sodium oxalate.

$CaCl_2 + Na_2C_2O_4 \rightarrow CaC_2O_4 + 2NaCl$

Method:

Solution of Calcium chloride (5mmol/L) and sodium oxalate (7.5mmol/L) were prepared in a buffer containing Tris –HCl 0.005mol/L and 0.15mol/L sodium chloride at PH 6.5. Different concentrations (10, 20, 40, 60, 80 and 100ug/ml) of ethanolic extract of *Ipomoea digitata* Linn was prepared by using buffer solution. Group was divided into VIII groups for experiment. Group I was negative control group in which 8ml of calcium chloride solution was mixed with 1ml buffer solution. Group II was positive control group in which 8ml of calcium chloride solution was mixed with Cystone tablet solution (4mg/ml). Cystone tablet was used as standard drug. Group III, IV, V, VI, VII, and VIII were groups of ethanolic extract of *Ipomoea digitata* Linn of different concentrations, 10, 20, 40, 60, 80 and 100ug/ml by adding of the 8 ml of calcium chloride respectively. Crystallization was started by adding of 940ug/mg of sodium oxalate solution. The temperature was maintained at 37°C. Absorbance was monitored at 620 nm (0-30 min every 3 second) by optical density method. The rate of nucleation was calculated in the presence or absence of inhibitors by comparing the induction time of Calcium oxalate crystallization. The percentage inhibition of nucleation was calculated using the formula [9-11].

Percentage inhibition = [1- optical density (experimental)/optical density (control)] x 100

RESULTS AND DISCUSSION:

In vitro nucleation assay was performed and result was shown in table 1, fig 1and 2. Increased oxalate acts as a predisposing factor for increase in production of reactive oxygen species (ROS) and decrease in endogenous antioxidant enzyme levels which is facilitating crystal nucleation.[12] Nucleation is the first step in the formation of CaOx crystals which grow small to bigger with aggregation in the urinary tract. Initial mineral phase stone formations are supersaturation of urine within the urinary tract by calcium oxalate and calcium phosphate crystals. They may be aggregate and form small, hard mass which is referred as crystal growth. Treatment of urolithiasis is decreased urinary supersaturation by altering the urine composition. There are no drugs that inhibit directly CaOx crystallization in urine. Drugs that would be able to inhibit the crystallization process would be new category of drugs.[13] Many herbal plants reduce urinary oxalate concentration and urinary calcium oxalate stones formation.[14 -18]

Ethanolic extract of *Ipomoea digitata* Linn was inhibited nucleation of calcium oxalate crystal. Inhibition of nucleation of calcium oxalate crystal means disintegrating into small particle. Nucleation of calcium oxalate crystals in solution was inhibited by adding different concentrations of ethanolic extract of *Ipomoea digitata* Linn. The absorbance values were found to decrease with the increase in concentration of ethanolic extract of this herbal drug indicating the decrease in nucleation of CaOx crystals. The optical density was highest absorbance (0.585) for control group and was lowest (0.134) at the maximum concentration of herb extract (100ug/ml). Anti crystallization was found according to the increasing concentration of ethanolic extract of *Ipomoea digitata* Linn. The results of anti urolithiasis activity indicated that percentage inhibition was more in ethanol extracts when compared to standard drug cystone tablets.

Extract of *Ipomoea digitata* exhibited significant in vitro anti lithiatic agent with increasing concentration of herb extract. *Ipomoea digitata* extract may be decrease the formation of ROS and increase endogenous antioxidant enzyme levels which is inhibited crystal nucleation. In conclusion *Ipomoea digitata* have inhibitory effect on calcium oxalate crystallization thus may be beneficial in the treatment of lithiasis.

Table 1 Antilithiatic activity of ethanolic extract of *Ipomoea digitata* Linn by optical density method

S. No	Experimental groups	Absorbance at 620 nm	Mean absorbance	% inhibition
1	Group-I (control group) Calcium chloride solution (8ml)+ buffer solution (1ml)+ Sodium oxalate solution (950ul)	0.585	0.585	
2	Group – II Calcium chloride solution (8ml)+ cystone tablet solution (1ml (4mg/ml))+ Sodium oxalate solution (950ul)	0.222	0.222	63
3	Group –III Calcium chloride solution (8ml)+ ethanolic extract of *Ipomoea digitata* of (1ml (10ug/ml))+ Sodium oxalate solution (950ul)	0.198 0.197 0.195 0.198	0.197	67
4	Group –IV Calcium chloride solution (8ml)+ ethanolic extract of *Ipomoea digitata* of (1ml (20ug/ml))+ Sodium oxalate solution (950ul)	0.190 0.191 0.189 0.190	0.191	68
5	Group-V Calcium chloride solution (8ml)+ ethanolic extract of *Ipomoea digitata* of (1ml (40ug/ml))+ Sodium oxalate solution (950ul)	0.185 0.185 0.186 0.184	0.185	69
6	Group-VI Calcium chloride solution (8ml)+ ethanolic extract of *Ipomoea digitata* of (1ml (60ug/ml))+ Sodium oxalate solution (950ul	0.180 0.182 0.178 0.181	0.180	70
7	Group –VII Calcium chloride solution (8ml)+ ethanolic extract of *Ipomoea digitata* of (1ml (80ug/ml))+ Sodium oxalate solution (950ul)	0.144 0.142 0.145 0.146	0.144	76
8	Group- VIII Calcium chloride solution (8ml)+ ethanolic extract of *Ipomoea digitata* of (1ml (100ug/ml))+ Sodium oxalate solution (950ul)	0.134 0.134 0.134 0.132	0.133	78

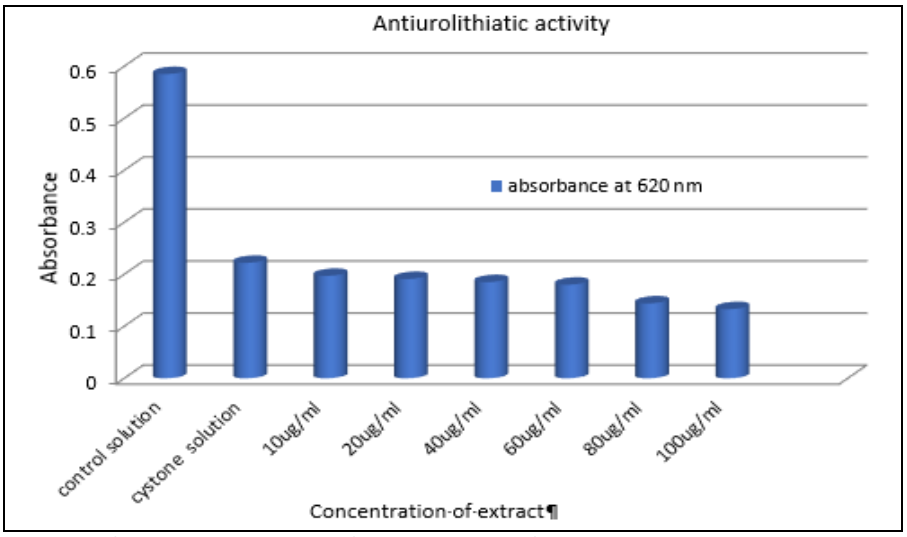

Fig1: Graphical presentation of antiurolithiatic activity of ethanolic extract of *Ipomoea digitata* Linn by optical density method

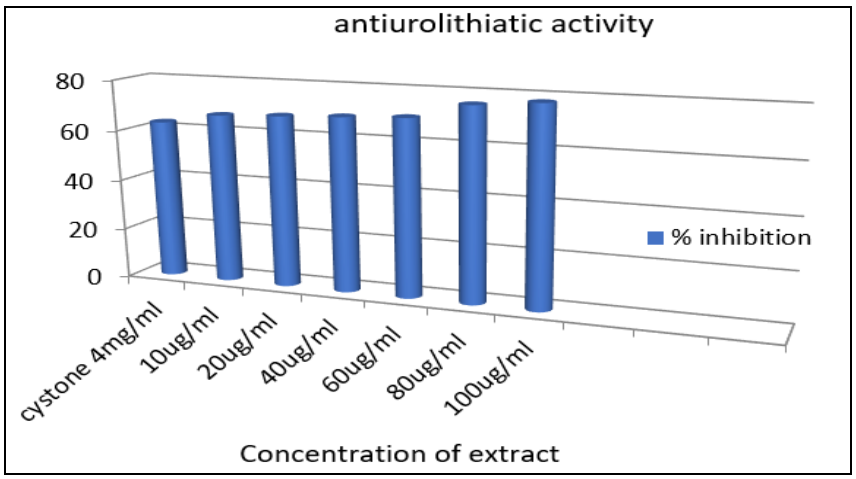

Fig2: Percentage inhibition of nucleation of ethanolic extracts of *Ipomoea digitata* Linn

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RESEARCH ARTICLE

Mesenchymal stem cells prove a significant role in Chronic non-healing ulcer progressive healing

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ABSTRACT:

Severity of non-healing ulcers depends upon both local and systemic factors. Available treatment options like wound dressing, compression therapy, and others have improved clinical outcomes, but chronic wounds continue to exist as the major clinical issues in the patients. As per previous reports, introduction of mesenchymal stem cells has a better treatment option for regenerative therapy. The purpose of this research work was to examine the histopathological and cellular level changes in chronic wound to access whether the application of autologous mesenchymal stem cells improved the wound site environment. Tissue samples from debrided tissue of the chronic wounds were taken before and after the mesenchymal stem cell treatment. Further histopathology and confocal microscopy analysis were also done. Characterization of MSCs was carried out by flow cytometer. We observed that autologous mesenchymal stem cell treatment improved the wound site environment Histopathological and cellular level changes supported the evidence that mesenchymal stem cell treatment reduces the inflammatory damage and facilitating wound area with angiogenesis. Autologous MSCs are positive for CD90, CD105 markers and negative for CD34, CD45 markers. We have found positive results for the treatment of chronic non-healing wounds using mesenchymal stem cells. It is suggested that autologous mesenchymal stem cell treatment has a positive impact on chronic wound healing. Further analysis should be performed to signify some more interactions among cells to facilitate chronic wound healing that would be a silver lining in regenerative medicine.

KEYWORDS: Non-healing ulcer, chronic, inflammation; mesenchymal stem cells; regenerative therapy.

INTRODUCTION:

Cutaneous wound healing is a complex process, requires a proper coordination of specific timely manner phases that involve hemostasis, inflammatory, proliferative, and maturation phase[1]. Chronic wounds develop when there is a disturbance in this controlled healing pattern coordination, result into open ulceration of various degree of severity. Pathological and physical conditions such as aging, infections, diabetes, vascular diseases, sickle cell disease, contribute to this situation.

Nearly 2-6 million people are affected from chronic wounds in United States alone, whereas world prevalence ranges from 1.9 to 13.1%[2]. In China,1.5% to 20.3% of surgically hospitalized patients suffered from chronic wounds. According to one study, 63% of total chronic wounds patients have lower extremities ulcer[3]. In India 4.5/1000 population is affected with chronic wounds. Acute wound healing incidence is 10.5/1000 and it is just more than double[4]. Of all leg ulcer cases, 70% are diagnosed with venous insufficiency, Foot ulcer is also a common complication, affecting 4%–10% persons with diabetes mellitus. It is estimated that diabetes affects 8.3% of the global population or 382 million people[5].

As traditional treatment approaches for chronic wounds are extending to their limits and frequently failing, improved therapies including skin substitutes, biological

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dressing and growth-factor based therapies are being implemented in chronic wound healing[6]. In recent years, stem cell administration has been implicating as a potential cellular therapy for regenerative medicine because of their self-renewing and multi-lineage differentiation capacity under favourable conditions[7]. The association of Mesenchymal Stem Cells (MSCs) in the wound-healing process is crucial, specifically for difficult non-healing wounds producing from trauma, diabetes, vascular insufficiency, and numerous other conditions[8]. MSCs have a role in the normal wound healing process, and their presence supports healthy physiologic functioning for healing. In addition to multi-lineage differentiation capacity, MSCs regulate immune response and inflammation and may enhance epithelialization, granulation tissue formation and neovascularization resulting in accelerated wound closure[8,9].

In the present study, we have given a treatment of autologous peripheral blood derived mesenchymal stem cells to the chronic non-healing ulcer patients and followed up the patients. Presence of mesenchymal stem cells was characterized by flow cytometry. On each follow-up visit wound photograph was taken. Histopathological analysis and membrane potential of the cells were analysed. After the mesenchymal stem cell treatment, improvements in wound healing were studied through various parameters. Moreover, mesenchymal stem cells appear to maintain a distinct phenotype in culture, suggesting that they can be used as a tool for potential therapeutic agent in wound healing.

MATERIAL AND METHODS:

Ethical approval and informed consent:

This study is approved from the Institutional Ethical Committee (NITRR/IEC/16/2015) of NIT Raipur, India. Informed written consent was taken from the patients. A total of 5 patients were enrolled for the study.

Selection of Patients:

Chronic Non-Healing Ulcer patients were selected that fit to the inclusion and exclusion criteria of the study.

i) Inclusion Criteria- a) those Chronic Non-Healing Ulcer patients, who have failed to respond all modes of available treatment, were selected for the study, b) patients with Chronic Non-Healing Ulcer of more than six months duration and post radiation burn injuries of less than six months were also included.
ii) Exclusion Criteria- ulcers of less than three months were excluded from the study.

Selection of study sample from the patients:

Wound biopsy samples from the Chronic Non-Healing Ulcer edges, before and after the treatment, were obtained from the selected chronic non-healing ulcer patients.

Histopathological Analysis:

A small portion of biopsy samples were fixed in 10% formalin and embedded in paraffin wax. Sections of 5µm thickness were cut and hematoxylin-eosin stained slides were prepared from formalin fixed, paraffin-embedded specimens[10]. Sections were then analysed under light microscopy.

Confocal Microscopic analysis:

Tissue slice of approximately 2mm thickness was prepared and protected from light. Staining with DAPI (invitogen) and MitoTracker® Red CMXRos (invitrogen) was done according to the standard protocol. Stained slides were incubated for 20 min at 4°C protected from light. Sections were then analysed by standard confocal microscope[11].

Mesenchymal Stem Cell isolation and administration:

10 ml of patient's venous blood was drawn in heparin containing sterile tube under aseptic condition. It was immediately centrifuged at 2500rpm at room temperature for 12 minutes with no break. White buffy coat was removed from the junction of packed red cells and plasma. Half of the white buffy coat along with 2 ml of plasma was injected in the wound margins and wound area[1213]. White buffy coat with some plasma was mixed with 30 grams antibiotic ointment (Soframycin). This ointment was dispensed in a sterile container. Patients were advised to apply the ointment locally three times per day after cleaning the wound with normal saline.Patients were followed up every 15 days till healing is complete or at least for 6months.At every visit patient was provided with freshly prepared ointment. Clinical photographs were taken during each visit for each of the patients to compare the healing area of ulcers (figure1) (table 1).

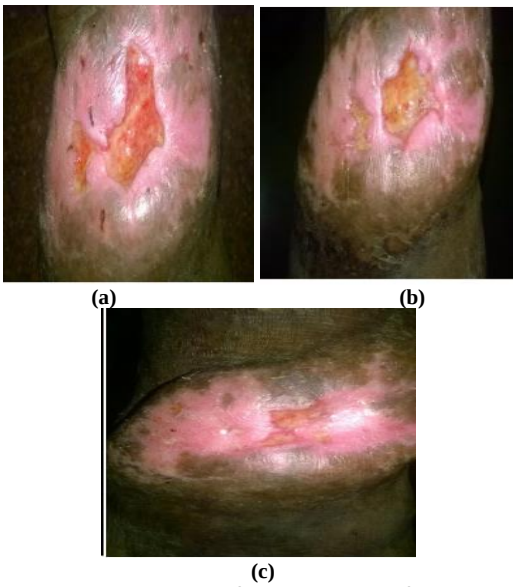
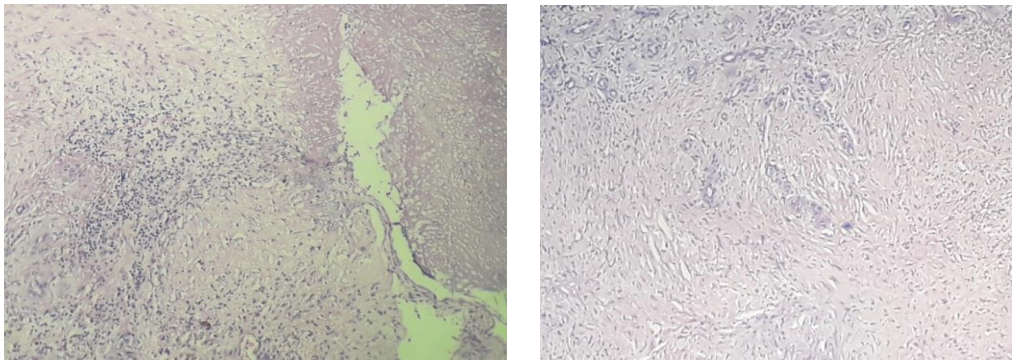


Figure 1: Clinical photographs of wound area (a) before treatment, (b) first follow-up (c) second follow-up.

Table 1: Data showing wound area, wound healing percentage and number of follow up of the patients.

S. No.	Age	Sex	Area (Cm^2)	Duration (Month)	Complete Healing	Incomplete Healing	% of Healing	No. of Follow Up
1	33	M	70	6	Y		100	4
2	50	M	2	60		Y	85	4
3	50	M	0.5	60		Y	90	4
4	53	M	12	12		Y	92	7
5	40	M	9	6	Y		100	7



(a) **(b)**

Figure 2: Histopathological slides showing improvement in wound area (a) before treatment, (b) after treatment.

Characterization of MSCs by Flow Cytometer:

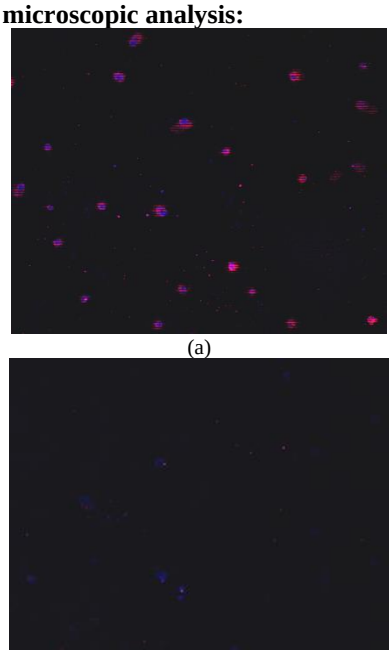
Mononuclear cells were isolated from peripheral blood according to the protocol. Presence of mesenchymal stem cells was characterized by specific MSCs CD markers. Cells were incubated with the markers CD90 (FITC), CD105 (PerCP-Cy5.5), CD34 (APC), and CD45 (PE) (BD Biosciences) in the dark. Result were analysed in flow cytometer.

RESULTS:

Histopathological analysis:

After the MSCT, there was a visible improvement in granular cells proliferation and angiogenesis. The wound was larger in size and breadth (Figure 1a), whereas in the treatment in was reducing significantly (Figure 1b and 1c). Cells from distinct locations within the wound have distinct migration capacities reflecting their specific phenotypes and effectivity of MSCT. Histopathological findings of tissue biopsies, before the MSCT, showed that chronic wound site mainly composed of inflammatory cells, disintegrated lymphocytes and fewer blood vessels. We found that chronic non-healing ulcers exhibited specific morphological changes in histopathological analysis. Tissue sections showed significant healing after treatment. Histopathological parameters such as inflammatory cells, angiogenesis, and new extracellular matrix deposition were evaluated. Significant increase in the levels of these parameters was shown after the treatment (Figure 2).

Confocal microscopic analysis:



(a)

(b)

Figure 3: Confocal microscopic slides stained with DAPI (blue) and Mitotracker (red) showing an increase in mitochondrial membrane potential of the cells (a) before treatment, (b) after treatment.

Due to decreased in mitochondrial membrane potential, chronic wound cells mainly stain with DAPI and weakly stain with mitotracker dye, whereas healthy cells properly stain with both the dyes. After MSCT, cells showed increased mitochondrial membrane potential, as more cells were seen properly stained with both the dyes (figure 3).

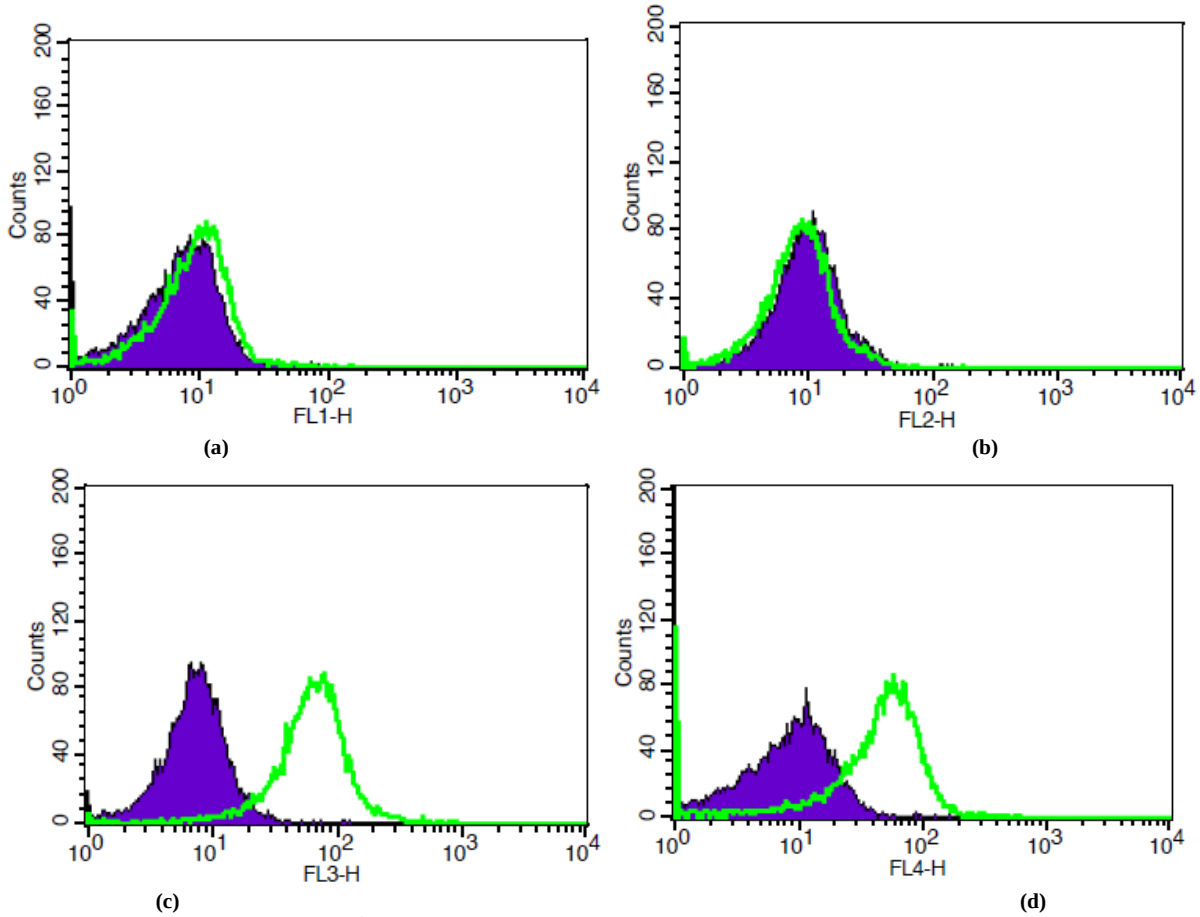


Figure 4: Positive and negative markers of MSCs (a) CD34 negative, (b) CD45 negative, (c) CD90 positive, (d) CD105 positive.

MSCs characterization by Flow cytometry analysis:
It was used to determine the integrity of MSC. MSC exhibited surface marker expression profiles. Isolated cells from autologous peripheral blood were positive for CD90 and CD105 markers and negative for CD34 and CD45 markers (hematopoetic markers) (figure4).

DISCUSSION:

It is known that chronic non-healing wounds are difficult to heal, and the available treatments are not very satisfactory. To address this issue, we have studied the mesenchymal stem cells treatment to these patients and observed the results. We have found positive results in the remodelling and proliferative phases of wound healing, also observed improved chronic inflammatory environment. These results showed the positive effect of mesenchymal stem cells to the chronic non-healing ulcer patients. Mesenchymal stem cells show a great effect on the healing of these ulcers. Histopathological analysis shows visible effect of granulation tissue formation, angiogenesis, collagen deposition, and epithelialization. Mesenchymal stem cells are prominent cells for the enhancement of wound band texture. Our results stipulate that mesenchymal stem cell therapy is enhancing the wound healing of different type of non-healing ulcer by reducing wound size, assisting the formation of granulation tissues, reducing pain thus increasing quality of life of patients. There are various studies to support regenerative and cellular level treatment[14].

Reactive oxygen species plays highly crucial role in different phases of wound healing. Proper concentration of reactive oxygen species is required for cell survival, and in combating microorganism invasion during wounding. Imbalance in this concentration causes disturbance in mitochondrial membrane potential, one of the causes that leads to the development of chronic non healing ulcer. Many studies support that mitochondrial targeting drugs regulate the mitochondrial membrane potential that further enhances efficient wound healing process in chronic wounds[15–19]. Our results provide insight into the potential use of autologous MSCs in the ex vivo expansion of wound healing. With these results it could be interpreted that Mesenchymal Stem Cells have a positive role in the improvement of healing process of chronic non-healing ulcer patients though further studies are still needed to explain the molecular mechanism involve in developing and healing of chronic wounds.

ETHICAL APPROVAL:

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional ethical committee under Approval No. NITRR/IEC/16/2015 and a written informed consent was obtained from patients.

CONFLICTS OF INTEREST:

Authors declare no conflicts of interest.

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RESEARCH ARTICLE

Development and Validation a New Stability Indicating LC method for the Quantification of Flucytosine

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ABSTRACT:
Flucytosine is a fluorinated analogue of cytosine. Flucytosine is an orally effective synthetic anti-mycotic agent used for the treatment of systemic fungal infections produced by yeasts. Flucytosine acts mainly against *Candida* and *Cryptococcus* and also against some of the species *Cladosporium* and *Phialophora*. A new reverse phase liquid chromatographic method has been proposed for the quantification of Flucytosine in pharmaceutical formulations and the method was validated as per ICH guidelines. Mobile phase consisting of a mixture of water: methanol: acetic acid (50:50: 0.1 v/v) with a flow rate 1 mL/min and UV detection at 264 nm was used for the assay of Flucytosine. The drug was exposed to different stress conditions such as acidic, alkaline, oxidation and thermal degradation. Linearity was observed over the concentration range 0.5–120 µg/mL with linear regression equation y = 74185x- 5852.7 (r^2 = 0.9999). The LOD was found to be 0.1393 µg/mL and the LOQ was found to be 0.4187 µg/mL. The present method can be applied for the pharmaceutical formulations, kinetics study and bioanalytical studies.

KEYWORDS: Flucytosine, RP-HPLC, Validation, Stability indicating, ICH guidelines.

INTRODUCTION:
Flucytosine is the only available nucleoside analog, acts as an antifungal by disrupting pyrimidine metabolism in the fungal cell nucleus. Flucytosine (Figure 1) is fungicidal in vitro against Candida species and C. *neoformans* but not against other commonly encountered fungi. Flucytosine is structurally related to the cytostatic fluorouracil and to floxuridine[1]. Flucytosine is chemically 6-amino-5-fluoro-1H-pyrimidin-2-one with molecular formula $C_4H_4FN_3O$ (CAS No. 2022-85-7) and molecular weight 129.09 g/mol. Flucytosine is a small, very water-soluble molecule and therefore it is rapidly and nearly completely absorbed from the intestine after oral administration. Flucytosine can cause bone marrow suppression and GI toxicity. Flucytosine does not have any significant drug interactions. Flucytosine has therapeutic effects related to its effects on nucleic acid synthesis. Its primary therapeutic use is in the treatment of serious cryptococcal and candidal infection. Flucytosine is well absorbed from the gastrointestinal tract and the peak plasma concentration is attained within 1 to 2 hours after oral administration [2-4].

Literature survey reveals that Flucytosine was estimated by various analytical techniques such as HPLC methods in biological fluids[5-7], fluorimetric method[8] and microbiological assay[9]. Milind Ubale[10] et al and Mathrusri[11] et al estimated the content of Flucytosine in bulk drug whereas Mohabbat Ullah[12] et al studied the process related impurities in injectable formulations of Flucytosine using stability indicating HPLC. The authors proposed a new stability indicating RP-HPLC method for the assay of Flucytosine in the present study and the method was validated as per ICH guidelines[13-14].

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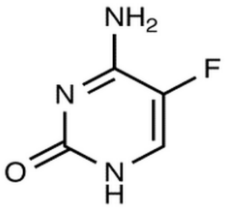


Figure 1: Chemical structure of Flucytosine

MATERIALS AND METHODS:

Chromatographic conditions

Shimadzu Model HPLC system (Shimadzu Co., Kyoto, Japan) equipped with PDA detector and C8 Luna column (250 mm × 4.60 mm i. d. 5µm particle size) was used for the chromatographic study. The system was maintained at 23ºC. A mixture of water: methanol: acetic acid (50:50: 0.1 v/v) was used as mobile phase with a flow rate 1 mL/min (UV detection at 264 nm) for the present study. The mobile phase was sonicated and filtered through 0.22µm membrane filter prior to use.

Materials and reagents

Flucytosine is available with brand name Fc fung (Glenmark Pharmaceuticals, India), BD-CYTOSIN (BDR Pharmaceuticals Intl Ltd) as tablets and also as ANCOBAN® (Lupin Pharmaceuticals, India) as capsules with label claim 500 mg. Flucytosine was obtained as gift sample from Glenmark Pharmaceuticals, India.

Solution preparation

25 mg of Flucytosine was accurately weighed and taken in to a clean 25 mL volumetric flask and made up to volume with HPLC grade methanol (MERCK) (1000 µg/mL) and further dilutions were made with mobile phase.

Method validation

The HPLC procedure was optimized with a view to develop an assay method for Flucytosine.

Linearity

Dilute solutions (0.5-120 µg/mL) of Flucytosine were prepared from the stock solution with mobile phase and 20 µL of each were injected in to the HPLC system. The mean peak area (n=3) of Flucytosine were calculated from the chromatograms obtained and a calibration curve was drawn by taking the concentration of drug solutions on the x-axis and the corresponding mean peak area values on the y-axis.

Precision, Accuracy and Robustness

Intraday and inter-day precision were studied using three different concentrations of Flucytosine on the same day and on three consecutive days respectively and the % RSD was calculated. The accuracy of the assay method was evaluated in triplicate at three concentration levels (50, 100 and 150 %) and the percentage recoveries were calculated. Standard addition and recovery experiments were conducted to determine the accuracy of the method for the quantification of the drug product and the percentage recovery was calculated. The robustness of the method was assessed by exposing the drug solution to different analytical conditions purposely changing from the original optimized conditions. The effects so obtained were summarized to calculate the % RSD and has to be less than 2.0% specifying that the proposed method was robust.

Forced degradation studies

Forced degradation studies were performed in the applied stress conditions. Flucytosine was exposed to different stress conditions such as acidic, basic, oxidation, thermal treatment.

Acidic degradation was performed by treating the drug solution with 1mL of 0.1N HCl, heated at 75 ºC for about one hour on a water bath. The stressed sample is then cooled neutralized with 1mL 0.1N sodium hydroxide solution and the solution was made up to volume to the required concentration with the mobile phase. 20 µl of the solution was injected in to the HPLC system.

Alkaline degradation was performed by treating the drug solution with 1mL 0.1 N NaOH heated at 75 ºC for about one hour on a water bath. The solution is then cooled and neutralized with 1mL 0.1N hydrochloric acid and diluted with mobile phase. 20 µl of the solution was injected in to the HPLC system.

Oxidation degradation was performed by treating the drug solution with 1ml of 30% v/v H_2O_2 heated at 75 ºC for about one hour on a water bath. The solution is then cooled and diluted with mobile phase. 20 µl of the solution was injected in to the HPLC system.

Thermal degradation was performed by heating the drug solution at 75 ºC for about one hour on a water bath. The solution is then cooled and diluted with mobile phase. 20 µl of the solution was injected in to the HPLC system.

Assay of Flucytosine tablets

Twenty tablets containing each 500 mg of two different brands were procured and the contents were powdered separately and powder equivalent to 25 mg Flucytosine was extracted using methanol and then with the mobile phase in two different 25 ml volumetric flasks. The solution was sonicated for half an hour and filtered through 0.45 mm membrane filter and 20 µL of this solution was injected in to the HPLC system. The peak area observed was noted at its retention time from the

resultant chromatogram and the mean peak area was calculated (n=3).

RESULTS AND DISCUSSION:

Method development and optimization

A new reverse phase stability indicating liquid chromatographic method was developed and validated for the estimation of Flucytosine in tablets. Mobile phase consisting of a mixture of water: methanol: acetic acid (50:50: 0.1 v/v) with a flow rate 1 mL/min and UV detection at 264 nm was used for the assay of Flucytosine using C8 Luna column (250 mm × 4.60 mm i.d. 5µm particle size). A symmetrical and sharp peak was observed at 2.98 ± 0.02 min (Run time 10 min). The observations were shown in Table 1. The chromatograms obtained during the optimization process were shown in Figure 2.

Table 1: Method Optimization

Mobile phase (v/v)	Flow rate (ml/min)	Rt (min)	Theoretical plates	Tailing factor	Observation	Figure
Methanol: Water (60:40)	0.8	3.829	2863	2.076	Tailing factor ≥1.5	2A
Methanol: Water (65:35)	0.8	3.711	3740	1.756	Tailing factor ≥1.5	2B
Methanol: Water (65:35)	1	3.111	3672	1.936	Tailing factor ≥1.5	2C
Methanol: Water (50:50)	1.2	3.167	3834	2.043	Tailing factor ≥1.5	2D
Methanol: Water: Acetic acid (50:50: 0.1)	1	2.977	6289	1.478	Method optimized	2E

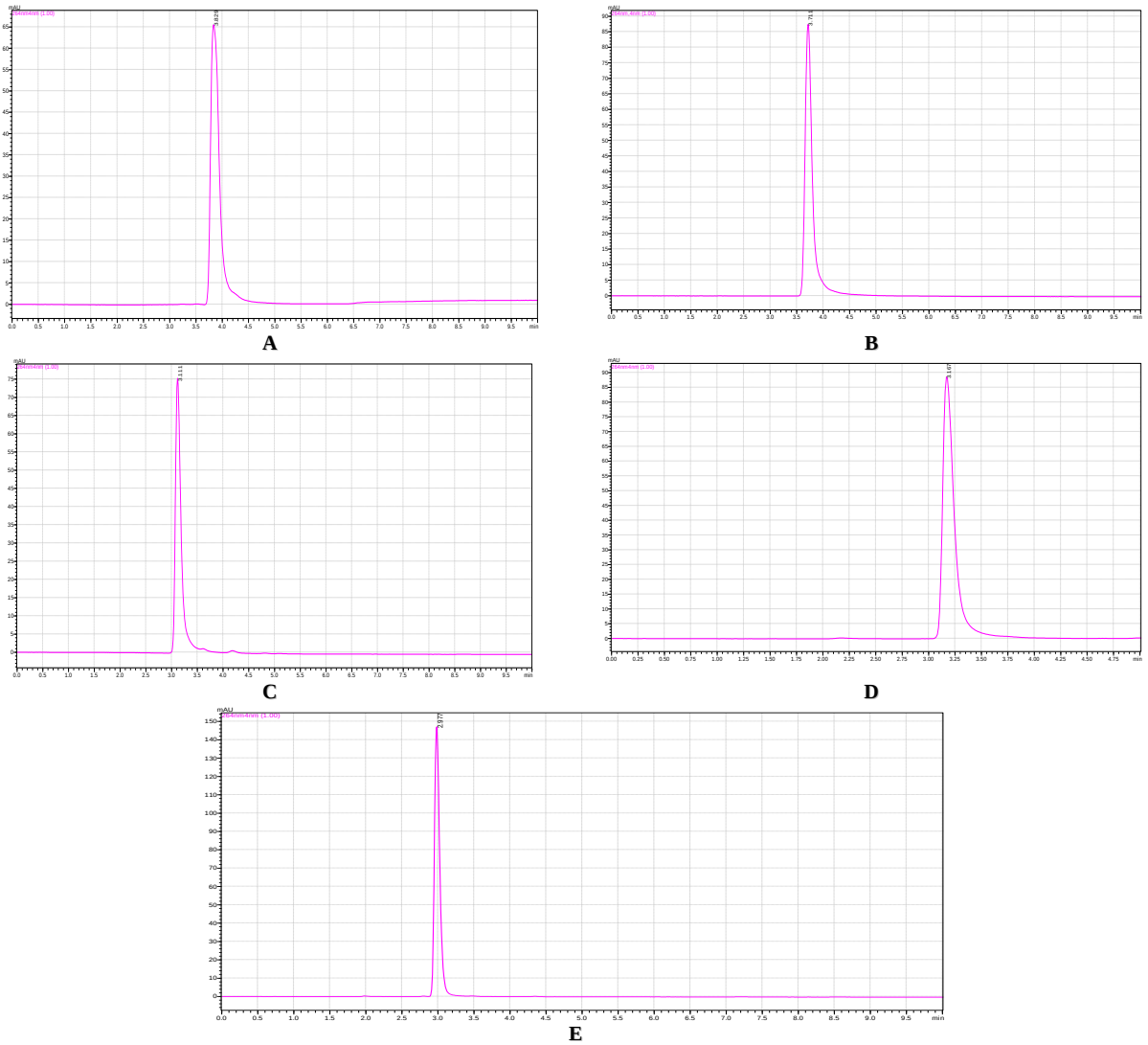


Figure 2: Representative chromatograms of Flucytosine standard (API) (10 µg/mL) obtained during the optimization

Method validation:

Linearity

Flucytosine obeys Beer-Lambert's law and follows linearity over the concentration range 0.5-120 µg/mL (Table 2) (% RSD 0.015-0.414) and the linear regression equation was found to be y = 74185x- 5852.7 (r^2 = 0.9999) (Figure 3). The LOD was found to be 0.1393 µg/mL and the LOQ was found to be 0.4187µg/mL.

Table 2: Linearity

Conc. (µg/mL)	*Mean peak area	% RSD
0.5	37837	0.043
1	75674	0.050
2	151349	0.030
5	378373	0.073
10	756747	0.414
20	1454531	0.034
40	2932520	0.115
60	4428780	0.071
80	5905040	0.015
100	7381300	0.048
120	8957560	0.064

*mean of three replicates

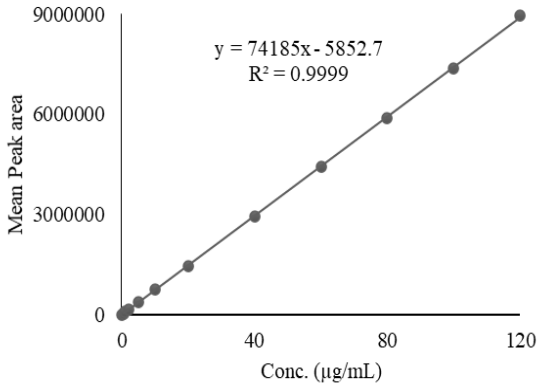


Figure 3: Calibration curve of Flucytosine

Precision, Accuracy and Robustness

Intraday and inter-day precision were studied at three different concentration levels of Flucytosine on the same day and on three consecutive days respectively and the % RSD was found to be 0.058-0.188 (Intraday) (Table 3) and 0.067-0.915 (Inter day) (Table 4) respectively (<2.0) demonstrating that the method is precise. The accuracy of the method was proved by the standard addition method and the recovery values were 0.02-0.06 (<2.0) with a recovery of 99.87-100.20 % (Table 5). The robustness of the assay method was established by introducing small changes in the chromatographic conditions which include detection wavelength (262 and 266 nm), percentage of organic phase i.e. methanol in the mobile phase (48 and 52%) and flow rate (± 0.1 ml/min). Robustness of the method was studied using 10 µg/mL of Flucytosine (Table 6) and the % RSD was found to be 0.19-1.51 (<2.0).

Table 3: Intraday precision study of Flucytosine

Conc. (µg/mL)	*Mean peak area	Statistical Analysis *Mean peak area ± SD (% RSD)
10	756747	757289.5 ± 442.949 (0.058)
10	757832	
10	758426	
20	1454531	1457898 ± 2748.736 (0.188)
20	1461264	
20	1473601	
30	2181796	2177189 ± 3762.008 (0.172)
30	2172581	
30	2163410	

*mean of three replicates

Table 4: Interday precision study of Flucytosine

Conc. (µg/mL)	*Mean peak area			*Mean ± SD (% RSD)
	Day 1	Day 2	Day 3	
10	758747	751281	755132	755014 ± 3047.982 (0.403)
20	1454361	1422124	1423120	1438243 ± 13160.70 (0.915)
30	2183186	2186816	2185861	2185001 ± 1481.941 (0.067)

*mean of three replicates

Table 5: Accuracy study of Flucytosine

Spiked Conc. ((µg/mL)	Formulation (µg/mL)	Total Conc. (µg/mL)	*Conc. obtained (µg/mL) ± SD %RSD)	% Recovery
5 (50%)	10 10 10	15 15 15	14.98 ± 0.004 (0.02)	99.87
10 (100%)	10 10 10	20 20 20	19.99 ± 0.012 (0.06)	99.95
15 (150%)	10 10 10	25 25 25	25.05 ± 0.012 (0.04)	100.20

*mean of three replicates

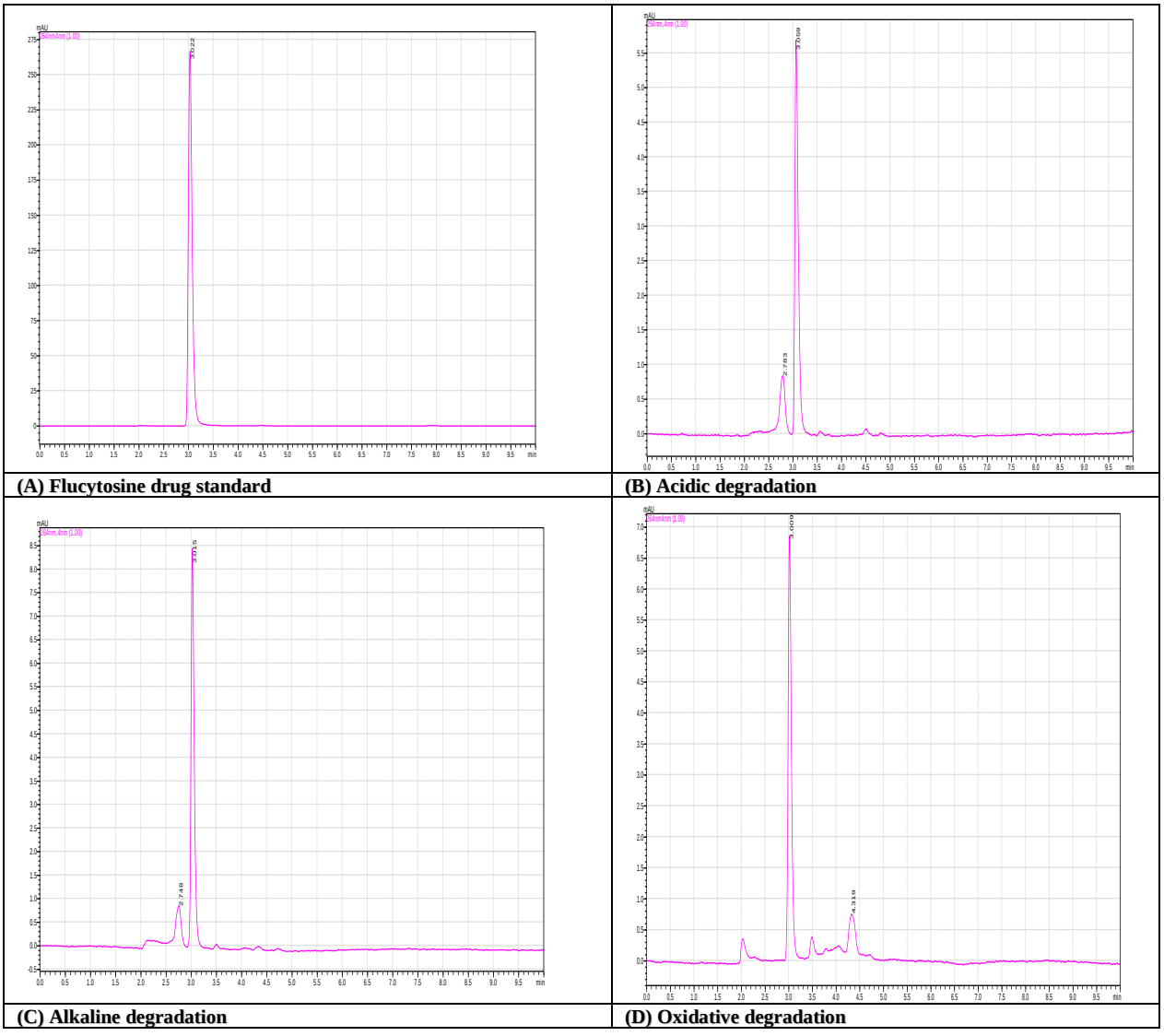
Table 6: Robustness study of Flucytosine

Parameter	Condition	*Mean peak area	*Mean peak area ±SD (% RSD)
Flow rate (± 0.1 ml/min)	0.9	757164	756962.33 ± 11430.13(1.51)
	1.0	756747	
	1.1	756976	
Detection wavelength (± 2 nm)	262	755978	755904.67 ± 1436.22 (0.19)
	264	756747	
	266	754989	
Mobile phase composition Methanol: water: Acetic Acid (50:50:0.1) (± 2 %, v/v)	52:48:0.1	756299	756645.67 ± 9079.75 (1.23)
	50:50:0.1	756747	
	48:52:0.1	756891	

*mean of three replicates

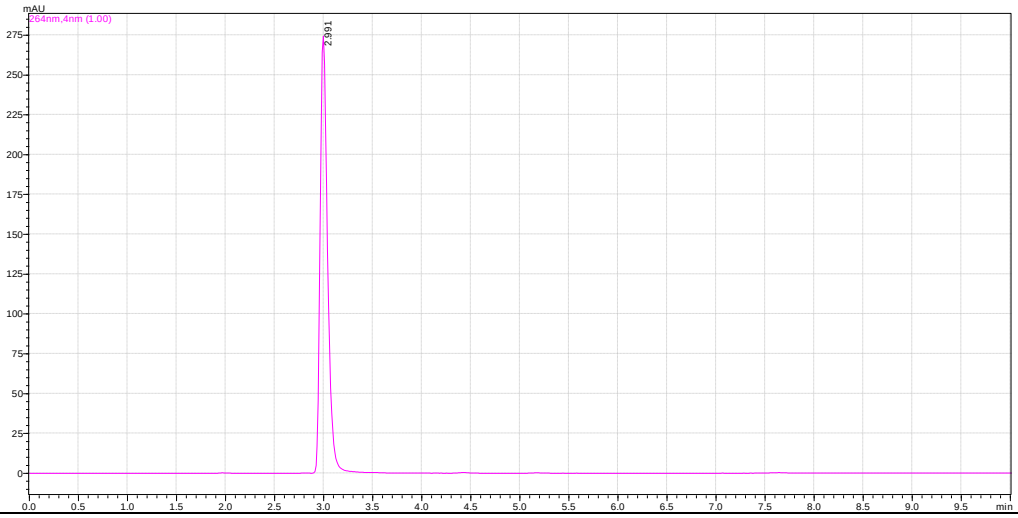
Forced degradation studies

Flucytosine (20 µg/mL) was eluted as a sharp peak at 3.022 min. During the acidic degradation the drug was eluted at 3.059 min and in alkaline degradation Flucytosine was eluted at 3.015 min. During the oxidation along with the drug peak (3.009 min) one more degradant was eluted at 4.196 min and in thermal degradation the drug was eluted at 2.991 min. Flucytosine was observed to be highly resistant towards all degradation conditions and the degradants were well separated. In all the degradation studies less than 3% degradation was observed and therefore it is confirmed that the method is selective and specific. The system suitability parameters has shown that the tailing factor was <1.5-2.0) and the theoretical plates were more than 2000. (Table 7). The individual chromatograms obtained during the forced degradation studies were shown in Figure 4.



(A) Flucytosine drug standard | **(B) Acidic degradation**

(C) Alkaline degradation | **(D) Oxidative degradation**



(E)Thermal degradation

Figure 4: Typical chromatograms of Flucytosine A) Drug standard (20 µg/mL) B) Acidic degradation C) Alkaline degradation D) Oxidative degradation E) Thermal degradation

Table 7: Stress degradation studies of Flucytosine

Stress condition Medium/temp./duration	Rt (min)	% Recovery	%Drug degradation	Theoretical plates	Tailing factor
Standard drug	3.022	100	------	5954	1.534
Acidic degradation 0.1N HCl/ 75°C/ 1 hour	3.059	1.93	98.07	7551	1.877
Alkaline degradation 0.1N NaOH/ 75°C/ 1 hour	3.015	2.56	97.44	8246	1.537
Oxidation 30%H_2O_2/ 75°C/ 1 hour	3.009	2.56	97.44	7271	1.461
Thermal degradation Water/ 75°C/ 1 hour	2.991	100.7	0.711	6053	1.520

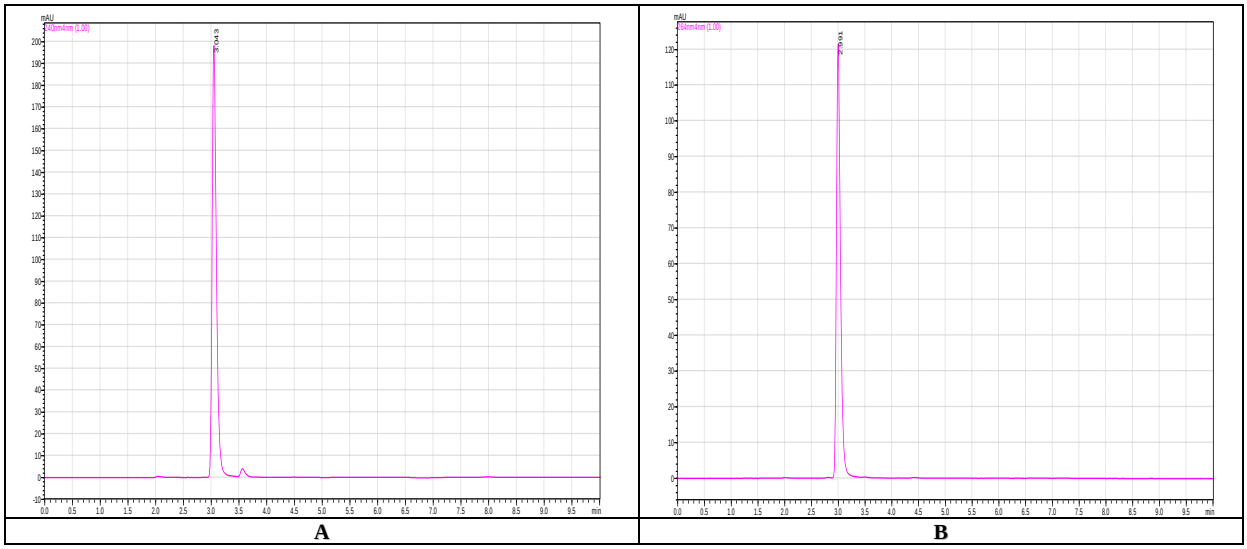
Assay of Flucytosine tablets

Assay was performed for two brands of Flucytosine tablets consisting of 500 mg API using the above proposed chromatographic method. The amount of Flucytosine was found to be 99.864-99.906 (Table 8) and no interference of excipients (Figure 5) was observed.

Table 8: Assay of Flucytosine tablets

Brand	Label claim (mg)	Observed amount (mg)	% Recovery*	Manufacturer (India)
I	500	499.53	99.906	Jolly Healthcare
II	500	499.32	99.864	Lupin pharmaceuticals

*mean of three replicates



A **B**

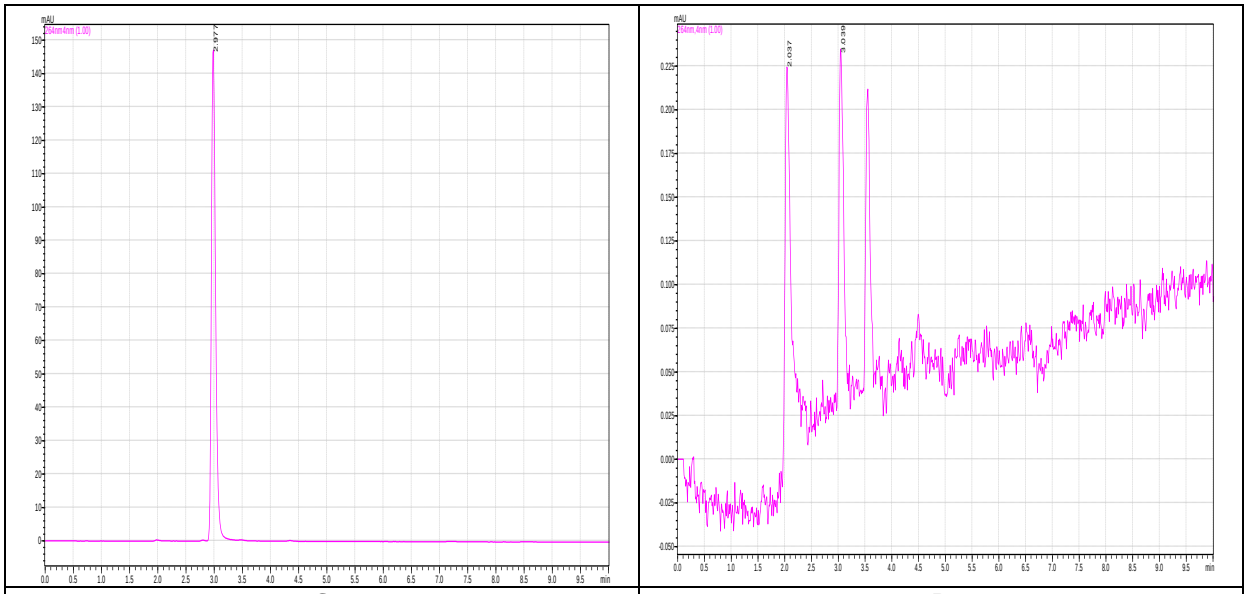


Figure 5: Representative chromatogram of A) Flucytosine standard (API) (10 µg/mL) B) Flucytosine tablets (Brand I) C) Flucytosine tablets (Brand II) D) Placebo

CONCLUSIONS:

The RP-HPLC method was validated as per ICH guidelines and observed to be simple, economical, simple and robust for the quantification of Flucytosine tablets.

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RESEARCH ARTICLE

Speciation of Cons Isolated from Nasal Swabs among Health Care Workers from A Tertiary Care Centre with special Reference to *S. caprae* and its Virulence Factor – Biofilm Production

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ABSTRACT:

CONS are one of the most frequent causes of nosocomial outbreaks in hospitals and are reservoirs of multiple antimicrobial resistant determinants. Identification and speciation of CONS has become important due to the growing recognition and medical importance to define their clinical significance. *S.caprae* has the ability to produce a biofilm in vitro which itself serves as a virulent factor for many infections. The study was to screen MRSA in nasal swabs among health care workers. Routinely isolation of genus staphylococci was done based on catalase test. For differentiating coagulase positive and coagulase negative staphylococci were done based on slide coagulase and tube coagulase test. CONS species was identified by Vitek 2 system Most important virulence factor in CONS spcies was biofilm production. So we checked biofilm production in cons species. Out of 82 nasal swabs that were collected from both right and left nostril, organisms were found in 72(87.8%) samples of both right and left nostril. *Staphylococcus caprae* was found predominantly in 56(77.7%) of 72 samples. 8(11.1%) samples showed the growth of *Staphylococcus lugdunensis. Staphylococcus schleiferi* and *Staphylococcus cohnii* were found in four sample each. Out of 56 *S.caprae,* 12(21.4%) samples showed strong biofilm production, 28(50%) showed moderate and 16(28.5%) showed weak. Our study shows a large number *Staphylococcus caprae* from the nasal swabs, that has got the significant property of biofilm production which itself serves as a virulent factor for causing infections. So, some of the CONS are considered pathogenic and it should be speciated to avoid deaths caused by infections

KEYWORDS: CONS, *S.caprae,* biofilm, nosocomial.

INTRODUCTION:

Coagulase negative staphylococci (CONS) are increasingly being recognized as significant nosocomial pathogens partly due to the growing reports of this group of organism causing opportunistic infections[1]. CONS are one of the most frequent causes of nosocomial outbreaks in hospitals and are reservoirs of multiple antimicrobial resistant determinants.

Identification and speciation of CONS has become important due to the growing recognition and medical importance to define their clinical significance[1,2]

The species include *Staphylococcus epidermidis, Staphylococcus haemolyticus, Staphylococcus lugdunensis, Staphylococcuss chleiferi,* Staphylococcus *saprophyticus*[3]. Coagulase Negative *Staphylococci* (CONS) are normal human microbiota and sometimes cause infections. Many times CONS were not considered pathogenic and left untreated which leads to death[4].

Staphylococcus caprae is a Gram-positive, coccus bacteria and a member of the genus *Staphylococcus*. *S. caprae* is coagulase-negative. *S. caprae* occurs as a commensal on human skin[5]. *Staphylococcus caprae* was first described by Devrise et al in 1983 based on a strain

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isolated from goat's milk. Surprisingly, this species also has been reported as a human-hospital acquired pathogen, mostly implicated in bone and joint infection and also studies prove that *S.caprae* has the ability to produce a biofilm in vitro which itself serves as a virulent factor for many infections[6].

Biofilm are a group of microbes which are encased in an exo-polysaccharide matrix on both biotic and abiotic surfaces. Various changes occur during their transition from a planktonic to a surface attached community. This causes a number of persistent infections which respond poorly to conventional antibiotic therapy[7].

The objective of the present study is to speciate CONS based on automated method and to check for biofilm production of isolated CONS species.

MATERIALS AND METHODS:

It is a prospective study involving 82 health care workers (hospital staffs, doctors and ICU team) working in various clinical departments in Saveetha Medical College Hospital. The study was done for 6 months from April 2017 to September 2017. The ethical clearance has been obtained for the study.

Statistical analysis:

Chi square test was used.

Identification of staphylococcus species:

Nasal swabs are collected and processed on Blood agar plates and gram staining was done. Based on gram staining result both Slide and Tube Catalase and Coagulase test was performed[8].

Speciation of cons by automated method:

The vitek 2 system was used according to the manufacturer's instructions, ID – Gram Positive Cocci cards (ID-GPC cards bioMerieux) were used for identification. The ID_GPC card is a 64 – well plastic card containing 18 empty wells and 46 wells for fluroscent and inhibitory tests that include pH change tests and derivatives to detect aminopepetidases and osidases. Substrates used for detection of aminopeptidases are usually coupled with 7-amino methylcoumarin (7AMC). Substrates used for detection of osidases are coupled with 4- methlybelliferone (4MU). Each organism suspension was prepared from the growth of pure cultures of bacteria cultivated on plates containing Trypticase soy agar with 5% sheep blood (bioMérieux, Marcy l'Etoile, France) and incubated overnight at 35°C. Bacterial cells were suspended in 2.5 ml of a 0.45% sodium chloride solution. The suspension used in the VITEK 2 system was adjusted to a McFarland standard of 0.5 by using a Densicheck (bioMérieux)[9]

Detection of biofilm formation for *S. caprae:*

Biofilm formation is detected using Tissue culture plate method and classified into strong, weak and moderate biofilm producers according to the values noted

Tissue culture plate method (TCP):

Isolates from fresh agar plates were inoculated in trypticase soy agar broth and incubated for 24 hrs at 37°C then diluted with fresh trypticase soy agar broth in 1 in 100 dilution. Tissue culture plate is sterile polystyrene 96 well flat bottom is filled with 0.2 ml aliquots of the diluted culture. The broth served as control to check sterility and nonspecific binding of media. The TCP was incubated for 24 hrs at 37°C. by tapping the plates, contents of each well was gently remove and washed 4 times 0.2 ml phosphate buffered saline to remove free floating planktonic bacteria, wells are stained with crystal violet. To remove excess stain, wells were washed with deionised water and the tubes were dried. Optical density (OD) was determined at a wavelength of 570 nm with micro ELISA auto reader. Experiment was repeated thrice and standard deviation was calculated. The mean OD value obtained from media control was deducted from all the test OD value[10].

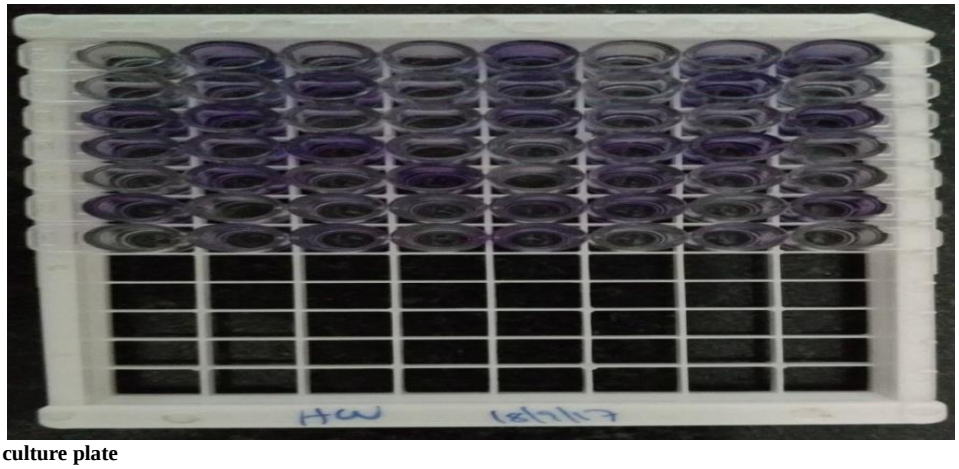

Figure 1 Tissue culture plate

RESULTS:

The study included 82 health care workers, of which Doctors were 36, staff nurse were 35 and CRRI comprised of 11 (fig 2). Out of 82 nasal swabs that were collected from both right and left nostril, growth were observed in 72 (87.8%) samples of both right and left nostril (fig 3). *Staphylococcus caprae* was found predominantly in 56(77.7%) of 72 samples. 8(11.1%) samples showed the growth of *Staphylococcus lugdunensis. Staphylococcus schleiferi* and *Staphylococcus cohnii* were found in four sample each (fig 4). Out of 56 *S.caprae*, 12 (21.4%) samples showed strong biofilm production, 28(50%) showed moderate and 16(28.5%) showed weak (tab 1).

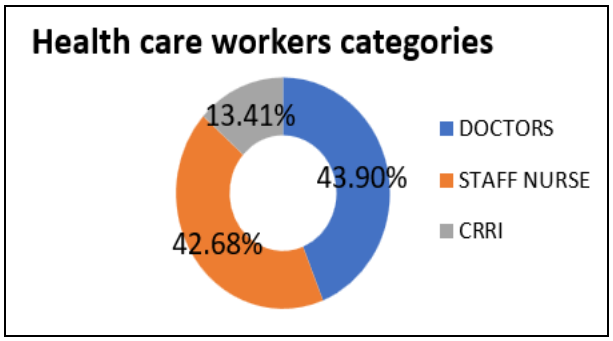

Figure 2 Population Involved

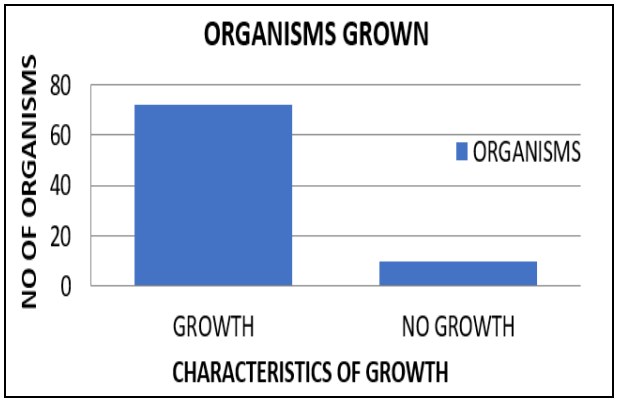

Figure 3 Growth detected in specimen

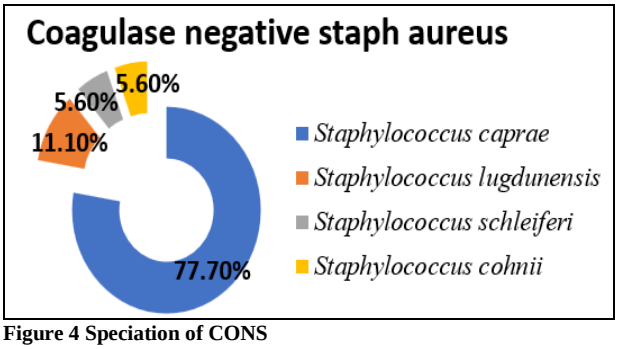

Figure 4 Speciation of CONS

Table 1 Biofilm production characteristics

Biofilm Production *S.caprae*	**%**
Strong	12 (21.4%)
Moderate	28(50%)
Weak	16(28.5%)

DISCUSSION:

Coagulase negative Staphylococci have emerged in recent years as pathogens in a growing number of nosocomial infections. Production of an exopolysaccharide, allowing adherence and subsequent formation of a multilayered biofilm, appears to be essential for the pathogenesis of Coagulase negative Staphylococcus species. Because many isolates are multi-drug resistant, their infections are difficult to treat and can even fatal. A detailed characterization of isolates of CoNS through speciation, genetics and antibiotic susceptibility may be necessary to distinguish infecting from contaminating isolates and to plan suitable therapy. In this study, the CoNS isolates were resistant to the common antibacterial used for wound infections; hence an accurate sensitivity pattern has to be determined before starting of the antibacterial therapy to prevent therapeutic failure. Hence, CoNS cannot be neglected as nonpathogenic as infections with these can be life-threatening and the biofilm producing isolates may be multi-drug resistant too. It is better to isolate the patients if they are infected with MRCoN for the better infection control measures.

Our study was started by screening for MRSA in nasal swabs of health care workers. By analyzing and processing the nasal swabs. we didn't get any MRSA but found CONS in 72 samples. So the species identification was done by an automated method Vitek 2 system.

Soumya et al identified 30 commensals isolated from the hands of laboratory workers. CONS species observed in the study were *S. epidermis* (60%), *S hominis* (33.3%), *S. hemolyticus* and *S. saprophyticus* (3.3%).[11]

Robert et al found 96 strains of coagulase negative staphylococcus aureus isolated from only single blood culture bottles and comprised of *S epidermis, S hominis, S. hemolyticus* and *S warneri*[12]

In the present study, *Staphylococcus caprae* was found predominantly in 56(77.7%) of 72 samples. 8(11.1%) samples showed the growth of *Staphylococcus lugdunensis. Staphylococcus schleiferi* and *Staphylococcus cohnii* were found in four sample each.

By DNA-DNA hybridization on microplates, Kawamura et al identified 1,230 strains of staphylococci from human clinical specimens and determined the distribution of species. The 10 *Staphylococcus* species isolated most often were *S. epidermidis* (31.3%), *S.*

aureus (23.3%), *S. haemolyticus* (12.2%), *S. caprae* (10.7%), *S. simulans* (4.4%), *S. hominis* (4.0%), *S. capitis* (3.9%), *S. saprophyticus* (3.6%), *S. warneri* (2.2%), and *S. lugdunensis* (1.3%). From these results, they realized that *S. caprae* strains were widely distributed in human clinical specimens. Among the identified species, *Staphylococcus caprae* comprised an unexpectedly large number of strains (132 of 1,230)[13].

In the present study, out of 56 *S.caprae*, 12 (21.4%) samples showed strong biofilm production, 28(50%) showed *moderate* and 16(28.5%) showed weak Ramakrishna et al found that CoNS 16% were non biofilm producers, 72% were moderate biofilm producers and 12% highly biofilm producers. It was found that CoN Staphylococci isolates were strong biofilm producer[14]

Thus, we conclude that the biofilm producing CoNS species are difficult to be eradicated from the site of infection using antibacterial drugs. These biofilm producing CoNS even though considered contaminant in the clinical specimens sometimes can cause serious infections in patient with reduced immunity. They can be multidrug resistant as biofilm restrict the entry of the drug to the point of action. Hence, when CoNS is isolated the biofilm producing capacity has to be evaluated before treating the patients with the antibacterial.

CONCLUSION:

Staphylococcus aureus was usually identified as the predominant species among nasal carries. Our study shows a large number *Staphylococcus caprae* from the nasal swabs, that has got the significant property of biofilm production which itself serves as a virulent factor for causing infections. So, some of the CONS are considered pathogenic and it should be speciated to avoid deaths caused by infections.

CONFLICT OF INTEREST:

None.

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RESEARCH ARTICLE

Influence of Sodium Alginate and Calcium Chloride on the Characteristics of Isoniazid Loaded Nanoparticles

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ABSTRACT:
The main objective of the current investigation was the evaluation of the influence of sodium alginate (SA) and calcium chloride concentration on the characteristics of isoniazid loaded nanoparticles (NPs). Isoniazid loaded NPs were fabricated using the ionotropic gelation technique. The NPs fabricated, were evaluated for average particle size, encapsulation efficiency, drug loading and *FTIR* spectroscopy along with *in vitro* drug release. The particle size, drug loading & encapsulation efficiency of fabricated nanoparticles were ranging from 230.7 to 532.1 nm, 5.88% to 11.37% and 30.29% to 59.70%, respectively. Amongst all batches studied, formulation F-8 showed the best-sustained release of drug at the end of 24 hrs.

KEYWORDS: Tuberculosis, Ionotropic gelation, nanoparticles, antitubercular drug, sodium alginate, Calcium chloride.

INTRODUCTION:
Tuberculosis is a global health problem as such every nation is fighting with the new cases especially among immigrant populations. Tuberculosis is second, next to HIV, in causing mortality from an infectious disorder which affects One-third of the total world population[1]. According to the World Health Organization (WHO), more people today are dying of tuberculosis than ever before. Tuberculosis kills 8,000 people per day globally. Tuberculosis is a disease of antiquity and was believed an inherited disease until Hermann Heinrich Robert Koch, a German physician, and microbiologist announced the discovery of the Mycobacterium the causative organism for tuberculosis on 24 March 1882[2].

Historically, TB is referred to as "Captain of All These Men of Death"[3]. The disease was also called consumption and phthisis in the 17th and 18th centuries until Johann Lukas Schonlein coined the term "tuberculosis" in the mid of the 19th century[4].

Tuberculosis is a potentially curable disease, still challenging, for which improved therapeutics are needed to shorten the course of treatment. The single most common reason for the failure of tuberculosis chemotherapy is poor patient compliance[5]. The useful technique to ensure patient compliance in tuberculosis patients is to supervise the administration of drugs, which is not always practically possible. Tuberculosis is still a serious public health care issue, because of this reason a new combined master plan, based on the improving drug treatment, diagnostic equipments, and prevention strategy, is required to completely eradicate the Mycobacterium Tuberculosis by the year 2050, as commitment made by the World Health Organization[6]. The daily dosing of antitubercular drugs is the demand of its biopharmaceutical and pharmacokinetic properties for the effective management of tuberculosis. The incidence of adverse effects, daily dosing and poor patient compliance may result in discontinuation of therapy and increase in cases of MDR tuberculosis[7,8]. Consequently, the goal of tuberculosis research is to improve the therapeutics to shorten the course of treatment. The pharmacokinetic profile of antitubercular drugs can be altered and by using various novel drug delivery systems that may improve the therapeutic strategy for the management of tuberculosis. For drug delivery systems, a variety of biodegradable polymers are employed which show desirable sustained,

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controlled, and targeted effect by keeping drug concentration within the therapeutic range.

Sodium alginate is a sodium salt of alginic acid, a natural occurring polysaccharide, widely obtained from the marine brown algae[9,10]. It is rich in the carboxyl group and is easily binds with a positively charged drug. Sodium alginate is a low toxic, biocompatible and inexpensive biopolymer, which enables it to be widely used in the medicine and food industry[11,12]. By reviewing the current and past research reports it was found that, the use of natural polymers as a formulation aid in sustained and controlled drug delivery systems has become an important area of research and development [13]. Therefore, Sodium alginate was used to fabricate nanoparticles entrapping an antitubercular drug[14].

Isoniazid is a first-line antitubercular drug used for the treatment of TB. Isoniazid was first synthesized in 1912 by Hans Meyer and Josef Malley as part of the work for their doctoral thesis in Prague. The antituberculosis properties of isoniazid were discovered in 1945. The investigations were conducted simultaneously and independently by three different pharmaceutical firms, Farbenfabriken Bayer,[15] Hoffmann La Roche[16,17] and Squibb Institute for Medical Research[18], led to the discovery of isoniazid as a potent antituberculosis drug regimen. In 1951 the first clinical trial was started at Sea View Hospital in Staten Island, New York and effective results were publicly reported in 1952[19]. After the discovery of isoniazid as an antitubercular drug, it was included in the therapeutic regimen for the management of tuberculosis in the year 1952[20,21]. Numerous Medicinal Chemistry research groups have recently reported isoniazid derivatives with strong antitubercular activity. Nevertheless, isoniazid continues at the top position as one of the leading options available for tuberculosis management even after 68 years of its discovery[22-25]. Isoniazid occupies a significant role in the present antitubercular therapeutic regimen and numerous clinical trials have underlined the importance of isoniazid in new therapeutic schemes under development [26-28].

Nanoparticle drug delivery systems have many advantages, including passive targeting, lowering drug toxicity, increasing drug stability, sustained and controlled release of drugs[29,30]. The nanoparticles have been studied to improve the efficacy and thus overcome the weakness of isoniazid in clinical applications. Various natural polymers, synthetic polymers and inorganic materials are used as carrier materials to prepare nanoparticles[31]. Recently, Characterization of doxorubicin nanoparticles prepared by ionic gelation[32] and the fabrication of sodium alginate nanoparticles as carriers for doxorubicin to increase the efficacy of active moiety has been reported[33]. The evaluation of the influence of sodium alginate (SA) and calcium chloride concentration on the characteristics of isoniazid loaded nanoparticles is reported here.

MATERIAL AND METHOD:

Material:

Isoniazid was purchased from High Purity Laboratory Chemicals (HPLC), Mumbai (India). Calcium chloride was purchased from SD Fine Chem Ltd., Mumbai (India). Sodium Alginate was procured from Himedia Laboratories Pvt. Ltd., Mumbai (India). Other reagents and chemicals of analytical grade were procured from manufacturers and used as received.

Experimental design:

The optimization technique was applied to obtain an appropriate formulation design in order to minimize the number of experiments. The experimental design of Sodium alginate nanoparticles is described in Table 1.

Table 1 Experimental design of Sodium alginate nanoparticles

Independent variables	Levels		
	Low (-1)	Medium (0)	High (1)
A Sodium alginate (%w/v)	2	2.5	3
B Calcium chloride (%w/v)	1	1.5	2

Method:

Preparation of nanoparticles:

The nanoparticles were prepared by a simple formulation approach, using a cost-efficient, biodegradable, and Food and Drug Administration-approved polymer. Ionotropic gelation technique with some modifications was used to fabricate isoniazid loaded Sodium alginate nanoparticles. Sodium alginate was used at various concentrations and dissolved in distilled water using a magnetic stirrer and allowed to stand for 30 minutes. Then the drug was suspended in above mentioned sodium alginate solution with continuous stirring. Various concentrations of Calcium chloride solution were added in a dropwise manner in the prepared solution of drug and polymer. The suspension of Isoniazid - Sodium alginate - Calcium chloride nanoparticles was formed. Calcium chloride was added as a cross-linking agent for the sodium alginate nanoparticles to achieve sustained drug release. It was kept for sonication for 25 minutes. After sonication, the centrifugation of nanoparticle suspension was carried out at 10,000 rpm (Remi, Mumbai) for 30 minutes and supernatant was discarded. The pellet was collected and redispersed in de-ionized water followed by sonification, centrifugation and lyophilisation[33, 34].

Characterization of sodium alginate nanoparticles:

Particle size:

Freeze-dried nanoparticles were dispersed into HPLC grade water. The particle size of the isoniazid loaded

sodium alginate nanoparticles was evaluated by Particle Size Analyzer (Malvern Instruments Ltd., Malvern, UK). The particle sizes of various fabricated nanoparticle formulations are shown in Table 2.

Determination of Encapsulation Efficiency (EE) of nanoparticles:

10ml suspension of nanoparticles was centrifuged at 10,000rpm (Remi, Mumbai) for 90 minutes at 10ºC. After centrifugation, the clear supernatant procured was diluted 10 times with double distilled water to quantify the amount of unbound isoniazid using the U.V-Visible spectrophotometer at λ_{max}=263nm. Encapsulation Efficiency and drug loading of nanoparticles were determined using the equation given below [35].

$$\text{Encapsulation efficiency} = \frac{\text{Total amount of isoniazid-Amount of free isoniazid}}{\text{Total amount of isoniazid}} \times 100$$

$$\text{Drug loading} = \frac{\text{Total amount of isoniazid-Amount of free isoniazid}}{\text{Total weight of nanoparticles}} \times 100$$

Fourier transform infrared spectroscopy (FTIR):

The infrared spectra of isoniazid, sodium alginate and physical mixture (sodium alginate and isoniazid) were examined to determine any drug-excipients interaction. The peaks of individual isoniazid drug, sodium alginate polymer and peak of isoniazid drug – sodium alginate polymer combination were compared to ascertain the interactions[36]. *FTIR* spectra of isoniazid drug and drug-polymer physical mixture were obtained by the *KBR* pellet method.

In vitro drug release studies of sodium alginate nanoparticles:

Drug release studies were carried out using dissolution medium (250ml of 7.4 pH phosphate buffer) at 50rpm at 37±0.5ºC temperature. The nanoparticles containing drug equivalent to 50mg were taken in a dialysis bag and put into the flask containing dissolution medium. 5ml aliquots of samples were withdrawn at specific interval i.e. 1, 2, 4, 6, 12, 14, 16, 18, 20, 22 and 24 hrs. After suitable dilutions, the absorbance of the samples was determined by UV-Visible Spectrophotometer at λ_{max} 263nm. Absorbance for the samples withdrawn was recorded and percentage drug release at different time intervals was plotted against time.

RESULTS AND DISCUSSION:

Optimization using Design-Expert®:

The factorial design was applied for the determination of the appropriate amount of sodium alginate (SA) and calcium chloride on the basis of loading capacity, encapsulation efficiency and average particle diameter measurements.

A total of 13 formulations were fabricated as per experimental design and influence of 2 factors, i.e. Sodium Alginate (A) and calcium chloride (B) was examined on 3 responses viz. loading capacity, encapsulation efficiency, and average particle diameter. The importance was designated to mean particle size, loading capacity and encapsulation efficiency. The variables according to Design-Expert® software for F-8 (optimized) nanoformulation were sodium alginate 3.0 % and calcium chloride 2 % which led to the formation of nanoparticles with an average particle diameter of 245.5nm.

Influence of sodium alginate (SA) and calcium chloride on the characteristics of isoniazid loaded nanoparticles:

Particle size:

The influence of sodium alginate (SA) and calcium chloride concentration on the characteristics of isoniazid loaded nanoparticles formulations is shown in Table 2.

Table 2 Experimental Design for Isoniazid nanoparticle and the measured response parameters.

Std	Run	Factor 1 Sodium alginate	Factor 2 Calcium chloride	Response 1 Drug loading (%w/w) ±SD	Response 2 Entrapment efficiency (%)±SD	Response 3 Average particle size (nm)
7	1	0	-1	7.97±0.18	41.25±0.08	230.7
6	2	1	0	10.82±0.19	56.53±0.20	386.8
8	3	0	1	10.40±0.13	54.34±0.29	284.2
9	4	0	0	8.99±0.13	46.78±0.19	231.5
13	5	0	0	9.91±0.10	51.55±0.82	285.3
1	6	-1	-1	5.88±0.09	30.29±0.25	253.7
3	7	-1	1	9.31±0.10	48.43±0.26	240.9
4	8	1	1	11.37±0.11	59.70±0.15	245.5
2	9	1	-1	9.62±0.05	50.06±0.07	416.3
5	10	-1	0	10.17±0.15	52.63±0.18	532.1
10	11	0	0	10.62±0.09	55.26±0.17	334.4
11	12	0	0	9.84±0.10	51.21±0.13	382.6
12	13	0	0	8.70±0.10	45.26±0.08	381.4

The obtained polynomial equation was employed for the calculation of the variance and responses were evaluated for parameters such as degree of freedom, F value, the sum of squares and mean sum of squares applying the software. The polynomial equation obtained through regression analysis for response is as follows:

Particle size = +324.40+96.63*A+25.72*B (1)

The observed particle size of the prepared nanoformulations was varying in the range of 230.7 to 532.1 nm. The formulation F-1 showed comparatively smaller size i.e. 230.7 nm with medium and low concentration of sodium alginate (SA) and calcium chloride respectively, whereas formulation F-10 showed comparatively large particle size i.e. 532.1 nm with low and medium concentration of sodium alginate (SA) and calcium chloride respectively. When the effect of both parameters was observed, the size was found increased with increased concentration of sodium alginate (SA) and calcium chloride. The increase in size was less in the case of calcium chloride as compared to sodium alginate. The response surface curve depicting the combined effect of both factors on the particle size of isoniazid nanoparticles is given below in Figure 1.

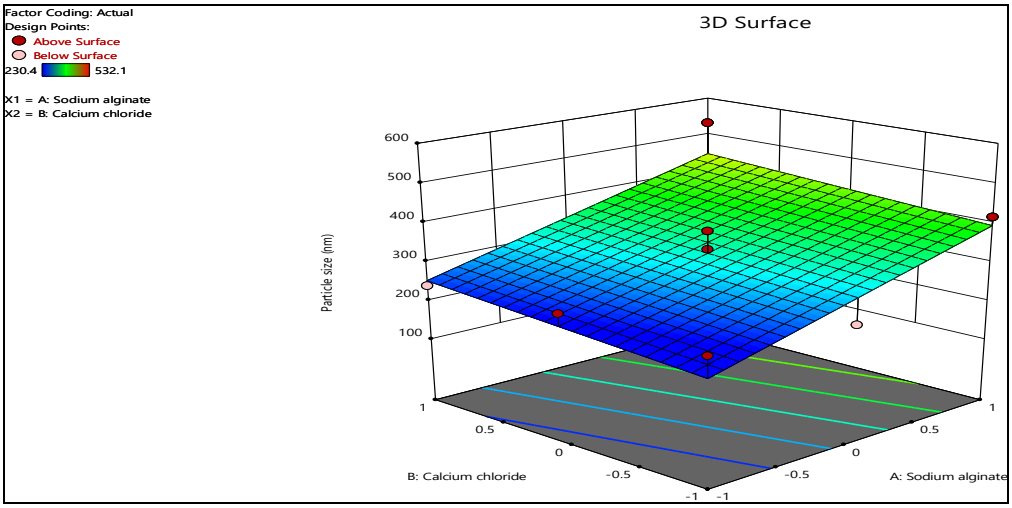

Figure 1: Response surface profile depicting the combined effect of Sodium alginate (SA) and Calcium chloride on the particle size of isoniazid nanoparticle

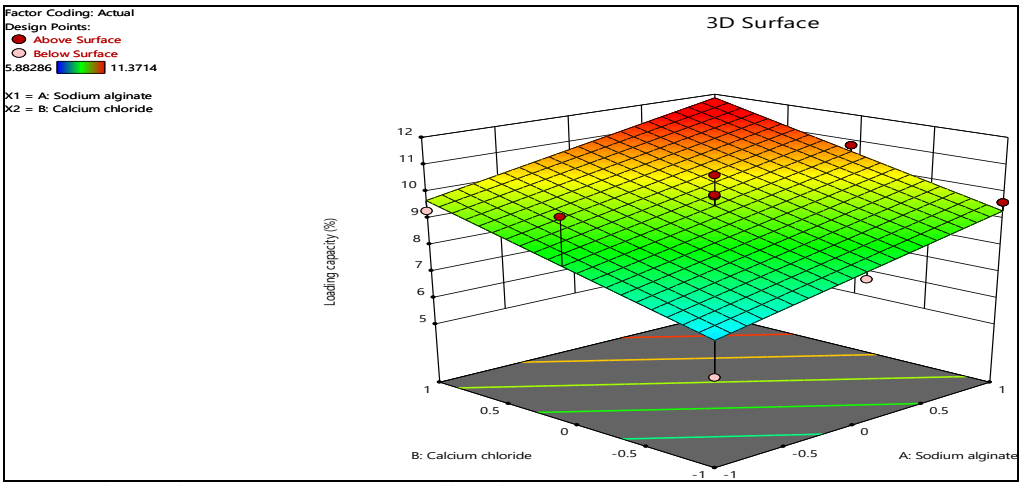

Figure 2: Response surface profile depicting the combined effect of Sodium alginate (SA) and calcium chloride on loading capacity (%) of isoniazid nanoparticles

Drug loading

The drug loading of fabricated nanoparticles was found in the range of 5.88% to 11.37% as shown in Table 2. The obtained polynomial equation was employed for the calculation of the variance and responses were evaluated for parameters such as degree of freedom, F value, the sum of squares and mean sum of squares applying the software. The polynomial equation obtained through regression analysis for response is as follows:

Loading capacity = +9.51-1.08*A+1.27*B (2)

The formulation F-6 showed minimum drug loading i.e. 5.88% with low concentration of Sodium alginate (SA) and calcium chloride, whereas maximum drug loading was observed in formulation F-8 i.e. 11.37% with high concentration of the sodium alginate (SA) and calcium chloride. Drug loading was increased as the concentration of sodium alginate (SA) and calcium chloride was increased. The response surface curve depicting the combined effect of both factors on the loading capacity of isoniazid nanoparticles is given below in Figure 2.

Encapsulation Efficiency (EE):

The encapsulation efficiency of the fabricated nanoparticles was found in the range of 30.29% to 59.70% as shown in Table 2. The obtained polynomial equation was employed for the calculation of the variance and responses were evaluated for parameters such as degree of freedom, F value, the sum of squares and mean sum of squares applying the software. The polynomial equation obtained through regression analysis for response is as follows:

Encapsulation efficiency = +49.49+5.82*A+6.81*B (3)

The formulation F-6 showed minimum encapsulation efficiency i.e. 30.29% with low concentration of sodium alginate (SA) and calcium chloride, whereas maximum encapsulation efficiency was observed in formulation F-8 i.e. 59.70% with high concentration of the sodium alginate (SA) and calcium chloride. Encapsulation efficiency was increased as the concentration of sodium alginate and calcium chloride was increased. The response surface curve depicting the combined effect of both factors on the encapsulation efficiency of isoniazid nanoparticles is given below in Figures 3.

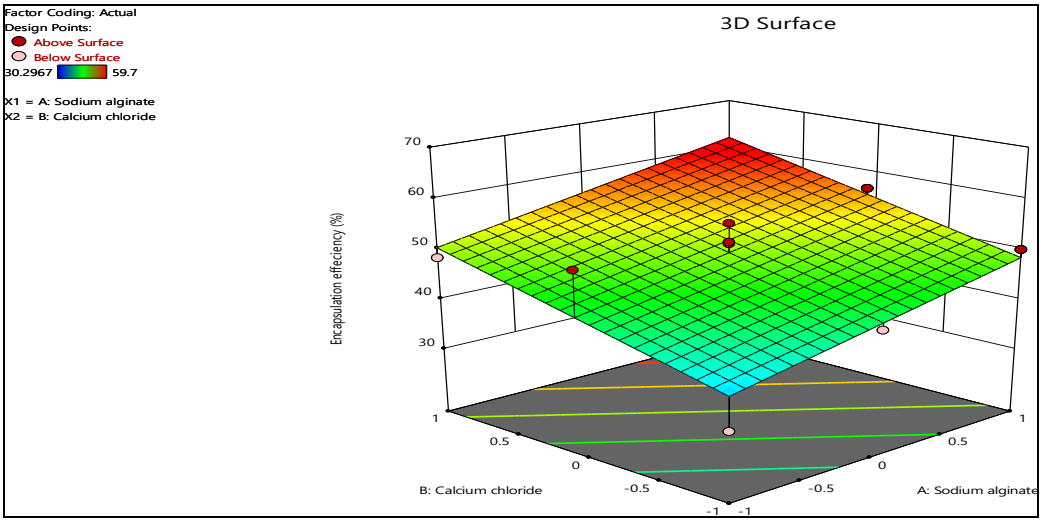

Figure 3: Response surface profile depicting the combined effect of Sodium alginate (SA) and Calcium chloride on encapsulation efficiency (%) of isoniazid nanoparticles

***In vitro* drug release studies:**

The pattern of drug release from the nanoformulations displayed cumulative drug release in the range 66.56%–83.53% as shown in Figures 4 and 5. The formulation F-11 showed maximum drug release i.e. 83.53% with medium concentration of sodium alginate (SA) and calcium chloride, whereas minimum drug release was observed in F-8 (optimized) formulation i.e. 66.56% with high concentration of the sodium alginate (SA) and calcium chloride during 24 hours study period as shown in Figure 5. The isoniazid loaded sodium alginate nanoparticles showed a biphasic drug release profile initially with rapid release of isoniazid followed by sustained release of isoniazid. The initial rapid release may be due to the association of isoniazid with the outer surface of nanoparticles. The initial release of isoniazid is linked with the isoniazid moieties which are present near the nanoparticle surface.

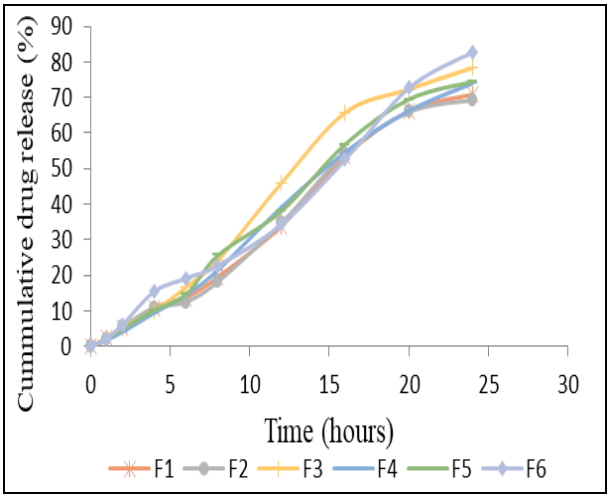

Figure 4. *In vitro* drug release profiles of Isoniazid nanoparticles (F1 –F6)

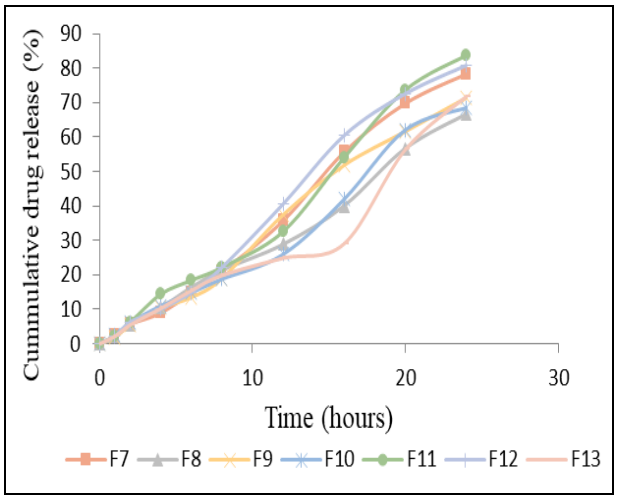

Figure 5. *In vitro* drug release profiles of Isoniazid nanoparticles (F7 –F13)

The drug release was found to depend upon the amount of sodium alginate present in the formulation. The F-8 (optimized) formulation showed a drug release of 66.56% within 24 hours showing a sustained release profile, during the first hour nanoparticle formulation gave outburst release and after that it showed a sustained. The macrophages take about 2 hours to attain their maximum engulfment capacity as reported in the literature[37]. Hence, it could be concluded that the maximum drug would be released inside the cell following endocytosis of the carrier system [38, 39].

Fourier transform infrared spectroscopy (FTIR):

Drug compatibility studies using FTIR were conducted for the drug, sodium alginate and the physical mixture. The spectral data are given in Figure 6-8. The results indicated no chemical incompatibilities between isoniazid and sodium alginate used in nanoparticles.

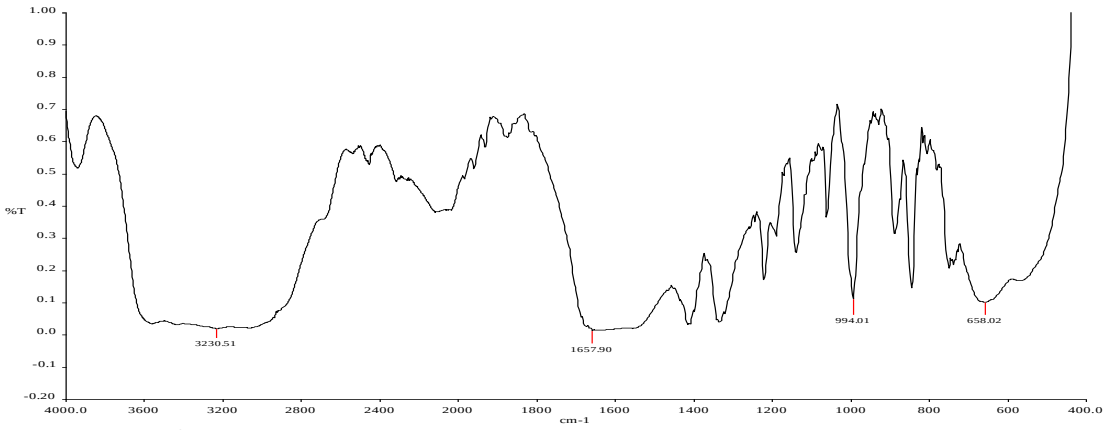

Figure 6. FTIR spectrum of the drug (Isoniazid).

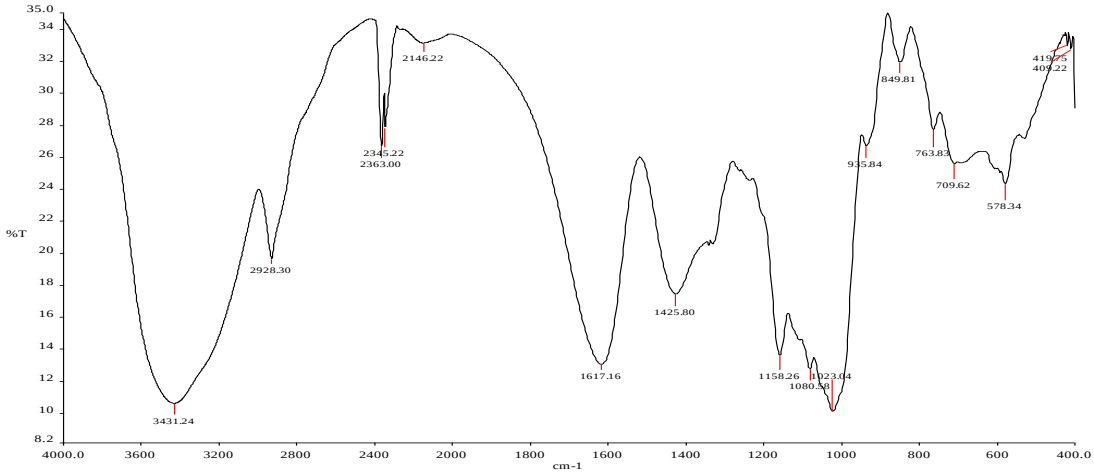

Figure 7. FTIR spectrum of the polymer (Sodium alginate)

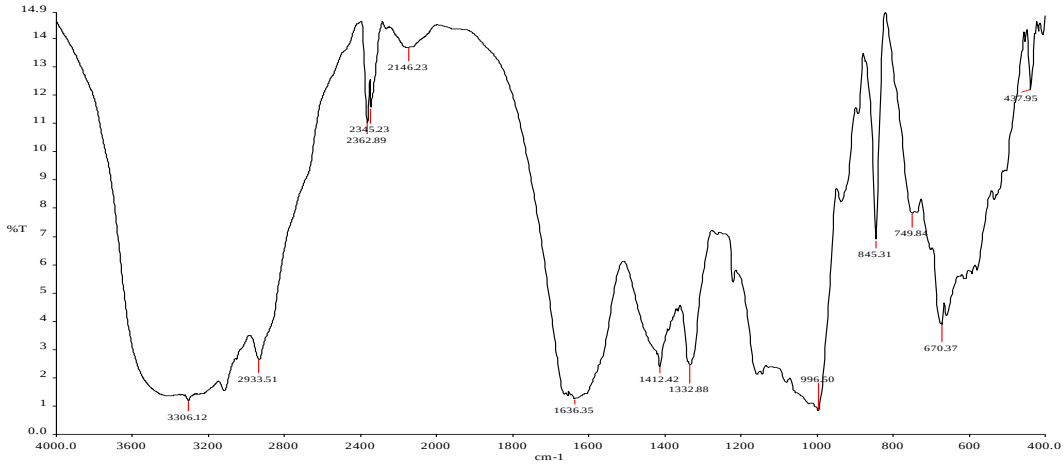

Figure 8. FTIR spectrum of the physical mixture of isoniazid and sodium alginate.

CONCLUSION:

The objective of the current investigation was the evaluation of the influence of sodium alginate and calcium chloride concentration on the characteristics of isoniazid loaded nanoparticles. The Isoniazid-loaded sodium alginate nanoformulations were evaluated for average particle size, encapsulation efficiency, drug loading, *in vitro* drug release and *FTIR* spectroscopy. Sodium alginate nanoparticles with properties like biodegradability, biocompatibility, more stability, low toxicity, convenient and simple preparation technique, offer an important and valuable tool for the administration of isoniazid through novel drug delivery systems. The influence of sodium alginate and calcium chloride concentration on loading capacity, encapsulation efficiency and particle size was evaluated by optimization. The observed parameters for formulation F-8 (optimized) were found significant as compared to software predicted values given by the Design-Expert®. The *in vitro* drug release for the formulation F-8 was 66.56% in 24 hours. Thus sodium alginate and calcium chloride have a significant influence on the characteristics of isoniazid loaded nanoparticle. The development of this isoniazid loaded sodium alginate nanoformulation has the potential to provide enhanced efficacy of isoniazid delivery.

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RESEARCH ARTICLE

Role of sToll-like receptors 2 and 4 in stage 2 periodontitis patients with and without type 2 diabetes: A Randomized clinical control trial

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ABSTRACT:

Objective: The role of TLRs as principal signaling receptors in recognizing endotoxins on gram positive and negative bacteria is facilitated by TLRs and further enhances its role as a potential biomarker in assessing periodontal disease. The study aimed at evaluating the expression of sTLR2 and sTLR4 among healthy, periodontal and diabetic patients and further if there a comparable expression among the TLRs among genders. **Material and Methods:** Patients were selected according to pocket depth, Clinical attachment loss and radiographic bone loss. Unstimulated whole saliva was collected. sTLR2 and sTLR4 quantification was estimated by ELISA. Comparison of sTLRs between the study groups were performed using ANOVA followed by Tukey post Hoc test. Independent sample t test was used to compare between the genders. ($p \leq 0.05$) **Result:** Expression of sTLR2 was higher among periodontitis patients compared to diabetic patients, this was also evident with relation to genders. sTLR4 showed significant expression among the three groups and also among the genders. **Conclusion:** The expression of sTLR2 was higher among periodontitis patients compared to diabetic patients. More studies need to be carried out to evaluate TLRs values among genders.

KEYWORDS: Toll-like receptors; sTLR2; sTLR4; Type 2 Diabetes Mellitus; Periodontitis.

INTRODUCTION:

Periodontal disease is a combination of multifactorial entities such as host immune response, bacterial virulence factors and causative factors that are directly and indirectly related to the causation of dysbiosis in the periodontal vasculature[1]. Impairment of homeostatic balance contributes to an accelerated inflammatory activity and responsible for dysbiosis of the biofilm complex[2,3]. Invading pathogens are neutralized by Innate Immunity which contributes to the first line of defense in the host, this is achieved by selective recognition of specialized receptors termed pathogen associated molecular patterns (PAMPs), the detection of agents

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contributing to infection is achieved by Toll-like receptors (TLRs), whose role is critical against invading pathogens[4].

The TLRs belong to a broad family of receptors categorized as pattern recognition receptors (PRRs). Cells of the innate immune system such as neutrophils, monocytes contain TLRs on their surface. Bacteria responsible for initiating inflammatory activity express PAMPs are localized by TLRs[5]. Upon recognition of a specific pathogen, specific member of the TLR family, upon activation initiates production of cytokine and adaptive immune response[6]. Currently, thirteen TLRs and their ligand have been identified[7,8,9]. The TLRs are comprised of both membrane and soluble form, with the soluble form present in tears, saliva, serum and urine. The soluble TLRs (sTLRs) have inhibitory action with relation to membrane associated TLRs and are hypothesized to have negative regulatory function[10]. Among the TLRs family, the two TLRs that have been identified as principal signaling receptors are TLR2 and TLR4. Recognition of PAMPs, lipoproteins and peptidoglycans present on gram positive and negative bacteria is achieved by TLR2[11]. Endotoxin such as lipopolysaccharide (LPS) and gingipains present on gram negative bacteria are recognized by TLR4[12].

It has been conceptualized that Porphyromonas gingivalis also referred to as the key stone pathogen[13,14], orchestrates polymicrobial synergy and dysbiosis in periodontal disease inflammation[15]. The transition from a symbiotic microflora to a predominantly gram negative dysbiotic flora is a synchronized step that features expression of select proteins and gene expression key to inflammatory and resorptive activity on the host cells, characteristic of any pathogenic resident microflora. Microbial related stimulation results in gene expressions that have different TLR-mediated pathways[3-17]. This further elucidates the fact that in periodontal tissue, the presence of TLRs, particularly TLR2 in connective tissue has been recorded. There is also presence of TLR4 and TLR9 in gingival epithelial cells and fibroblasts present in gingival and periodontal ligament cells[15,18,19]. which determines the progression of periodontal disease in inflammatory conditions such as rheumatoid arthritis and diabetes.

Metabolic alterations that contribute to cardiovascular disease predominates with the presence of Diabetes Mellitus Type 2 which predominantly is responsible for insulin resistance, and inflammation[20]. Most of the population who have Type 2 Diabetes Mellitus (T2DM), have increased prevalence of cardiovascular conditions[21]. The mortality rate increases with the uncontrolled progression of the condition[22]. One of the root causes for patients with T2DM is the presence of chronic hyperglycemia. This is mainly due to the inadequate release of Insulin coupled with insulin resistance[23]. The increased modification of biochemical reactions together with protein and enzyme changes is associated with the presence of advanced glycation end products (AGEs) which contributes to the changes in tissue proteins further leading to diabetic complications[24]. Periodontitis is considered one of the sixth complication of Diabetes which also includes microangiopathy, neuropathy, nephropathy, and delayed wound healing and macrovascular disease[25].

In Diabetic conditions more so with relation to T2DM, TLRs play a detrimental role in relation to insulin resistance, chronic hyperglycemia and contributes to atherosclerotic plaque buildup[20,26]. Heat shock proteins 60,70, (HSP60,70), endotoxins, high mobility group B 1 protein (HMGB1P), AGEs and extracellular components are specific ligands present in TLR2 and TLR4 that bind to both gram positive and negative pathogens[27,28]. The presence of both TLR2 and TLR4 in increased concentration in skeletal muscle tissue and adipose tissue of T2DM patients where it is characteristic for insulin resistance has been seen[27-30]. Inflammatory pathways in systemic conditions plays a critical role in the comorbidities and etiological factors in T2D. Various animal studies have recently implicated the role of both TLR2 and TLR4 in cytokine production and consequently development of diabetes. The role of TLR4 in recent times has also been justified by its presence as an important bridge between innate immune system and free fatty acid Inflammation[31-33].

Though there are animal and human tissue data which suggest role of TLR2 and TLR4 in diabetic patients, especially T2DM[33]. The presence of inflammation in diabetic patients due to alteration in TLR2 and TLR4 pathways need to be further understood. Periodontopathic bacteria is acted on by sTLR2 and sTLR4 initiated by oral epithelial cells. Apart from membrane bound receptors of TLR2 and TLR4, soluble forms of TLR2 and TLR4, (sTLR-2 and sTLR-4) play a detrimental role in binding to microbial receptors. The purpose of our study is to determine whether there is a comparable difference between sTLR2 and sTLR4 values among male and female cohorts as no previous study has reported this variation and if any notable expression among sTLR2, 4 values among healthy, periodontitis and diabetic cohorts is seen.

MATERIALS AND METHODS:

This interventional study was a randomized clinical trial. The duration of the study was 8 months, from May 2019 to December 2019. Informed consent was obtained from all the subjects, the study was approved by the Deanship of graduate studies and research ethical committee,

Ajman University (Ref no- D-F-19-03-03), and it is also registered in Clinical Trials. gov PRS, ID no- NCT04201912. The research manuscript follows consolidated standards of reporting trials (CONSORT) guidelines as well as the Helsinki declaration for human research as revised in 2013.

Study Population:
The study population involved healthy, periodontal patients without diabetes and diabetic patients with periodontitis with an age group of 20-60, healthy (mean 35.467), periodontitis (36.893) and Diabetic with periodontitis (38.234). The assessment for grading and staging of periodontal diseases was done according to the criteria set by Tonetti et al[34].

Selection criteria for healthy subjects:
Inclusion Criteria-40 individual (n-20 male, n-20 female) with no clinical evidence of gingivitis or periodontitis were selected. Further patients with no family history of chronic systemic disease or diabetes and fasting blood glucose less than 100mg/dl were included in the study.

Exclusion criteria-Smokers, pregnant women, patients on antioxidants in the past three months or consumption of omega-3 fatty acid capsules were excluded from the study.

Patients with Periodontitis:
The study included 40 individuals (n-20 male, n-20 female) having clinical features of generalized moderate chronic periodontitis (CP) stage 2, (Clinical attachment loss CAL 3-4mm, probing depth of ≤ 5mm, it was validated by radiographic evidence of horizontal bone loss, and graded by calculating percentage of bone loss divided by age, 0.25-1 was categorized as grade B), the patients are systemically healthy and have no habits such as smoking, betel nut chewing. Patients who have undergone surgery in the past three months or under medication for any infection were also excluded from the study

Type 2 diabetes mellitus patients with Periodontitis:
Inclusion criteria for selection of T2DM patients with periodontitis clinically and radiographically were done using the same protocol as for patients with periodontitis without systemic condition, but inclusive factors were more elaborate considering medical factors. The study included 40 individuals (n-20 female, n-20 male) with controlled diabetes, HbA1C between 7-8%. Patients with thiazolidinedione, ACE inhibitors, angiotensin receptor blockers, insulin or statin were excluded from the study as they interfere with TLR expression.

Saliva collection:
Unstimulated whole saliva (UWS) was collected from the participating subjects according to procedure by prakasam et al[35]. All the subjects were asked not eat or drink 1 hour prior to saliva collection. The UWS was collected by asking the subjects to tilt the head gently so as to initiate passive release of saliva. The position was maintained for a period of 15 minutes, whereby the saliva was collected in 15mL Eppendorf tube (Thomas Scientific, Swedesboro, NJ, USA). The samples were sent immediately to the laboratory in an ice box. The samples were centrifuged at 2000-3000rpm for 20 min to collect the supernatant.

Quantification of sTLR2 by ELISA:
ELISA for identification of sTLR2 is a double sandwich one-step assay. The assay range for identification employed was between 0.78ng/ml- 50ng/ml. A standard, test sample and HRP (Horse radish peroxide) labeled antibody were placed to enzyme wells which were pre-coated with TLR2 antibody, the incubation was carried out for a period of 1 hr and the uncombined conjugate was removed. 50ul of chromogen solution was added and incubated for 10min at 37 ^{0}C, the color will change to blue, and further the reaction with acid (stop solution) causes the color to become yellow. The depth of the color and concentration was determined for a positive significance with relation to sTLR2.

The final measurement was carried out by setting the OD (Optical density) at 450nm wavelength and performed 15 min after adding the stop solution.

Quantification of sTLR4 by ELISA:
Identification for sTLR4 was also performed using a double sandwich enzyme linked immunosorbent one step assay (ELISA). The assay range was at 6.25ng/ml-200ng/ml. Similar to sTLR2, a standard, test and HRP labeled antibody wells were added to wells pre-coated with sTLR4 antibody, the incubation was carried out for a period of 1 hr at 37°C. 50ul of chromogen solution was added and incubated for a period of 10 min at 37°C. The color initially change to blue and then to yellow by adding the stop solution. Positive significance was determined for sTLR4 using depth and concentration.

The final measurement was carried out by setting the OD (Optical density) at 450nm wavelength and performed 15 min after adding the stop solution.

Statistical method:
Sample size was calculated keeping the confidence level at 95%, with a confidence interval at 1%. The population prevalence was assumed at 120 for a period of eight months. The sample size obtained was 119 which was rounded up significantly to 120. The data collected were entered into Microsoft excel spreadsheet and analyzed using IBM SPSS Statistics, Version 22(Armonk, NY: IBM Corp). Descriptive data were presented in the form of mean and standard deviation. Comparison of sTLR2

and sTLR4 between the study groups were performed using ANOVA followed by Tukey post Hoc Test. Independent sample t test was used to compare sTLR2 and sTLR4 between the genders. P value < 0.05 was considered as statistically significant.

RESULTS:

Comparison of sTLR2 and sTLR4 between the study groups:

sTLR2 levels were significantly higher in Diabetic (21.47±5.26) ng/mL and Non-Diabetic (23.32±3.73) ng/mL group as compared to Healthy subjects. (Table 1) There was no difference in TLR2 levels between the Diabetic and Non-Diabetic group.

Level of sTLR2 showed statistical significance between the three groups (p<0.001) with an interesting observation seen of the mean with relation to Periodontitis patients (23.32±3.73) ng/mL compared to diabetic patient (21.47±5.26) ng/mL. (Table 1, Figure 1) This could be attributed to TLR ligation, which is a trait normally seen in diabetic patients. There was significant expression of sTLR2 values between healthy and diabetic cohorts.

sTLR4 levels were significantly higher in Diabetic (96.75±10.76) ng/mL group followed by Non-Diabetic (73.62±15.54) ng/mL group and least in Healthy subjects (64.37±9.16) ng/mL. (Table 1, Figure 1)

sTLR4 showed a significant increase between the three groups with means significantly higher comparing diabetic (96.75±10.76) ng/mL and control cohorts (64.37±9.16) ng/mL. Means of cohorts in the periodontitis group (73.62±15.54) ng/mL also showed significant expression with diabetic patients. (Table 1)

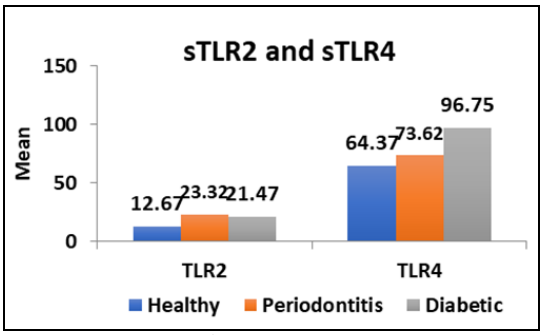

Figure 1: Comparison of sTLR2 and sTLR4 among healthy, periodontitis and diabetic patients

Comparison of TLRs between study groups among gender:

sTLR2 among the male cohorts demonstrated significant expression among the three groups, with periodontitis patients exhibiting a mean (21.45±3.15) ng/mL higher than diabetic cohorts (19.07±4.19) ng/mL. (Table 2, Figure 2, 3) sTLR4 levels between the three groups in males showed significant expression with each other. There was significant variation between the TLRs among the male cohorts.

Among the three groups in the female cohorts, there was significant relation between the three groups with diabetic patients exhibiting a mean (23.87±5.20) ng/mL lower than periodontitis cohorts (25.20±3.35) ng/mL similar to males. (Table 2, Figure 2, 3)

Table 1: Comparison of TLR2 and TLR4 between the study groups

	Study groups	N	Mean	SD	Min	Max	ANOVA	
							F	p-value
TLR2	**Healthy**	40	12.67	3.49	5.65	19.35	72.34	<0.001*
	Periodontitis	40	23.32	3.73	15.81	31.23		
	Diabetic	40	21.47	5.26	10.29	32.34		
TLR4	**Healthy**	40	64.37	9.16	46.65	89.84	75.65	<0.001*
	Periodontitis	40	73.62	15.54	30.79	97.95		
	Diabetic	40	96.75	10.76	66.84	131.31		

*p<0.005 Statistically Significant, p>0.05 Non-Significant, NS

Table 2: Comparison of TLR2 and TLR4 between the study groups in each gender

Gender		Study groups	N	Mean	SD	Min	Max	ANOVA	
								F	p-value
Male	TLR2	Healthy	20	11.33	3.30	5.65	16.65	43.84	<0.001*
		Periodontitis	20	21.45	3.15	15.81	27.75		
		Diabetic	20	19.07	4.19	10.29	28.93		
	TLR4	Healthy	20	59.88	5.80	46.65	69.35	32.02	<0.001*
		Periodontitis	20	70.97	18.55	30.79	97.95		
		Diabetic	20	93.66	13.34	66.84	131.31		
Female	TLR2	Healthy	20	14.02	3.20	8.75	19.35	46.09	<0.001*
		Periodontitis	20	25.20	3.35	18.50	31.23		
		Diabetic	20	23.87	5.20	12.21	32.34		
	TLR4	Healthy	20	68.86	9.81	53.45	89.84	57.58	<0.001*
		Periodontitis	20	76.28	11.70	45.56	96.54		
		Diabetic	20	99.83	6.28	86.66	115.05		

*p<0.005 Statistically Significant, p>0.05 Non-Significant, NS

There was statistically significant value among the three groups with relation to sTLR4. Comparing sTLR2 and sTLR4 between each other, there was significant relation.

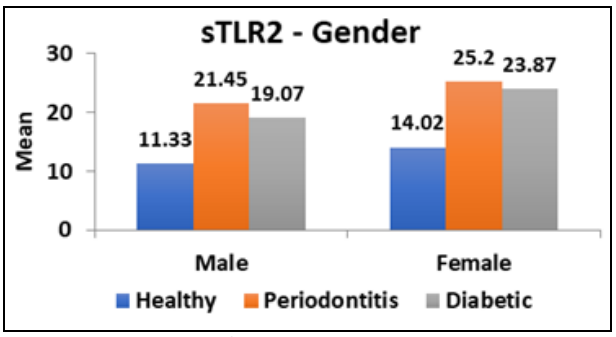

Figure 2: Comparison of sTLR2 among Male and Female cohorts with healthy, periodontitis and diabetic patients

Evaluating TLRs between genders among study groups:

In the healthy cohorts in males, there was statistical significant relation to both sTLR2, with the healthy cohort exhibiting (11.33±3.30) ng/mL, periodontitis patients with (21.45±3.15) and diabetic patients having (19.07±4.19) and sTLR4, healthy cohort (59..88±5.80)ng/ml, periodontitis patients (70.97±18.55)ng/ml and diabetic patients with (93.66±13.34)ng/mL. (Table 2, Figure 3, 4) Among the females, sTLR2 levels among the healthy cohorts (14.02±3.02)ng/mL, periodontitis patients (25.20±3.35) and diabetic patients with (23.87±5.20)ng/mL, (Figure 3) with sTLR4 levels showing healthy with (68.86±9.81)ng/mL, periodontitis patients with (76.28±11.70)ng/mL and diabetic patients with (99.83±6.28)ng/mL. (Table 2, Figure 4, 5) sTLR2 levels in both diabetic and periodontitis cases, there was marked significance among both male and female cohorts. With relation to sTLR4, the values were non-significant among male and females in both periodontitis and diabetic patients.

Comparing sTLR2 and sTLR4 levels among healthy cohorts, there was significant expression with sTLR2 showing ($p \leq 0.01$) and sTLR4 showing ($p \leq 0.001$).(Table 3) Among the diabetic patients, sTLR2 showed a significant expression of ($p \leq 0.001$), but sTLR4 expressing a non-significant value ($p \geq 0.29$). In the periodontitis category, sTLR2 showed a significant expression of ($p \leq 0.003$) and sTLR4 showing a ($p \geq 0.07$) non-significant value. (Table 3)

Table 3: Comparison of TLR2 and TLR4 between the genders in each study group

Study groups		Gender	N	Mean	SD	Mean Difference	95% Confidence Interval of the Difference		t	df	p-value
							Lower	Upper			
Healthy	**TLR2**	**Male**	20	11.33	3.30	-2.69	-4.78	-0.61	-2.62	38	0.01*
		Female	20	14.02	3.20						
	TLR4	**Male**	20	59.88	5.80	-8.98	-14.14	-3.82	-3.53	38	0.001*
		Female	20	68.86	9.81						
Diabetic	**TLR2**	**Male**	20	21.45	3.15	-3.75	-5.83	-1.67	-3.65	38	0.001*
		Female	20	25.20	3.35						
	TLR4	**Male**	20	70.97	18.55	-5.31	-15.24	4.61	-1.08	38	0.29(NS)
		Female	20	76.28	11.70						
Periodontitis	**TLR2**	**Male**	20	19.07	4.19	-4.80	-7.82	-1.78	-3.21	38	0.003*
		Female	20	23.87	5.20						
	TLR4	**Male**	20	93.66	13.34	-6.17	-12.84	0.51	-1.87	38	0.07(NS)
		Female	20	99.83	6.28						

*p<0.005 Statistically Significant, p>0.05 Non-Significant, NS

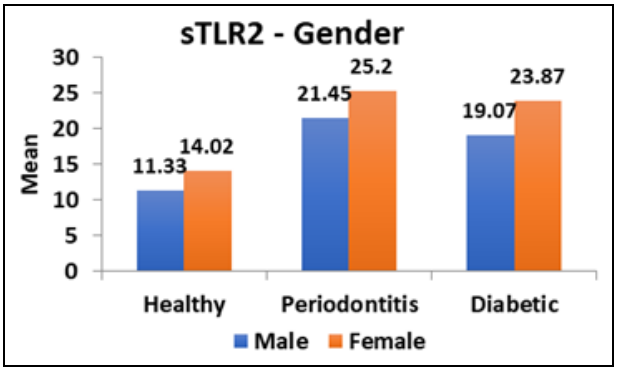

Figure 3: sTLR2 among Male and Female cohorts with healthy, periodontitis and diabetes patients

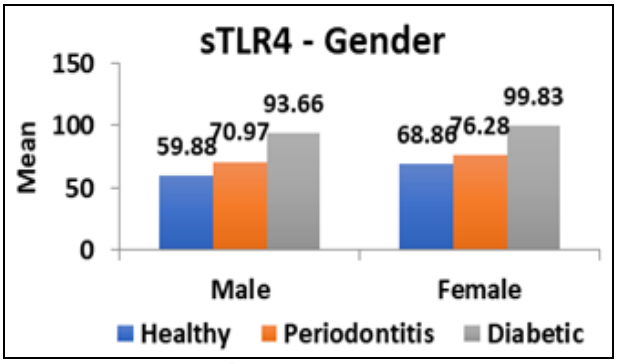

Figure 4: Comparison of sTLR4 among Male and Female cohorts with healthy, periodontitis and diabetic patients

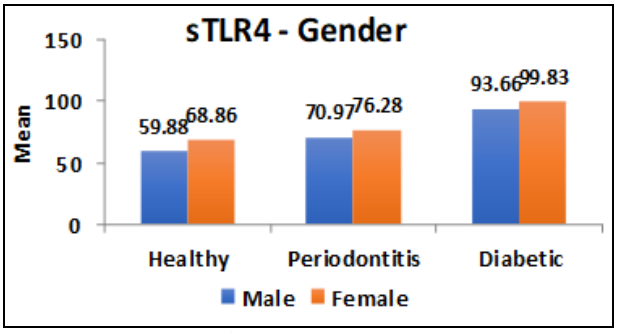

Figure 5: Comparison of sTLR4 among Male and Female cohorts with healthy, periodontitis and diabetic patients

DISCUSSION:

Periodontal inflammation, proliferation of cell family and innate immunity initiation are detrimental roles played by TLRs[36,37,38]. Bone metabolism and periodontal disease inflammation progression is believed to be orchestrated by sTLR2 and sTLR4[39,40]. However, little is known about sTLR2 and TLR4 variation among gender. In the present study we evaluated whether there are significant differences among control, diabetic and periodontitis cohorts and among this population if there is any comparable distribution of sTLR2 and sTLR4 levels among genders.

Saliva was used to measure both sTLR2 and sTLR4 owing to the probability that both these TLRs function as potential deceptors for PAMPs. Elevated TLR2 levels has also been confirmed by immunofluorescent studies in periodontitis cases[41]. In our study there was significant expression of sTLR2 levels among healthy, periodontitis and diabetic cohorts. Contrary to some studies which was carried out among gingivitis patients, they reported a negative expression related to TLR2[41,42]. In our study there was a positive expression in TLR 2 levels when comparing periodontitis cohorts (23.32±3.73) with diabetic patients (21.47±5.26). This could be attributed to the elevation of pathogen associated molecular pattern in the saliva of periodontitis patients (PAMPs), which is in agreement with a study by Lappin et al[43].

The probability of sCD14 in saliva influencing elevated production of sTLR2 in periodontitis patients is also a possibility and has been seen in various periodontal conditions[44,45]. Saliva was used to measure both sTLR2 and sTLR4 owing to the probability that both these TLRs function as potential deceptors for PAMPs. Elevated TLR2 levels has also been confirmed by immunofluorescent studies in periodontitis cases[46]. In our study T2DM (Type 2 diabetes mellitus) cohorts selected were controlled and the potential for an active periodontal disease progression was limited as validated by other studies[47,48]. This could also be the reason for a reduced sTLR2 expression among diabetic patients in the presence of an inflammatory condition[49]. The presence of elevated sTLR2 and sTLR4 levels in T2DM patients was elaborated by ex vivo studies done which involved gingival tissues from periodontitis patients[50-52]. This further demonstrates the down signaling pathway of TLRs in an active inflammatory condition. Significant results for sTLR2 were demonstrated in our study while evaluating periodontitis cohorts with diabetic patients.

sTLR4 levels showed significant increase in expression from health to diabetes. The striking observation seen was the increase of sTLR4 in diabetic patients, though the HbA1C (glycated haemoglobin) levels were controlled for the period of the study. This could probably be due to an increased level of circulating endotoxins which contributes to a proinflammatory burden and spirals signaling pathways downwards[53]. The presence of HSP (Heat Shock Protein) also contributes to the elevation in sTLR4 levels in T2DM by activating proinflammatory cytokines[54,55]. Though some of the studies advocate the increased presence of HMGB1 (High Mobility Group Box 1) leading to increased presence of sTLR4, these studies have evaluated T1DM. In the present study, there could be a possibility of high titer of sTLR4 in T2DM diabetic patients as a result of an altered metabolism or due to a metabolic stressor contributing towards a pro inflammatory environment. It could also be due to nature of the salivary sample, quality, and disease status at the time of collection and comparable differences in the specificity and sensitivity of the antibodies added to the kit wells. Though majority of the patients having DM (Diabetes Mellitus) fall into the Type 2 category, it is still categorized as a proinflammatory state like T1DM. In a study done by Mythily et al where sTLR4 levels were elevated in gingivitis cases when compared to periodontitis cases, further corroborating the pro inflammatory status of T2DM[43]. The hyper responsiveness of TLR4 in high glucose states in presence of microbial endotoxins like LPS (Lipopolysaccharides) also is a cause for elevated levels.

The literature regarding comparison of TLRs among genders with relation to periodontitis and diabetic cohorts is scarce. Maria et al recently reported no difference in TLRs profiles when comparing women with PCOS and control women. In the same study Fasting sTLR2 expression in neutrophils was higher in men than women[56]. Contrary to this, in our study there was a positive correlation with sTLR2 expression, with values more in women when compared to males in both T2DM ($p<0.001$) and periodontitis cohorts ($p< 0.003$). With relation to sTLR4, there was a negative correlation with relation to both T2DM and periodontitis patients among female cohorts. From our study, we could presume that the elevated expression of sTLR2 in periodontitis was due to the presence of pro inflammatory activity working in a preliminary stage.

We could also see that sTLR4 expression was influenced by the presence of glucose[56]. sTLR2, sTLR4 levels showed significant difference when comparing T2DM and periodontitis cohorts with controls among both men and women. Reduced sTLR4 expression among both male and female represents a redemptive process, which is characteristic of LPS (Lipopolysaccharide) a characteristic endotoxin released by gram negative bacteria *Aggregatibacter actinomycetemcomitans* which is an auto shut mechanism seen in individuals already prone for further host bacterial inflammatory insults. This is characteristically seen in relation to sTLR2 and not TLR4[57-59]. This could be one of the reason for a lack of congruence in our study comparing sTLRs results between healthy, T2DM and periodontitis cohorts. In addition sTLRs expression has been influenced by periodontal inflammation, dysbiosis due to host-bacterial interaction and sex hormones[60,61,62]. Further it can be assumed that probably fasting among the cohorts could also have led to discrepancy in sTLR2 results in both men and women where in sTLR2 values greatly increased at the time of salivary sample delivery. In our study men presented with reduced sTLR4 expression when compared to females, probably owing to an inhibitory effect of testosterone on sTLR4 which was also in agreement with some studies[56,63]. Contrary to a study by Jitprasertwong et al where men had higher expression of sTLR2 compared to females and which indicated higher sTLR4 levels upon a pro inflammatory condition and reduced sTLR2 expression on estrogen influence[64]. In our study the levels of sTLR2 in males were lower compared to females in both T2DM and periodontitis cohorts. Further studies evaluating the role of TLRs during menstrual cycle have also determined the presence of higher titers of sTLR2/sTLR4 ratio in the follicular phase compared to the luteal phase[65,66]. This disparity in values of TLRs among women could be due to the presence of ectodomains created by splicing and proteolytic bifurcation[67]. Further, the presence of lower sTLR2 has been seen in chronic diseases such as URTI(Upper respiratory tract infection) and more specifically colitis[68,69]. A positive correlation of sTLR2 in periodontitis patients in both male and female cohorts and a direct correlation of sTLR4 in T2DM patients among both male and female cohorts suggest that both could function as a viable diagnostic marker.

The limitations in our study is given the complexity of our study design, we feel the sample size for each groups were relatively small to come to a conclusive hypothesis and probably enervated to detect changes within the groups. In our opinion, these limitations were compensated by the presence of a robust healthy cohorts, periodontitis patients and controlled T2DM patients. The results gathered from our study will open questions for the discrepancy in values found among male and female cohorts and the reasons which could have led to these changes.

CONCLUSION:

We observed an increase in sTLR2 levels in periodontitis patients when compared to diabetic cohorts among both male and female samples. There was positive correlation in both sTLR2 and sTLR4 levels when comparing female with male patients. Increase in sTLR4 levels were possibly due to relationship of TLR4 with endotoxin released by gram negative bacteria that are among the predominant microflora in both periodontal and diabetic patients. These values could possibly suggest a transient discrepancy in actual readings or could be a normal immunologic response to prevent an increased inflammatory load, which was modulated by T2DM. Further investigations need to be carried out to rule out possible mechanisms in this disparity. Studies involving different parameters for female cohorts need to be designed to bring a possible explanation to these changes.

Data Availability:

The data used to support the findings of this study are included in the article.

CONFLICTS OF INTEREST:

The authors declare that they have no conflicts of interest.

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RESEARCH ARTICLE

Assessment of Anticonvulsant properties of Caloric vestibular stimulation in Pilocarpine-induced status epilepticus mice model

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ABSTRACT:

Objective: The present study was undertaken to assess the anticonvulsant properties of caloric vestibular stimulation in mice pilocarpine model of epilepsy. **Materials and methods:** 24 healthy, adult male Swiss albino mice with body weight ranging between 25 - 40g were used in the study. Animals were assigned into four groups by simple random sampling that is control, Status epilepticus mice, and hot water and cold water caloric vestibular stimulation groups. Bilateral hot and cold water vestibular stimulation was given to the mice as an intervention for 30 days. On 31st day status epilepticus mice model was induced by administration of i.p. Injections of pilocarpine hydrochloride (300 mg/kg, i.p.). Occurrence of seizures that is number of the rats in which seizures occurred, seizure latency in seconds, and duration of the clonic phase of the seizure in seconds was recorded manually. Assessment of neurotransmitters like acetyl choline and glutamate was performed. **Results:** Caloric vestibular stimulation showed positive impact on seizure occurrence, latency and duration. Epilepsy induced changes in acetylcholine and glutamate was limited followed by the intervention. **Conclusion:** The results of the study showed beneficial effect of vestibular stimulation in mice pilocarpine model of epilepsy. The study recommends further detailed studies.

KEYWORDS: Anticonvulsant properties, Caloric vestibular stimulation, Epilepticus mice model.

INTRODUCTION:

The vestibular system is considered the gateway to the brain and is said to have the most important influence on everyday functioning. Several recent studies associate the function of vestibular system beyond maintaining balance, equilibrium and/or reflexes with advanced functions such as improving cognition, improving general health and reducing stress. As the vestibular system remains enigmatic among the human senses, it is fast becoming one of the most thoroughly and broadly investigated topic of this era[1]. It was reported that caloric vestibular stimulation does not accentuate the abnormal brain wave pattern in seizure-prone children [2]. The link between epilepsy and vertigo was well documented in the literature[3,4].

The pharmacotherapy for management of epilepsy leads to vertigo and nystagmus and even other vestibular disturbances like Meniere disease. Further, decrease in the dosage of the drugs reversed the condition[5]. Epileptic patient exhibited vide range of vestibular sensation followed by the cortical stimulation. Further, the responses varied based on the area of stimulation and intensity of the stimulation[6]. It was reported that vestibular stimulation produces beneficial effects when it is applied optimally. Over stimulation may leads to nausea, vomiting and other symptoms[7]. It was hypothesized that vestibular stimulation may be effective in limiting the effects of epilepsy. As a first step, the pilot study was undertaken to assess anticonvulsant properties of caloric vestibular stimulation in Pilocarpine-induced status epilepticus mice model.

MATERIALS AND METHODS:

Experimental animals:

24 healthy, adult male Swiss albino mice with body weight ranging between 25 - 40g were used in the study. The animals were housed in polypropylene cages, in the standard environmental conditions with food and water

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provided ad libitum. All efforts were made to minimize the number of animals used and their suffering.

Animals were assigned into four groups by simple random sampling.

Group 1 (n=6): Control group – Mice were administered intraperitoneal (i.p.) injections of normal saline (0.9%) per day. (Neither i.p. Injections of pilocarpine hydrochloride (300mg/kg i.p.) nor caloric vestibular stimulation was administered)

Group 2 (n=6): Status epilepticus mice – No vestibular stimulation was given to the mice for 30 days + on 31st day administration of i.p. Injections of pilocarpine hydrochloride (300mg/kg, i.p.).

Group 3 (n=6): Hot water vestibular stimulation group- Bilateral hot water vestibular stimulation was given to the mice once in a day for 30 days + on 31st day administration of i.p. Injections of pilocarpine hydrochloride (300mg/kg, i.p.).

Group 4 (n=6): Cold water vestibular stimulation group- Bilateral cold water vestibular stimulation was given to the mice once in a day for 30 days + on 31st day administration of i.p. Injections of pilocarpine hydrochloride (300mg/kg, i.p.).

Pilocarpine-induced status epilepticus mice model:
Pilocarpine-induced status epilepticus mice model was prepared by administering the intra peritoneal injection of pilocarpine hydrochloride (300mg/kg, i.p.). To reduce the side effects, the mice were administered atropine methyl nitrate (2mg/kg, i.p.) and terbutaline hemisulfate salt (2mg/kg, i.p.) 30 min before the injection of pilocarpine hydrochloride. The Racine scale was used to determine the stages of seizure (Jeong KH et al., 2013) [8].

Caloric vestibular stimulation:
Caloric vestibular stimulation was administered by irrigating the middle ear cavity with hot water with a temperature of 45°C with the help of syringe for 30 days and 60 days[9]. 2ml of hot water is taken in a 2ml syringe. Hold the rat in supine position in the hands of the helper and tilt the head slightly. After titling the head, slowly push the hot water from the syringe steadily at a constant rate for 2 minutes. In the present study, bilateral vestibular stimulation was used as an intervention. Caloric water caloric vestibular stimulation started giving between 9am-12 every day for 30 days.

Outcome measures:
Seizure:
Occurrence of seizures that is number of the rats in which seizures occurred, seizure latency in seconds, and duration of the clonic phase of the seizure in seconds was recorded manually.

Neurotransmitter analysis:
Acetyl choline and glutamate were measured by spectrophotometry. The animals were sacrificed after treatment by euthanasia. The whole brain was removed immediately and placed in ice-cold saline. The tissues weighing 0.5g were homogenized with motor driven Teflon coated homogenizer with 5mL of ice-cold 0.1 M phosphate buffer pH 8.0 to get 10% homogenate. The homogenate was centrifuged at 10,000rpm for 20 min at 5C. The supernatant was collected and used for the ex vivo neurotransmitter estimation.

Acetyl choline assay:
Acetylcholine content in all the tissues was estimated by Sectrophotometry (Spectrophotometer -Shimadzu-model 1800) and based on the method of Hestrin (1949) [10]. After isolating and weighing, the tissues were transferred to the tubes and kept it in the boiling water bath for 10 minutes inner to inactivate the Acetyl choline esterase enzyme and to release the bound Ach. The tubes were then cooled and the contents were homogenized in 2ml of distilled water. 2ml of Alkaline hydroxylamine reagent is prepared freshly by mixing equal amount of hydroxylamine hydrochloride and sodium hydroxide (stored in room temperature for 3 hours). 1ml of homogenized brain sample is added to 2ml of Alkaline hydroxylamine reagent. After one minute, added 1ml of conc. HCL in order to bring back the pH into 1.2+0.2. The content was centrifuged and then 1ml of ferric chloride is added. The intensity of brown colour developed (Ferric-Acethydroxamic acid complex) which was measured at 540nm in spectrophotometer against blank. The blank consists of 2ml of alkaline hydroxylamine hydrochloride,1ml diluted Hcl and 1ml of ferric chloride. Ach values were expressed as mole/minute/mg tissue.

Glutamate:
The level of Glutamate was estimated by spectroflurometry (spectroflurimeter - JASCO MODEL FP 6200)[11]. At the end of experiment, rats were sacrificed and the whole brain was dissected out. 0.25g of tissue was weighed and was homogenized in 5mL HCl–butanol with motor driven Teflon coated homogenizer for about 1 min. The sample was then centrifuged for 10 min at 2000rpm. An aliquot supernatant phase (1mL) was removed and added to centrifuge tube containing heptane (2.5mL) and 0.1 M HCl (0.31mL). After 10 min of vigorous shaking, the

tube was centrifuged under the same conditions in order to separate the two phases, and the overlaying organic phase was discarded. The aqueous phase was then taken for glutamate assay.

Study setting:
The present study was conducted at Little Flower Medical Research Centre, Angamaly, Kerala, India.

Ethical consideration:
The institutional animal ethical committee approved the study of Little Flower Hospital and Research Centre, Angamaly, Kerala, India.

Data analysis:
Data was analyzed by using SPSS 20.0 version. One way ANOVA followed by Tukey HSD post hoc test was applied to observe the significance of the difference between the groups. A probability value of less than 0.05 was considered significant.

RESULTS:

Seizure occurrence was 6/6 in group 2, 5/6 in group 3 and 4/6 in group 4. There was significant increase in the seizure latency (sec) followed by the intervention in group 3 and 4. Increase in the seizure latency (sec) in the hot water vestibular stimulation (group 3) was statistically significant P<0.0001 when compared with group 2. Increase in the seizure latency (sec) in the cold water vestibular stimulation (group 4) was statistically significant P<0.0001 when compared with group 2. Increase in the seizure latency (sec) in the cold water vestibular stimulation (group 4) was statistically significant P<0.0001 when compared with hot water vestibular stimulation (group 3). There was a significant decrease in the seizure duration followed by both hot water and cold water vestibular stimulation (P<0.0001) when compared with group 2. There was a significant decrease (P<0.0001) in the seizure duration followed by cold water vestibular stimulation when compared with hot water vestibular stimulation. There was a significant increase (P<0.0001) in the acetylcholine levels in group 2 when compared with group 1. Pilocarpine induced changes in acetyl choline levels were not observed in both hot and cold water vestibular stimulation groups. There was a significant decrease (P<0.0001) in the acetylcholine levels in group 2 and group 4 when compared with group 1. Pilocarpine induced changes in glutamate levels was limited in hot water vestibular stimulation group.

Table 1: Seizure details and Acetylcholine and glutamate levels in the animals before and after the intervention

	Group 1 (n=6)	Group 2 (n=6)	Group 3 (n=6)	Group 4 (n=6)	F value
Seizure occurrence	-	6/6	5/6	4/6	
Seizure Latency (sec)	-	130±2.1	420±5.5	484±7.82	6685.31
Seizure duration (sec)	-	38±0.58	22±0.63	17±0.83	1522.99
ACH (mole/minute/mg tissue)	0.14±0.001	0.22±0.01	0.15±0.003	0.14±0.006	245.20
Glutamate (ng/g tissue)	0.42±0.01	0.32±0.02	0.41±0.01	0.22±0.01	298

(ACH- Acetyl choline)

DISCUSSION:

Epilepsy indicates excessive activity of either one particular area of brain or entire brain[12]. One of the critically affected areas of the brain due to epilepsy is the hippocampus where there is severe neuronal loss that leads to atrophy. Further, atrophied hippocampus triggers the development of epilepsy[13-15]. It was well reported that the theta activity of hippocampus was modulated followed by vestibular stimulation[16]. Interestingly, it was reported that normal functioning of hippocampus depends on vestibular inputs[17]. Another important area involved in the epilepsy is basal ganglia. The neurotransmitter dopamine released from the basal ganglia has a role in the seizures control[18]. Stimulating vestibular system was reported to modulate the secretion of dopamine[19]. Further, deregulation of the hypothalamic-pituitary-adrenal axis was reported in majority of epileptic cases[20,21]. Lesions of thalamus were commonly reported in the epileptic patients and they were also associated with atrophy of the limbic system[22]. Vestibular system was reported to have extensive connections with thalamic nuclei[23]. Further, vestibular system is well connected with the limbic structures and plays a key role in the regulation of emotions and behavior[24]. The pilot study was undertaken to assess anticonvulsant properties of caloric vestibular stimulation in Pilocarpine-induced status epilepticus mice model. Positive response was observed in the Pilocarpine-induced status epilepticus mice model followed by the vestibular stimulation.

CONCLUSION:

The results of the study showed beneficial effect of vestibular stimulation in mice pilocarpine model of epilepsy. The study recommends further detailed studies.

CONFLICTS OF INTEREST:

None declared.

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RESEARCH ARTICLE

Phytochemical Evaluation and in-vitro Antioxidant Potential of Whole Plant of *Hyptis suaveolens*

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ABSTRACT:

The present study was to investigate total phenol and flavonoid content, the antioxidant potential of various extracts of *Hyptis suaveolens* whole plant using various in vitro systems were quantified by colorimetric methods. The Chloroform extract exhibited potent antioxidant activity as determined by 2,2-diphenyl-1-picrylhydrazyl (DPPH), nitric oxide scavenging and ferric reducing antioxidant power assays (FRAP). The total phenolic content and flavonoid content of chloroform extract of plant was found to 86.16±0.877 and 64.66±1.201mg of GAE and Quercetin equivalents respectively.

KEYWORDS: *Hyptis suaveolens*, Antioxidant activity, DPPH, Nitric oxide scavenging activity, FRAP.

INTRODUCTION:

Naturally occurring bioactive molecules commonly found in vegetables, herbs, and other plants promotes various health benefits.[1] Antioxidants are mainly composed of phenolic acids, flavonoids, and catechins, that suppresses the lipid peroxidation, prevents DNA oxidative damage, and scavenge the reactive oxygen species (ROS), such as superoxide, hydrogen peroxide, and hydroxyl radicals.[2] ROS causes oxidative damage to cellular compartment, leads to cell injury and death. It leads to various chronic diseases like coronary heart disease, carcinogenesis, and other health problems related to advancing age.[3] An increased antioxidant intake in humans through diet can reduce such oxidative damage. The commercially available synthetic antioxidants are known to exhibit serious toxicity. Hence, efforts are made in search of natural, economical, and effective antioxidants.

Hyptis suaveolens (L.) Poit commonly known as ‘‘Wilaiti tulsi’’ belongs to the family Lamiaceae and is a common weed of roadsides and waste grounds, is an ethnobotanically important medicinal plant. The plant is distributed throughout the tropics and subtropics. All parts of this plant are being used in traditional medicine to treat various diseases. *Hyptis suaveolens* has both medicinal as well as insecticidal properties. Literature of *Hyptis* indicates that leaf extracts cure swellings, abscesses and haemorrhoids. In India the plant is considered to be stimulant, carminative, sudorific and lactogogue. Infusion is used in infections of the uterus; leaf juice is taken in cases of colic and stomach ache.[4] Based on the traditional knowledge and recent pharmacological studies, the objective of the present study was to investigate the chemical constituents and antioxidant potential of various extracts of H. suaveolens by various in vitro assays.

METHODS:

Collection of Plant material and extraction:

The whole plant of *H. suaveolens was* collected from the forests of Maisammaguda, Secunderabad situated in the state of Telangana (India) and shade dried and powdered mechanically. The plant specimen was authenticated by botanist of Osmania University and authenticated voucher specimen Number 276 of the plant has been

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preserved in department for future reference. The dried plant powder was extracted with various solvents based on polarity (Pet ether, Chloroform, Ethyl acetate, Methanol AND Aqueous) by hot continuous extraction in Soxhlet's apparatus and the extracts were evaporated to dryness under vacuum, dried in vacuum desiccators and stored in refrigerator.

Phytochemical Evaluation:
Phytochemical investigation of alkaloids, saponins, carbohydrates, tannins, phenolics, flavonoids, steroids and glycosides were carried out for various extracts of plant using standard protocols.

Total phenol content Estimation:
The total phenolic content of *H.suaveolens* was assessed using Foline Ciocalteau phenol reagent method described by Singleton et al.[5] Briefly, 1.0mL of the extract at various concentrations was mixed with 2.5mL of 10% Foline Ciocalteau reagent and 2.5mL of 7.5% sodium carbonate. The contents were thoroughly mixed and allowed to stand for 30 minutes. The absorbance was read at 750nm in a spectrophotometer. The total phenol content was expressed as gallic acid equivalents in milligram per gram of the extract.

Total Flavonoid estimation:
The flavonoid content of *H.suaveolens* was determined using aluminum chloride colorimetric method described by Chang et al.[6] Briefly, 0.5mL of the extract at various concentrations was mixed with 3mL of 95% methanol, 0.1mL of 10% (weight/volume) aluminum chloride, 0.1 mL of 1M potassium acetate, and 2.8mL of distilled water. The reaction mixture was allowed to stand at room temperature for 30 minutes and absorbance was measured at 415nm against a blank sample. A calibration curve was prepared using quercetin in methanol. The flavonoid content was expressed as quercetin equivalents in milligram per gram of the extract.

In-vitro Antioxidant activity:
DPPH antiradical capacity
The antiradical potential of *H.suaveolens* was determined spectrophotometrically as described by Ilahi et al.[7] Six different concentrations of various extracts of plant material (100, 200, 400, and 800 and 1000µg/ml) were mixed with 100µL of DPPH radical solution in a 96-well microplate and incubated for 20 min at room temperature. The resultant mixture was read spectrophotometrically at 517nm against a methanol blank and the following equation was used to calculate the % inhibition of each extract:

$$\% \text{ inhibition} = (A_{Control} - A_{Sample}) / (A_{Control}) \times 100$$

where $A_{Control}$ and A_{Sample} indicate the absorbance of the DPPH solution and the reaction mixture, respectively. The effective dose of plant extract needed to neutralize 50% of the DPPH radical solution (IC50) was obtained from a plot comparing percent inhibition to extract concentration.

Nitric oxide scavenging activity:
Nitric oxide radical scavenging activity was determined according to the method reported by Garrat.[8] Sodium nitro prusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions, which can be determined by the use of the Griess Illosvoy reaction. 2 ml of 10mM sodium nitroprusside in 0.5ml phosphate buffer saline (pH 7.4) was mixed with 0.5ml of extract at various concentrations and the mixture incubated at 25°C for 180 min. From the incubated mixture 0.5ml was taken out and added into 1.0ml sulfanilic acid reagent (33% in 20% glacial acetic acid) and incubated at room temperature for 5 min. finally, 1.0 ml naphthylethylenediaminedihydrochloride (0.1% w/v) was mixed and incubated at room temperature for 30 min, the absorbance at 540nm was measured with a spectrophotometer. The nitric oxide radicals scavenging activity was calculated.

Reducing power Determination:
The Fe^{2+} reducing power of plant extract was determined by the method of Oyaizu[9] with slight modification. Various concentrations of plant extract (0.75mL) was mixed with 0.75ml of phosphate buffer (0.2 mole, pH 6.6) and 0.75mL of potassium ferricyanide $K_3Fe(CN)_6$ (1%w/v), followed by incubating at 50°C for 20 mins. The reaction was stopped by adding 2.5mL of 10% (w/v) trichloroacetic acid followed by centrifugation at 3000 rpm for 10min. Finally, 1.5mL of the upper layer was mixed with 1.5mL of distilled water and 0.5mL of $FeCl_3$ (0.1%) and the absorbance was measured at 700nm. Higher the absorbance of reaction mixture indicated greater the reducing power. Ascorbic acid is used as reference compound.

RESULTS AND DISCUSSIONS:

Phytochemical Constituents:
Phytochemical screening was carried out for various extracts and revealed the presence of the following constituents shown in Table 1. '+' indicate the presence and '-' indicates the absence of phytoconstituents.

Table 1: Phytochemical Constituents in various extracts of *H. suaveolens*:

	Pet. Ether	Chloroform	Ethyl Acetate	Methanol	Aqueous
Alkaloids	+	+	+	+	+
Saponin	-	-	-	-	+
Glycosides	-	-	+	+	+
Carbohydrates	+	+	+	+	+
Tannins and Phenolics	+	+	+	+	+
Flavonoids	+	+	-	+	+
Steroids	+	+	_	_	_
Proteins and Amino acids	+	+	+	+	+

Total phenol content Estimation:

Phenolic compounds are the key phytochemicals with high free radical scavenging activity. It has generated a great interest among the scientists for the development of natural antioxidant compounds from plants. In the current work, phenolic content of the various extracts of *H. suaveolens* were measured and listed in (Table2). The Chloroform extract of *H.suaveolens* showed higher amount of phenolic compounds when compared to other extracts. The concentration of the phenolic compounds was increased with an increase in the dose. The results are described as Gallic acid equivalents (GAE) (Figure 1).

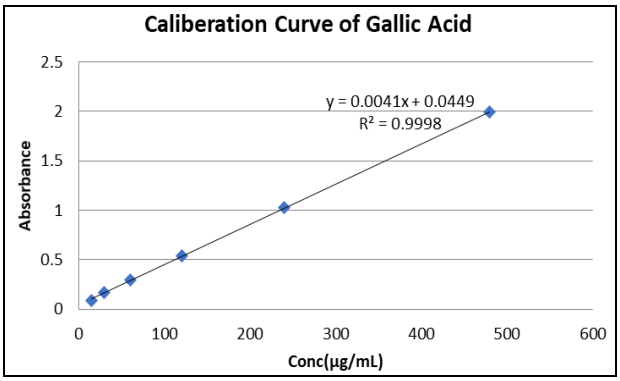

Figure 1. caliberation Curve of gallic acid.

Total Flavonoid estimation:

The flavonoid content of various extracts of *H.suaveolens* was determined using aluminum chloride colorimetric method. In the current study, the total flavonoid content were measured and listed in (Table 2). The total flavonoid content in Chloroform extract was found to be high when compared to other extracts. The flavonoid content was expressed as quercetin equivalents in milligram per gram of the extract (Figure 2).

Table 2: Total Phenolic and Total Flavonoid content in various extracts of *H.suaveolens*

S. No	Extract	Total Phenolic Content (Gallic acid equivalents in mg /gm extract)	Total Flavonoid Content (Quercetin equivalents in mg/ gm extract)
1	Pet Ether	17.66 ±0.381	11.22±0.838
2	Chloroform	86.16±0.877	64.66±1.201
3	Ethyl Acetate	30.66±0.629	--------
4	Methanol	69.33±0.144	21.44±0.509
5	Aqueous	76.08±0.629	34.22±1.170

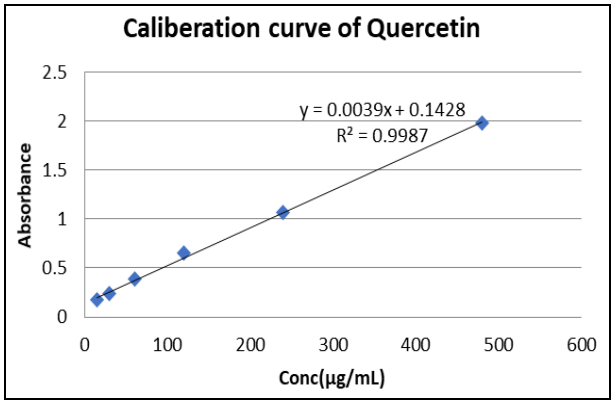

Figure 2: Calibration curve of Quercetin

DPPH antiradical capacity:

DPPH antioxidant assay is based on the ability of DPPH, a stable free radical, to decolorize in the presence of anti oxidants. The DPPH radical contains an odd electron which is responsible for the absorption at 517nm and also for visible deep purple colour. When DPPH accepts an Electron, donated by an antioxidant compound, results in decolorisation which can be quantitatively measured from changes in the absorbance. DPPH radical scavenging activity of various extracts of *H. suaveolens* in comparison with ascorbic acid are reported in (Figure 3). IC_{50} values for the various extracts were mentioned in (Table 3). Chloroform Extract showed better antiradical activity than the remained extracts.

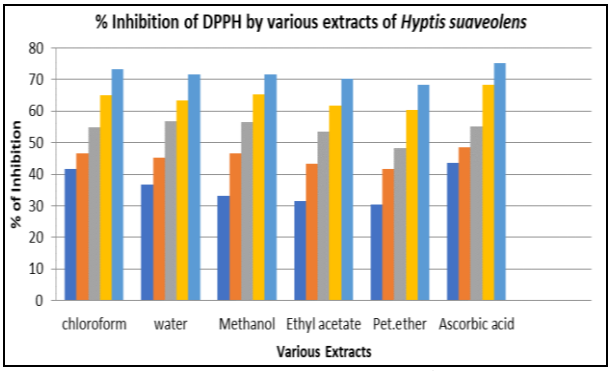

Figure 3: DPPH radical scavenging activity of various extracts of *H. suaveolens*

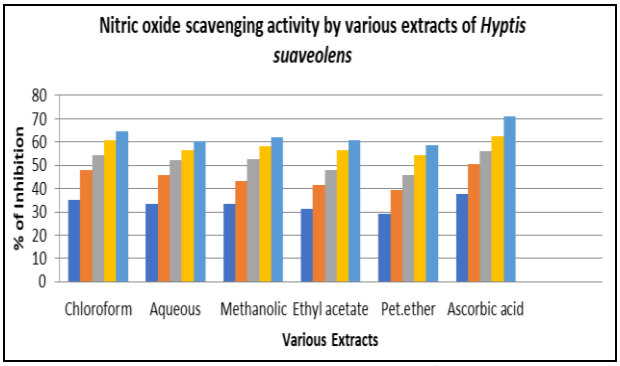

Figure 4: The nitric oxide scavenging activity of various extracts of *H. suaveolens*

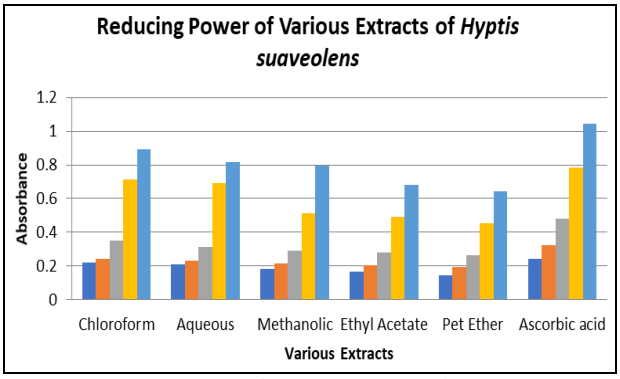

Figure 5: Reducing power of Various Extracts of *H. suaveolens*:

Nitric Oxide scavenging activity:

Nitric Oxide is a potent pleiotropic mediator of physiological processes playing vital role in various biological systems. The nitric oxide scavenging activity of various extracts of *H. suaveolens* in comparison with ascorbic acid are reported in (Figure 4). IC_{50} values for the various extracts were mentioned in (Table 3).

Table 3: IC_{50} values for the various extracts of *H. suaveolens*:

S. No	Extract	DPPH Method	Nitric oxide scavenging activity
1	Pet Ether	311.334 ±5.138	765.324±3.605
2	Chloroform	204.759±1.005	217.616±0.583
3	Ethyl Acetate	253.794±1.669	310.629±1.361
4	Methanol	223.743±0.521	254.182±3.246
5	Aqueous	218.456±0.635	237.152±0.536
6.	Ascorbic acid	194.99±1.258	193.420±2.099

Reducing Power:

Reducing power experiment is a good reflector of antioxidant activity of the plant. The plant having high reducing power generally reported to carry high antioxidant potential too. In this experiment, Ferric ions are reduced to ferrous ions which is identified by colour change from yellow to bluish green. The results for ferric reducing power activity of various extracts of *H.suaveolens* in comparison with ascorbic acid are reported in (Figure 5). Chloroform extract showed high reducing power than that of other extracts. Reducing power potential of extracts increase with the dose, however the extracts exhibited low reducing power than that of ascorbic acid.

CONCLUSION:

Plant source is considered to be the good source of natural antioxidants for curing the diseases. In the present study, it is revealed that the plant *H.suaveolens* is rich in phenolic and flavonoid content in it. Chloroform extract has shown better antioxidant profile when compared to the other extracts and less potent than the synthetic antioxidants like ascorbic acid and quercetin.

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RESEARCH ARTICLE

A Qualitative study on commercially available: Sleep Monitoring Systems and Sleep Analysis Tools

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ABSTRACT:

Background: A sound sleep is the key parameter for healthy life. The monitoring of such vital parameter is very complex as it is associated with electrical waves generated by brain. In this paper, study focuses on comparison of various sleep monitors available commercially based on technique used and complexities involved- in, to analyze the quality of sleep. **Method**: This qualitative study consolidates the information from 1913 to 2017, about sleep, its importance and sleep monitoring systems. The conventional sleep monitoring method Polysomnography (PSG) to the wearable devices reviewed comparatively. The study shows that sleep is a function of brain. **Results**: The data collected from various sources are compared and its result shows that 85% of commercially available system are not using the important parameter of brain signals, only 15% present commercial sleep monitoring system are based on Electroencephalograph. **Conclusion**: The study reveals that there is an actual need of sleep monitoring system. The available sleep monitors have a great scope for research to improve the quality of sleep analysis in daily life.

KEYWORDS: Polysomnography (PSG), Non Rapid Eye Movement (NREM), Rapid Eye Movement (REM), Electroencephalograph (EEG).

INTRODUCTION:

In the 19th century, researcher had started the study about important biological function of human body that is sleep, to improvise the performance and efficiency of human brain. The sleep quality and quantity impact directly on human life style, occupation and health by improving one's memory, maintaining blood pressure, keeping proper functioning of endocrine system and metabolism. Sleep loss and sleep disorders results in cardiovascular diseases, Obesity, Cancer, less productive at work place, restless, short temper, depressed and increased likelihood of accidents in the lives. To live quality of life it is necessary to understand sleep, its function, monitoring of sleep and sleep behavior of an individual. A daily sleep monitoring system is required for self-care.

Sleep is the fundamental physiologic activity of a body controlled by the central nervous system (CNS). It is a reversible behavioral state characterized by closed eyes, muscles relaxed, mobility decreased, minimal movement, low attention to the environment and lessened consciousness in which body becomes inactive but brain and other organs of body work in continuation for our physical, mental and emotional health**.** Sleep is necessary for physical health, for proper functioning of nervous system, neurocognitive function, energy conservation, neural plasticity and ecological adaptation. Sleep performance and its importance can be realized by the effects of its deprivation in which mood becomes imbalanced, learning becomes impaired and periods of temporary loss of consciousness, become clear[1-3]. In addition, researchers found correlations between sleeplessness and diseases like diabetes and cardiac[4]. A complete sleep is needed for smooth functioning of all biological processes to live quality of life. It gives support in healthy functioning of various organ systems in a body. Sleep conserves energy which cannot be attainable from quiet wakefulness[5].

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The researchers study the effect of sleep on the various organ system of human body to improve quality of life. The important role of sleep is in the functioning of the brain by improving memory and learning[6–11], enhancement in attention, creativity and aid in making decisions. The ability to recall spoken language[12], spatial memories[13,14] auditory patterns[15], motor skills[16,17] and factual information[18,19] has been raised by taking different types of sleep[20], and exceptions are found in the patterns of sleep[21,22] from which the data is compelling. The ability of the brain to reorganized and reactivated its memories is enhanced by sleep[23]. Lack of sleep leads to depression, suicide and risk-taking behavior.

During sleep, immune system of body release cytokines to kill bacteria, viruses and defend our body against illness[24]. Sleep restores the immune system and its disturbance take part in the risk of infectious disease, cardiovascular disease, cancer, and major depression[25].

Feelings of hunger and fullness has been controlled and maintained by hormones named leptin and ghrelin which are chemically balanced by taking proper sleep. Furthermore, decreased glucose tolerance, increased risk of weight gain, hunger and appetite increases due to short sleep duration.[26,27], and type 2 diabetes mellitus (T2DM) come into sight in the last decade[28,29]. Sleep is required to repair cell damage caused by metabolic processes, recovery and recuperative process of the body[30,31,32].

Sleep plays vital role to heal and repair the blood vessels and heart. It maintains body blood sugar, blood pressure, and inflammation levels. Sleep loss-induced changes in cellular metabolic status can be reversed by taking balanced sleep[33]. Short term sleep directly increases risk of cardiovascular disease[34,35,36, 37]

Sleep produces testosterone, growth hormone, insulin hormone to build muscle mass and repair cells and tissues. Sleep debt results in normal ageing and age-related chronic disorders[38].

Irregular life style and demand of job like call centers impact directly on sleep hours of people. In long term, night shift workers found an increased risk for colon, prostate, breast and endometrial epithelial malignancies together with non-Hodgkin's lymphoma which contribute to all-cause mortality[39–42 ,43, 44, 45,46].

The performance of sportsperson depends on its physical and mental fitness[47], and is affected by the sleep awake time, temperature of body, and hormone regulation[48]. Currently, exercise performance due to sleep loss is limited[49,50,51,52,53–55].

A definite quantity and quality of sleep have reversed the impairments caused due to its deprivation[56]. The quality of sleep is monitor by identifying the main stages of sleep and their time span as it changes due to brain activity and physiological functions of a body. In this manner, sleep occurrence can be detected. Normally human sleep is divided into Non Rapid Eye Movement (NREM) which is further subdivided into NREM1, NREM2, NREM3 and NREM4, and Rapid Eye Movement (REM) stages[57]. Figure1 shows the different sleep stages with respect to time called hypnogram.

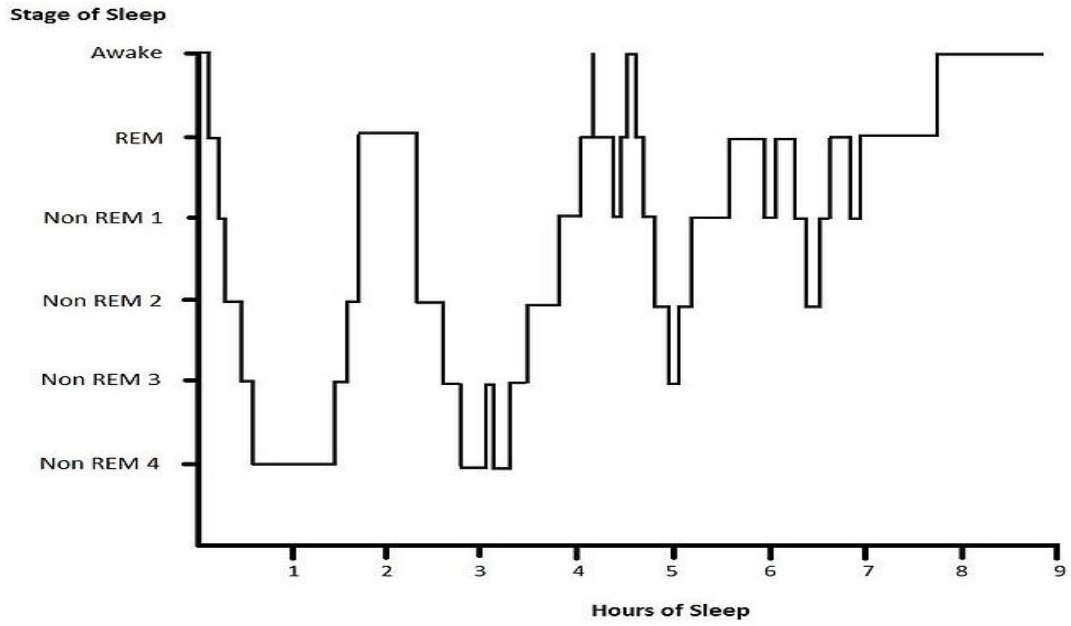

Fig. 1. Hypnogram.

The recorded patterns of sleep help in identification of different types of sleep disorders. The amount of time you spend in each stage also depends on your age. In this way one can check the status of sleep and its quality to improvise their medical and general wellness.

MATERIALS AND METHODS:

The present study was conducted from 1913 to 2017. In1913, Henri Pieron, a French psychologist, published a book called *Le Probleme Physiologique Du Sommeil*, the first physiological examination of sleep. His text is officially regarded as the start of modern sleep research. "The proposed mechanisms for the origin of sleep are varied and include the classical concept of central sleep regulation by thalamocortical relays[58], the brainstem[59], and the hypothalamus[60]; local network regulation of sleep through sleep regulatory factors[61–63]; and decentral regulation involving cortical contributions[59,64] and states of individual cortical neurons being either active or inactive[65]". In 1929 German psychiatrist Hans Berger first recorded human electrical brain waves and noted differences between activity patterns in the sleeping and waking brain. In 1936 Loomis explained different stages of sleep, based on electroencephalography (EEG). In 1950, Aserinsky and Klietman (1953) discovered REM sleep. It is related to dreaming in living organism. Finally, sleep has been split up into two types NREM and REM sleep. In 1957 Stanford University Psychiatrist William C. Dement, the "father of sleep medicine" teamed up with klietman, his former teacher, and created a sleep-stage classification system involving REM sleep and four stages of NREM sleep. Barring a few tweaks, the classification system remains in use today. Dement would also define sleep medicine as "the branch of medicine that deals with the sleeping brain and all manifestations and pathologies deriving there from."[66]. The sleep stages were standardized in 1968 by Rechtschaffen and Kales (*R&K*) based on EEG changes, dividing NREM sleep into a four further stages (stage I, stage II, stage III, stage IV)[56]. It should be noted that some dreaming has been discovered during NREM sleep. Sleep and its stages are measured by using Polysomnography (PSG), Electroencephalography (EEG), Electro-oculogram (EOG), Electromyography (EMG), Pulse oximetry, Electrocardiogram (ECG) and Actigraphy.

In 1970 Dement established the Stanford Sleep Clinic, the country's first full-scale sleep research center. This would pave the way for the creation of more than 1000 sleep clinics now in operation across the country. The commercial clinical PSG are developed and available in 1980. Multiple Sleep Latency Test (MSLT) introduced in the year 1982 as a diagnostic tool to determine sleep disorders having excessive daytime sleepiness. At the same time another test called maintenance of wakefulness test (MWT) is used to measure person alertness during day time. The recent development in electronics technology has change the complete medical electronic testing systems. In 1990 commercial sleep monitor Emfit QS is founded in market. Emfit is ballistocardiography based sleep tracker for the bed to monitor sleep. The Evalution of Microelectro Mechanical System (MEMS) Acceleration Transducer in year 2004 has introduced actigraphy based commercial sleep monitoring system in market.

In 2004, the American Academy of Sleep Medicine (AASM) standards commissioned the AASM Visual Scoring Task Force to review the *R&K* scoring system[57]. Cardiac, arousals and respiratory events were also added to the scoring. This document resulted in the most significant being the combining of stages NREM 3 and NREM 4 into *Stage NREM3*[57]. In 2005, the International Classification of Sleep Disorders (ICSD) classify eight types of sleep disorders in humans[57].

In 2007, the revised scoring was published as The AASM Manual for the Scoring of Sleep and Associated Events. It is found that NREM and REM sleep are alternating cycles. In 2008, a University of Wisconsin research team found that there is a high risk for life when person cannot take breathe during sleep. It is also considered as one of sleep disorder. Elsewhere, researchers used epidemiological data to link sleep deprivation with obesity and diabetes. The last decade has seen the emergence of sleep as a public health concern, with an increasing focus on the connection between poor sleep quality, chronic disease and socioeconomic factors[66,67]. Sleep monitoring could help people to understand need of quality sleep and its behavior while sleeping. There are two types of sleep monitors available for monitoring the sleep pattern Clinical sleep monitors and Commercial sleep monitors.

In clinical sleep monitors PSG, Multiple Sleep Latency Test (MSLT) and Clinical Actigraphy (2007) are widely used. These monitors are complex, required medical trained person and are expensive too for long-term (i.e., weeks, months) treatment for sleep monitoring[68]. Especially for PSG, spending whole night in a hospital with dozens of wires hooked up is not comfortable idea for restful sleep[69]. The daytime sleepiness is detected by MSLT test at early stage of treatment[70]. In actigraphy a device called an actigraph, is worn on a wrist, ankle, or trunk to monitor movement, sleep wake patterns and circadian rhythm abnormalities[71]. It is widely used to detect sleep disorders in a person suffering from neurological diseases such as Parkinson's disease, Alzheimer's disease, cerebral infarction, seasonal affective disorder, restless leg syndrome, vascular dementia[72]. The advantage of actigraphy over traditional PSG is that it is less complicated, dozens of

wires are not used and can continuously record pattern of sleep for 24-hours or even longer[73]. In the diagnosis of children, actigraphy is widely accepted[74].

In 2006, the advancement to technology, miniaturization and lack of availability of time leads to portable sleep monitoring devices. These commercially available monitors allow individuals to record and analyze their own sleep pattern in real-world environment that is home. Our study includes both non-wearable and wearable and sleep monitoring systems.

Table-1: Comparative study of technology of Commercially Available Sleep Monitors

Sr. No.	Device	Wearable	Non Wearable	Sensor on	Year	Web Link
1	aXbo Single	X	√	Nightstand	2006	https://www.axbo.com/products/axbo-singlecarbon-metallic
2	Beddit	X	√	Under bed Mattress	2006	http://www.beddit.com/
3	Sleeprate	√	X	Chest or Mattress	2009	http://www.sleeprate.com/
4	Zeo	√	X	Head	2009	https://www.amazon.com/Zeo-Model-ZEOBP01-Personal-Sleep-Manager/dp/B002IY65V4
5	iBrain	√	X	Head	2010	http://neurovigil.com/index.php/technology/ibrain-device
6	Peak	√	X	Wrist	2010	http://www.mybasis.com/
7	Actiwatch	X	√	Night stand	2011	http://www.healthcare.philips.com/main/homehealth/sleep/actiwatch/default.wpd
8	Sleep as Android	X	√	Mattress	2011	http://sleep.urbandroid.org
9	Somnus Sleep Shirt	√	X	Torso	2011	http://www.nyxdevices.com/product/
10	Dream ON	X	√	Mattress	2012	http://www.dreamonapp.com
11	Fuelband	√	X	Wrist	2012	http://www.nike.com/us/en_us/c/nikeplus-fuelband
12	Dreem	√	X	Head	2013	https://sleeptrackers.io/qa-dreem-active-eeg-sleep-wearable/
13	Fitbit One	√	X	Wrist	2013	https://www.fitbit.com/one
14	Sense	X	√	Nightstand or Pillow	2013	https://hello.is/3
15	Sleep Cycle	X	√	App	2013	http://www.sleepcycle.com/
16	Sproutling	√	√	Ankle or Nightstand	2013	http://www.sproutling.com/
17	Aurora	√	X	Head	2014	https://iwinks.org/
18	Withings Aura	X	√	Mattress	2014	https://www.withings.com/us/en/products/aura
19	Eight	√	X	Arm	2014	http://eightsleep.com/
20	Jawbone UP (2/3/Move)	√	X	Wrist	2014	https://jawbone.com/store/buy/up3
21	Orbit	√	X	Wrist	2014	https://www.runtastic.com/orbit
22	Proactive Sleep	X	√	Pillow	2014	http://www.proactivesleep.com/
23	S+	X	√	Nightstand	2014	http://www.resmed.com/us/en/consumer/s-plus.html
24	Sleep Genius	√	X	Wrist	2014	http://sleepgenius.com/sleepgenius-apps/wearables/
25	Vivofit	√	X	Wrist	2014	https://buy.garmin.com/en-US/into-sports/healthfitness/vivofit-/prod143405.Html
26	Vue	√	X	Wrist	2014	https://www.ifit.com/vue
27	Band 2	√	X	Wrist	2015	http://www.microsoftstore.Com/store/msusa/en_US/pdp/Microsoft-Band-2/productID.324438600
28	BAM Labs	X	√	Mattress	2015	http://www.bamlabs.com/
29	Kokoon	√	X	Head	2015	https://sleepjunkies.com/blog/kokoon-eeg-headphones/
30	Sleep Image	√	X	Chest	2015	http://www.sleepimage.com/
31	Sleep Sense	X	√	Mattress	2015	https://news.samsung.com/global/be-a-better-youwith-samsung-electronicssleepsense
32	Fusion Bio	√	X	Wrist	2016	https://www.striiv.com/pages/striiv-fusion-bio
33	Lucid Catcher	√	X	Head	2016	https://lucidcatcher.com/#/
34	Neuroon	√	X	Multi sensor	2016	http://sleeptrackers.io/neuroon-review/
35	Sleep Time +	X	√	Mattress	2016	http://www.azumio.com/s/sleeptime/index.html
36	Smart band 2	√	X	Wrist	2016	http://www.sonymobile.com/global-products/smartwear/smartband-2/
37	FitbitAlta HR	√	X	Wrist	2017	https://www.fitbit.com/altahr
38	Nokia Steel	√	X	Wrist	2017	https://health.nokia.com/es/en/steel
39	Pillow	X	√	Nightstand	2017	https://neybox.com/pillow/
40	Polar A370/ Polar A360	√	X	Wrist	2017/ 2015	https://www.polar.com/us-en/products/sport/A370-fitness-tracker

The study of available commercial sleep monitors implicates that in near future smart sleep tracking device market is expected to grow at its highest level because of awareness and health concern pupil numbers are increasing in our society[75]. Table-1 shows the classification of commercially available sleep monitors (2006-2017) based on techniques and sensors position.

RESULT:

The study shows that wearable device captures 65% of market share and 37.5% are non-wearable devices. The Pie Chart 1 (Figure2) implicates the study of above 40 type of popular Sleep monitors. This shows that 37.5% of sleep monitors are based on actigraphy technique, 22.5% are on Force sensor-based system, on the third rank Nightstand systems scores 17.5%, while sleep is a function of brain but EEG based system is on fourth rank with 15% contribution in commercial sleep monitoring market. The comparative study of these sleep monitors shows the demand for a new technically validated scheme of sleep monitoring system, at low cost so that person can monitor such a vital and complex parameter of life by its own at home.

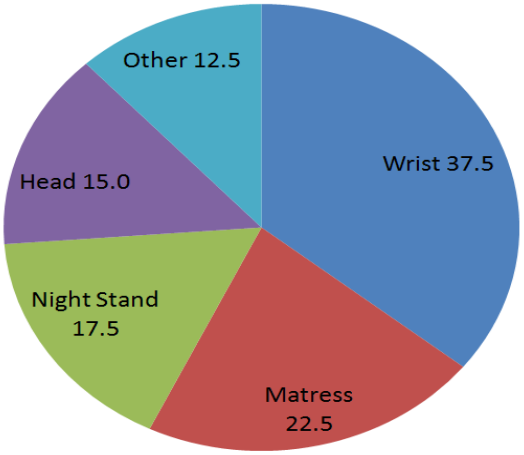

Figure 2: Pie Chart-1 Sleep Monitor Market Share Based on Different Technologies.

DISCUSSION:

Our study related to sleep, its disorders and deprivation shows that 20% of world population is sleep deprived and percentage of Indians are 93%[76]. People come in depression and think of suicide due to problems arise from disturbances of sleep and its disorders[56], and taking pills either for treatment or sleep well[77-79]. Secondly, sleep has significant impact on economy that is estimated about tens of billions of dollars annually, so its treatment is costly and available sleep monitors are also expensive. Furthermore, sleep is a function of brain but very few products are based on EEG technique[80-82]. Third, a wide range of various design sleep monitoring devices available in the market are based on ECG and actigraphy which are obtrusive to patients[83-90] belongs to America, China, Japan, Korea etc and none of them belongs to India.

In conclusion, the paper delivers information about sleep and need of sleep monitoring system. There is wide range of sleep monitoring system with different technologies available in the market in the form of wearable and non-wearable devices. These devices are having large number of features to monitor individual health in all aspects. However, the information about their data processing technique is not disclosed as a trade secret and none of the device provides raw data to process it further. The validation of such devices is only on individual's experience with it, no technical correlation data or information is provided. Very few products have such clinical analysis information. Daily quality of sleep monitoring is essential in today's lifestyle to avoid futuristic health related issues. A clinically proven compact household device is required which gives close correlation to the medical test set up, to set bench mark for the further research.

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RESEARCH ARTICLE

Development and validation a new stability indicating RP-UFLC method for the quantification of Voriconazole

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ABSTRACT:
Voriconazole is anti-fungal agent. Voriconazole is used for treating invasive candidiasis, invasive aspergillosis and emerging fungal infections. A new stability indicating RP-UFLC method has been proposed for the quantification of Voriconazole in pharmaceutical formulations and the method was validated as per ICH guidelines. Mobile phase consisting of a mixture of Acetonitrile: 0.01% Acetic acid (50: 50, v/v) (pH 5.0) with a flow rate 1 mL/min and UV detection at 240 nm was used for the assay of Voriconazole. The drug was exposed to different stress conditions such as alkaline, acidic, thermal and oxidation degradation. Linearity was observed over the concentration range 0.5-50 µg/mL with linear regression equation y = 23554x- 774.8 (r^2 = 0.9999). The LOQ and LOD were found to be 0.3987 µg/mL was found to be 0.1313 µg/mL. The present method can be useful for the quantification of pharmaceutical formulations such as tablets and injections and also for the bioanalytical studies.

KEYWORDS: Voriconazole, RP-UFLC, Validation, Stability indicating, ICH guidelines.

INTRODUCTION:
Voriconazole (Figure 1) is a triazole anti-fungal agent and chemically known as (2R, 3S)-2-(2, 4-difluro phenyl)-3-(5-fluoro-4-pyrimidinyl)-1-(1H-1, 2, 4 triazol1-yl)-2-butanol. Voriconazole is used to treat serious fungal infections. Voriconazole has a very low aqueous solubility and its maximum solubility is in acidic conditions. It is used to treat invasive fungal infections that are generally seen in immune compromised patients. These include invasive candidiasis, invasive aspergillosis and emerging fungal infections[1-4]. Voriconazole is an extended spectrum triazole developed specifically to target Aspergillus[4]. Voriconazole was determined by spectrophotometry[5-10], HPTLC[11-12], LC-MS[13] and HPLC[14-22]. In the present study the authors have proposed a new stability indicating reverse phase RP-UFLC method for the assay of Voriconazole and the method was validated[23-24].

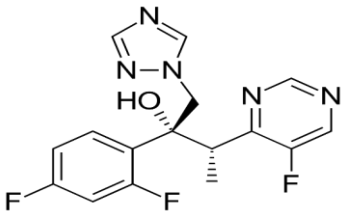

Figure 1: Chemical structure of Voriconazole

MATERIALS AND METHODS:
Shimadzu Model HPLC system (Shimadzu Co., Kyoto, Japan) equipped with PDA detector and C8 Luna column (250 mm × 4.60 mm i. d. 5µm particle size) was used or the chromatographic study. The system was maintained at 25 ºC. A mixture of Acetonitrile: 0.01% Acetic acid (50: 50, v/v) (pH 5.0) was used as mobile phase with a flow rate 1 mL/min (UV detection at 240 nm) for the present study. The mobile phase was sonicated and filtered through 0.22µm membrane filter prior to use.

Voriconazole was obtained as gift sample from Glenmark Pharmaceuticals Ltd. Voriconazole is available as tablets with brand names Voraze (Sun Pharma), Vorizol (Natco Pharma), Vonaz (United

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Biotech Pvt. Ltd.), Voritek (Cipla Limited) and Vosicaz (Glenmark Pharmaceutical Ltd) (Labelled claim 200 mg) and as I.V. injection with brand name Vfend (Pfizer).

Preparation of Voriconazole drug solution

Voriconazole (25 mg) was weighed accurately and dissolved in a 25 mL volumetric flask, sonicated and made up to volume with HPLC grade acetonitrile (MERCK) (1000 µg/mL) and further dilutions were made with mobile phase, sonicated and filtered through 0.22 µm membrane filter prior to injection.

Method validation

Linearity

Dilute solutions (0.5-50 µg/mL) of Voriconazole were prepared from the stock solution with mobile phase Acetonitrile: 0.01% Acetic acid (50: 50, v/v) (pH 5.0) and 20 µL of each were injected in to the UFLC system. The mean peak area (n=3) of Voriconazole was calculated from the chromatograms obtained and a calibration curve was drawn by taking the concentration of drug solutions on the x-axis and the corresponding mean peak area values on the y-axis.

Precision, Accuracy and Robustness

Intraday and inter-day precision were studied using three different concentrations (10, 20 and 30 µg/mL) of Voriconazole on the same day and on three consecutive days respectively and the % RSD was calculated. The accuracy of the assay method was evaluated in triplicate at three concentration levels (50, 100 and 150 %), and the percentage recoveries were calculated. Standard addition and recovery experiments were conducted and the percentage recovery was calculated. The robustness of the method was assessed by exposing the drug solution to different analytical conditions purposely changing from the original optimized conditions.

Stress degradation studies

Forced degradation studies were performed in the applied stress conditions. Voriconazole was exposed to different stress conditions such as acidic, basic, oxidation and thermal treatment.

Acidic degradation was performed by treating the drug solution with 1mL of 0.1N HCl, heated at 80 ºC for about 30 minutes on a water bath. The stressed sample is then cooled neutralized with 1mL 0.1N sodium hydroxide solution and the solution was made up to volume to the required concentration with the mobile phase. 20 µl of the solution was injected in to the UFLC system.

Alkaline degradation, was performed by treating the drug solution with 1mL 0.01 N NaOH heated at 80 ºC for about 30 minutes on a water bath. The solution is then cooled and neutralized with 1mL 0.01N hydrochloric acid and diluted with mobile phase. 20 µl of the solution was injected in to the UFLC system.

Oxidation degradation was performed by treating the drug solution with 1ml of 30% v/v H_2O_2 heated at 80 ºC for about 30 minutes on a water bath. The solution is then cooled and diluted with mobile phase.20 µl of the solution was injected in to the UFLC system.

Thermal degradation was performed by heating the drug solution at 80 ºC for about 30 minutes on a water bath. The solution is then cooled and diluted with mobile phase. 20 µl of the solution was injected in to the UFLC system.

Assay of Voriconazole tablets

20 Tablets of Voriconazole (Label claim: 200 mg) were procured and powdered. Powder equivalent to 25 mg Voriconazole was extracted using acetonitrile and then with the mobile phase in a 25 ml volumetric flask. The solution was sonicated for half an hour and filtered through membrane filter and 20 µL of this solution was injected in to the UFLC system. The peak area observed was noted at its retention time from the resultant chromatogram and the mean peak area was calculated (n=3).

RESULTS AND DISCUSSION:

A new stability indicating RP-UFLC method was developed and validated for the estimation of Voriconazole in tablets. A review of analytical methods so far published for the quantification of Voriconazole was given in Table 1.

Table 1: Literature survey

Mobile phase or Reagent / Detection (nm)	Column	Linearity (µg/mL)	Remarks	Ref
Spectrophotometric methods				
Water / 252	-	5-80	-	5
0.1N HCl / 256	-	10-70	-	6
Water / 255	-	5-35	-	7
0.1N HCl / 256	-	10-60	-	8
Methanol / 256	-	5-30	-	9
Sodium Hydroxide Phosphate buffer pH 2.0 Phosphate buffer pH 4.0 / 256 Phosphate buffer pH 6.8 Phosphate buffer pH 7.0	-	5-60	-	10
HPTLC Methods				
Toluene: Methanol: triethylamine (6: 4: 0.1) / 254	Silica gel 60 F254	50–400 ng/band	Human plasma Cephalexin (Internal standard)	11
Acetonitrile: Water (60:40) / 257	Silica gel 60RP-18F-$_{254}$S	200–1200 ng/band	Cream	12
LC-MS Method				
Acetonitrile: 0.1% Formic acid in 10 mM Ammonium acetate (50:50)	Poroshell 120 EC-C18	0.05-10	Human plasma (Protein precipitation) Fluconazole (Internal standard)	13
Liquid chromatographic methods				
50 mM Ammonium phosphate dibasic buffer (48:52) (pH adjusted to 6.0 with dilute ortho phosphoric acid): Acetonitrile / 250	Agilent Zorbax SB-C18	10-100	HPLC Related substances (4 impurities)	14
Water: Acetonitrile: Methanol (50:25:25) / 256	Hypersil BDS C18	20-400	HPLC Rt is more (12.986 min)	15
Acetonitrile: Water: Acetic acid (55:45:0.25) (pH 4.0) / 256	Diamonsil C18	0.1-50	HPLC Rat & Beagle dog plasma	16
Acetonitrile: Water (70:30) / 255	Symmetry C18	0.2-15	HPLC Plasma (Protein precipitation)	17
Acetonitrile: Water (60:40) / 256	enable C18G	10-50	HPLC Not stability indicating method	18
0.05M Ammonium acetate: Acetonitrile: Methanol (40:20:40) / 256 (Voriconazole) / 310	Microsorb MV- C18	0.1-10	HPLC Plasma Serum Clonazepam (Internal standard)	19
Methanol: Water (45:55)	Hypersil ODS C_{18}		HPLC Chiral Separation & Analysis of enantiomers	20
Acetonitrile: Water (40:60) / 256	C18 Hypersil BDS	5-25	HPLC Rt is more (8.0 min)	21
Acetonitrile: Water (50:50) / 256 (pH 3.0)	RP C18	5-50	HPLC Experimental design Famciclovir (Internal standard)	22
Acetonitrile: 0.01% Acetic acid (50: 50) (pH 5.0) / 240	C8 (2) 100A (Luna)	0.5-50	UFLC Stability indicating Rt is less (6.45 min)	Present method

Mobile phase consisting of a mixture of Acetonitrile: 0.01% Acetic acid (50: 50, v/v) (pH 5.0) with a flow rate 1 mL/min and UV detection at 240 nm was used for the assay of Voriconazole using C8 Luna column (250 mm × 4.60 mm i.d. 5µm particle size). A symmetrical and sharp peak was observed at 6.45 ± 0.05 min. The chromatograms obtained were shown in Figure 2.

Linearity

Voriconazole obeys Beer-Lambert's law and follows linearity over the concentration range 0.5–50 µg/mL (Table 3) (% RSD 0.19-1.65) and the linear regression equation was found to be y = 23554x- 774.8 (r^2 = 0.9999) (Figure 3). The LOQ and LOD were found to be 0.3987 µg/mL was found to be 0.1313 µg/mL.

Table 2: Linearity of Voriconazole

Conc.(µg/mL)	*Mean peak area	% RSD
0.05	1173	1.42
1	23513	0.62
5	117377	0.92
10	235839	0.87
15	343778	1.65
20	471654	0.54
25	589550	1.34
30	707503	1.52
40	943657	0.63
50	1175195	0.19

*mean of three replicates

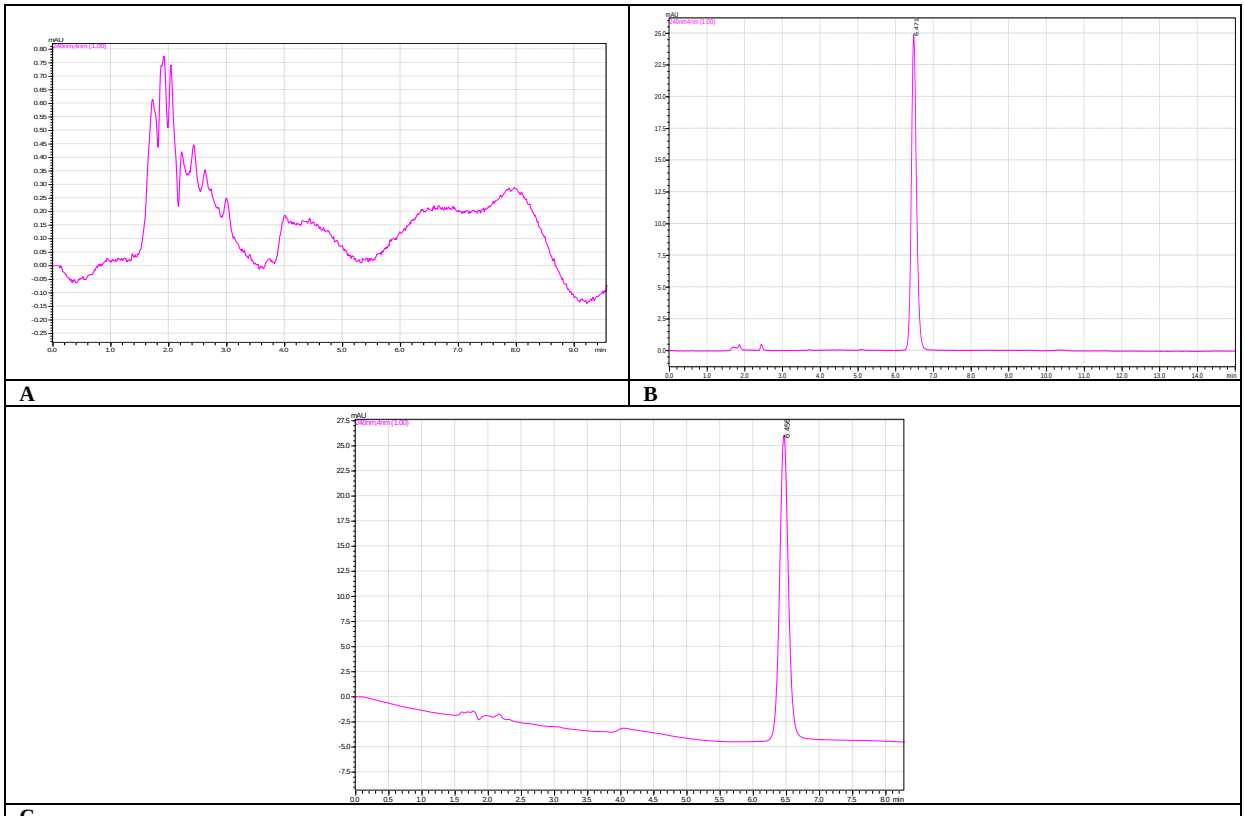


Figure 2: Representative chromatograms of Vorconazole A) Placebo B) Vorconazole standard (API) and C) Vosicaz tablet (Labelled claim: 200 mg)

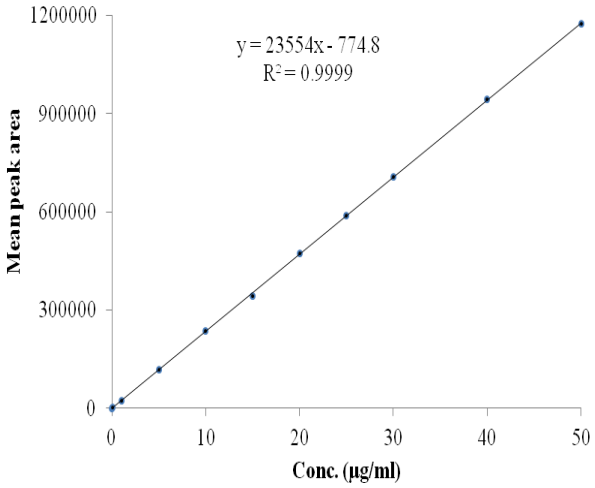


Figure 3: Calibration curve of Voriconazole

Precision, Accuracy and Robustness

Intraday and inter-day precision were studied at three different concentration levels of Voriconazole on the same day and on three consecutive days respectively and the % RSD was found to be 0.73-1.42 (Intraday) (Table 4) and 0.52-1.54 (Inter day) (Table 5) respectively (<2.0) demonstrating that the method is precise. The accuracy of the method was proved by the standard addition method and the % RSD values were 0.36-1.15 (<2.0) with a recovery of 99.72-99.93 % (Table 6). The robustness of the assay method was established by introducing small changes in the chromatographic conditions which include detection wavelength (242 and 238 nm), percentage of organic phase i.e. Acetonitrile in the mobile phase (48 and 52%) and flow rate (± 0.1 ml/min). Robustness of the method was studied using 10 µg/mL of Voriconazole (Table 7) and the % RSD was found to be 0.43-1.23 (<2.0).

Table 4: Intraday precision study of Voriconazole

Conc. (µg/mL)	*Mean peak area	Statistical Analysis *Mean ± SD (% RSD)
10	235883	235857.67 ± 3372.76 (1.42)
10	235839	
10	235851	
20	471734	471732 ± 3962.55 (0.84)
20	471671	
20	471793	
30	707498	707521.67 ± 5164.91 (0.73)
30	707504	
30	707563	

*mean of three replicates

Table 5: Interday precision study of Voriconazole

Conc. (µg/mL)	*Mean peak area			*Mean ± SD (% RSD)
	Day 1	Day 2	Day 3	
10	235839	235756	235799	235798 ± 3631.29 (1.54)
20	471634	471539	471698	471623.67 ± 4055.96 (0.86)
30	707513	707629	707436	707526 ± 3679.13 (0.52)

*mean of three replicates

Table 6: Accuracy study of Voriconazole

Spiked conc.((µg/mL)	Formulation (µg/mL)	Total Conc. (µg/mL)	*Mean Conc. (µg/mL) ± SD (%RSD)	% Recovery
5 (50%)	10 10 10	15 15 15	15.01 ± 0.163 (1.09)	99.93
10 (100%)	10 10 10	20 20 20	19.97 ± 0.231 (1.15)	99.85
15 (150%)	10 10 10	25 25 25	24.93 ± 0.090 (0.36)	99.72

*mean of three replicates

Table7: Robustness study of Voriconazole

Parameter	Condition	*Mean peak area	*Mean peak area ± SD (% RSD)
Flow rate (± 0.1 ml/min)	1.1	235712	235710 ± 2899.23 (1.23)
	1.0	235839	
	0.9	235579	
Detection wavelength (± 2 nm)	242	235847	252524.33 ± 1085.86 (0.43)
	240	235839	
	238	235887	
Mobile phase composition Acetonitrile: 0.01% Acetic acid (50: 50, v/v) (pH 5.0) (± 2 %, v/v)	48:52	235698	235772.67 ± 1626.83 (0.69)
	50:50	235839	
	50:48	235781	

*Mean of three replicates

Assay of Voriconazole tablets

Two different brands of Voriconazole tablets consisting of 200 mg API were procured from the pharmacy store and the method developed was applied. It was found that the amount of Voriconazole found in tablets was 98.93-99.49 (Table 8) and there is no interference of excipients.

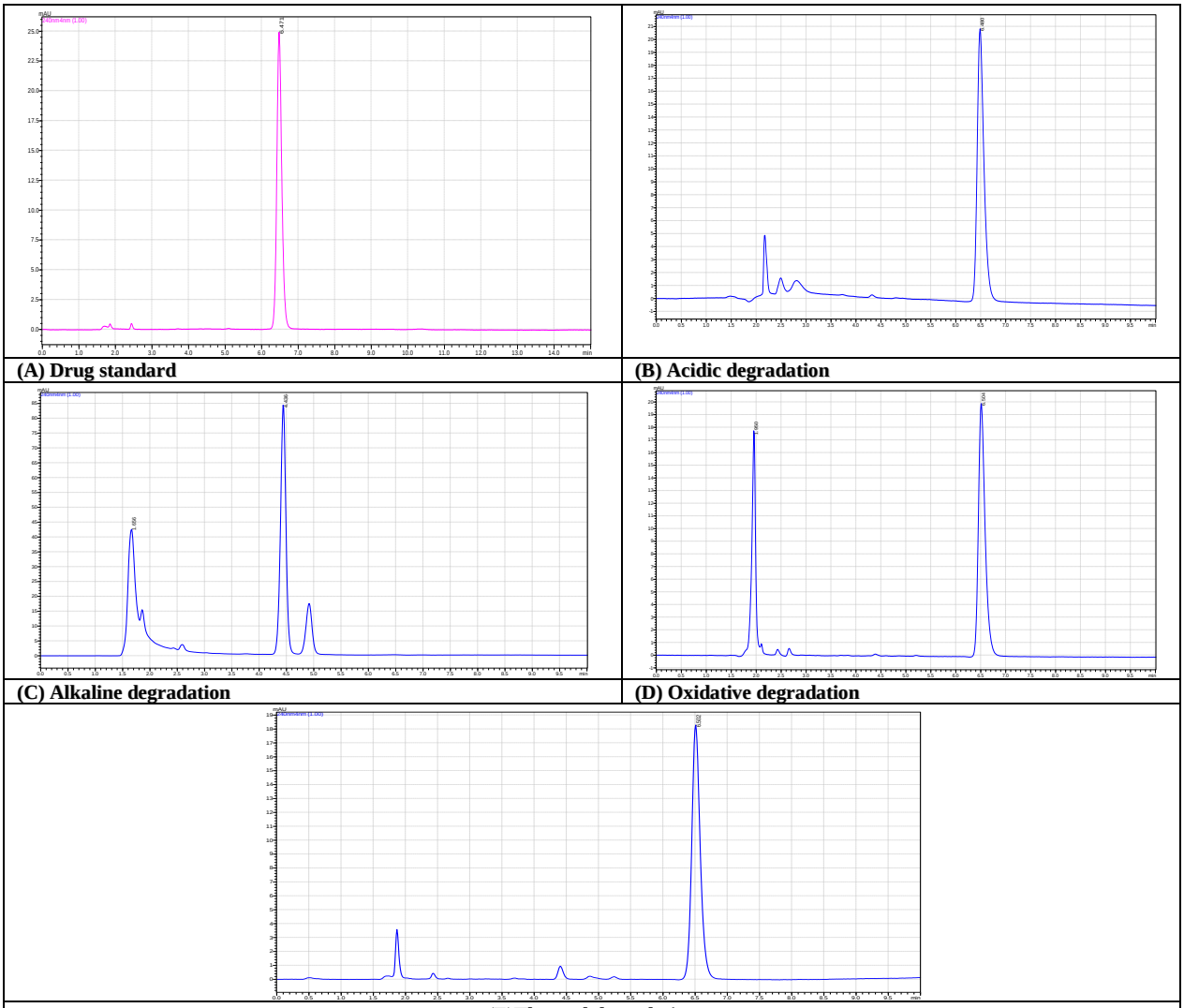
Table 8: Assay of Voriconazole tablets

Brand	Label claim (mg)	Observed amount (mg)	% Recovery*	Manufacturer (India)
I	200	198.97	99.49	Glenmark Pharmaceutical Ltd
II	200	197.86	98.93	Cipla Limited

*Mean of three replicates

Stress degradation studies

Voriconazole (10 µg/mL) was eluted as a sharp peak at 3.022 min. During the acidic degradation the drug was eluted at 6.480 min and in alkaline degradation Voriconazole was eluted at 4.436 min. During the oxidation along with the drug peak was eluted at 6.504 min and in thermal degradation the drug was eluted at 6.502 min. Voriconazole was very sensitive towards alkaline degradation conditions. Initially the degradation was performed by using 1 ml of 0.1N NaOH but the total amount of drug has undergone degradation and therefore the alkaline degradation was continued by using 0.05, 0.01 N NaOH but even then total drug was decomposed and two degradants were eluted at 4.436 and 1.656 min. The fluoro phenyl moiety may be responsible for the complete degradation of the drug due to the alkali effect. In all the degradation studies the degradants were well separated without interfering with the main drug peak indicating that the method is selective and specific. The system suitability parameters were within the acceptable criteria i.e. the tailing factor was less than 1.5 and the theoretical plates were more than 2000 (Table 9). The individual chromatograms obtained during the stress degradation studies were shown in Figure 4.



(A) Drug standard

(B) Acidic degradation

(C) Alkaline degradation

(D) Oxidative degradation

(E)Thermal degradation

Figure 4: Typical chromatograms of Voriconazole A) Drug standard (10 µg/mL) B) Acidic degradation C) Alkaline degradation D) Oxidative degradation E) Thermal degradation

Table 9: Stress degradation studies of Voriconazole

Stress condition Medium / Temp. / Time	Rt (min)	% Recovery	%Drug degradation	Theoretical plates	Tailing factor
Standard drug	6.471	100	-	9856	1.196
Acidic degradation 0.1N HCl/ 80°C/ 30 min	6.480	84.63	84.63	10033	1.404
Alkaline degradation 0.01N NaOH/ 80°C/ 30 min	4.436 1.656	-	100.0	7846	1.032
Oxidation 30% H_2O_2/ 80°C/ 30 min	6.504 1.950	79.69	20.30	10298	1.411
Thermal degradation 80°C/ 30 min	6.502	71.72	28.27	10572	1.224

CONCLUSION:

The RP-UFLC techniques were validated as per ICH guidelines and found to be simple, precise, accurate and robust for the quantification of Voriconazole tablets. There is no interference of excipients and the degradants were well separated without interfering with the drug peak. The method is specific and the system suitability parameters are within the acceptable criteria. The method can be successfully applied or the determination of Voriconazole in pharmaceutical formulations.

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RESEARCH ARTICLE

Formulation of Oral Dosage Form of Antidiabetic Drug Glipizide using Mixed Solvency Method

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ABSTRACT:

Glipizide is an antidiabetic drug which is used to treat type 2 diabetes. Glipizide belongs to BCS class 2, which have low solubility and high permeability. Due to the solubility problem it has low bioavailability. In this experimental work attempts are made to enhance the solubility of glipizide by mixed solvency technique which consists of cosolvency and hydrotrophy methods. In cosolvency method four cosolvent namely polyethylene glycol, propylene glycol, glycine and ethanol were considered and In Hydrotropy method, three different hydrotropes, sodium benzoate, sodium citrate and sodium salicylate in concentration with % (5,10,15,20,25,30,35 & 40) were used. In cosolvency method PEG was found to be most suitable compare to other three. There was a rise in solubility of glipizide with increase in concentration of PEG but considering its toxic effect 40% of PEG was taken for further concentration. Out of the three different hydrotropes sodium salicylate showed best result with 40%. The monophasic dosages form was formulated and evaluated. The parameters like pH, viscosity, surface tension and specific gravity etc were also performed.

KEYWORDS: Glipizide, Antidiabitic, Solubility, Cosolvency and Hydrotropy**.**

INTRODUCTION:

Most of the therapeutic agent newly synthesized are weak electrolytes or non-electrolytes. These drugs possess low or unpredictable bioavailability which limits their pharmacological action. Different methods are employed to overcome the problem related to solubility and permeability. Some of the currently used method are solid dispersion method, complexation method, hydrotropic solubilization, fluctuating the pH and using cosolvents. In this research work the objective is to enhance the solubility of Glipizide an antidiabetic drug belonging to the sulfonylurea class used to treat type 2 diabetes and develop syrup formulation of Glipizide[1]. According to biopharmaceutical classification Glipizide is categorized under BCS class II. It has low aqueous solubility and short half-life of 2 hours.

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Mixed solvency is used to enhance the solubility of glipizide, both hydrotrophy and co-solvency method are applied. Hydrotrophy is a process of solubilization in which a large amount of second solute is added to increase the aqueous solubility of another solute. Solute which are alkali metal salts of different organic acids can be used as hydrotropic agents and these substances are called as "Ionic organic salts". Achievements of science that involves in the increment of solubility in the given solvent are known as "salt in" the solute. Some of the water-soluble additives known as hydrotropes like sodium benzoate, sodium ascorbate, sodium citrate, niacinamide, urea[2-4].

Cosolvency method improves solubility of poorly water soluble drugs by increasing their miscibility in the given solvent by interfacial tension between the solvent and the hydrophobic solute. It have been used in parenteral preparation but focus has to be given on the safety of the cosolvent used and it should not cause precipitation. Some examples of cosolvents are Glycerine, Peg, Diethyl acetamide, Ethyl lactate, Ethanol and Propylene glycol. Using this mixed solvency method various appropriate dosage forms viz. solutions, syrups, injections, topical solutions etc)[5,6].

The objective of the present work is to use the mixed solvent system to formulate monophasic dosages form of glipizide. Inadequate aqueous solubility of the drug offers obstacles in formulation of syrup of glipizide. First the solubility of drug in various concentration of co-solvent is determined. The best suitable concentration of cosolvent is selected which offers maximum solubility to the drug. To this hydrotropes are added to further enhance the solubility of the drug and after fixing the amount of suitable hydrotropes the monophasic dosages form is formulated. Various preformulation and post formulation evaluations are conducted[7,8].

MATERIAL AND METHOD:

Glipizide and polyethylene glycol 400 and Propylene glycol was purchased from CDH -R.K Enterprises Meerut, U.P. India. were purchased by CDH- R.K Enterprises, Meeru U.P., India and remaining cosolvents Glycine and ethanol as well as hydrotropes sodium benzoateμ, sodium citrate and sodium salicylate were purchased from Ambay Biotech, Greater Noida U.P. India. All solvents and other chemical were of HPLC /analytical grade.

Experimental:

Estimation of glipizide:

Stoke solution was prepared by adding 10mg of drug in 100ml and further by dilution with distilled water 10μg/ml, 20μg/ml, 30μg/ml, 40μg/ml, 50μg/ml concentration was obtained and standard curve of glipizide was plotted after UV spectroscopic analysis at 278nm (UV- 1700, Shimadzu, Japan.)[9].

Solubility determination:

Equilibrium solubility method was used to determine the solubility of glipizide in various solubilizers solution. Excess amount of glipizide was added to 10ml screw-capped glass vials containing 5ml of aqueous solution of individual co-solvent ratios at two different temperatures of 25^0C and 37^0C (Table 1) and the vails were subjected to thermostatic mechanical shaking for 6 hours. Finally, the solutions were let to equilibrate for the next 24 hours. The supernatants of each vial are filtered through Whatman filter paper of 0.45μm and the filtrate collected and analysed for drug content after suitable dilution by UV visible spectrophotometer at 278nm[10,11].

The effect of various pH from 1.2, 2.2, 4.0, 5.8, 8.0, 9.0 and 10.0 on solubility was also determined according to the above-mentioned method.

Enhancement ratio = Solubility of drug in the solubilizer solution\ Solubility of drug in water.

Determination for additive or synergistic effect on solubility in mixed solvent blends:

Depending on the value of solubility power the the appropriate cosolvent along with the ratio of co- solvent and water would be decided. To this fixed solubilizer solution different concentration of various hydrotropes as per table 3 were added and the solubility of glipizide was ascertained by equilibrium solution method[12,13].

Evaluation of Physicochemical properties of the mixed solvent solution:

Different physicochemical properties like pH, viscosity, surface tension and specific gravity. The pH of preparation was determined by using pH meter (LI614, Elico Ph analyzer). Viscosity was dictated by Ostwald viscometer. Surface tension was calculated using by stalagmometer. Specific gravity of the drug by pycnometer.[14,15].

Compatibility drug-excipient studies by FTIR analysis:

The interaction between the drug and solubilizers were studied. All the components were physically mixed with the drug in a ratio of 1:1. The KBr was first dried in microwave oven and then KBr and drug excipient pellets were prepared and analyzed by FTIR spectrometer[16].

Formulation of oral syrup of Glipizide:

Glipizide syrup was formulated based on solubility analysis result obtained from the final blends of mixed solvent. All the solubilizers were added in necessary quantities to 50ml of warm water taken in 100ml volumetric flask. To this 1g of drug and 40g of sucrose was added and shaken vigorously then the volume was made up to 100ml. The flask was set aside to bring it to the room temperature and then transferred to an airtight container. The glipizide content in the oral syrup formulation is 20mg per 5ml.

Evaluation of oral syrup formulation of Glipizide:

a. **Drug content analysis:** 1ml of the syrup was taken and after appropriate dilution the sample was spectrophotometrically analysed and the drug content was estimated using the regression equation y = 0.0072x + 0.0154.
b. Evaluation of physical properties of glipizide oral syrup formulation

The physical parameters analysed were absorbance, viscosity, specific gravity and pH. The pH was measured using Brookfield viscometer.

RESULTS:

Glipizide is an N-sulfonylurea, a member of pyrazines, it has an aromatic amide along with monocarboxylic acid amide. The solubility analysis data reveals that with

increase in pH (Fig 2) the solubility of glipizide increases, this may be attributed to the acidic nature of the drug molecule.

The aqueous solubility of glipizide was 35mg/L at 25°C and 40.67mg/L at 37^{0}C. This indicates an increase in solubility of glipizide with increase in temperature suggesting an endothermic process. The solubility and solubility enhancement ratio of glipizide in different co-solvent in decreasing order were PEG> PG>glycerin> ethanol. The enhancement of solubility don't have a linear relationship with co-solvents. The PEG was selected as the suitable co-solvent as max solubility compare to others. From the literature review on safety and toxicity amount of PEG has to be considered while fixing its concentration for formulating a dosages form. Here 40% v/v of PEG is used. Then to the above solution of water and cosolvent various concentration of different hydrotropes are added. From the solubility data it was found that the Glipizides solubility in hydrotropes ranked in decreasing order sodium salicylate> sodium benzoate> sodium citrate. The highest of glipizide can be described based on the reason given by Poochikian and Gradock's (1974) in the presence of one hydrotrope over other. In case of sodium salicylate and sodium benzoate they have the parent benzene nucleus and mobile Electron cloud which forms non-bonded and Vander Waal's interaction with water-cosolvent system as well as with glipizide. 40% w/v of sodium salicylate is considered for the formulation of glipizide syrup.

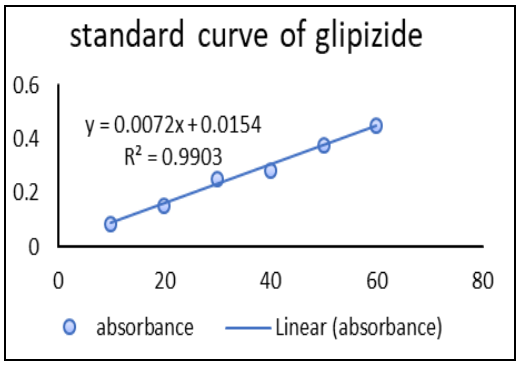


Fig.1. Standard Curve of Glipizide.

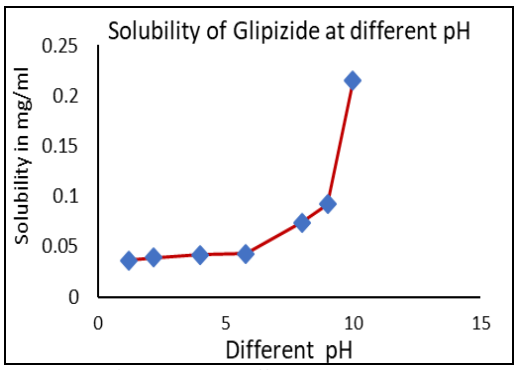


Fig.1. Solubility of Glipizide at different pH.

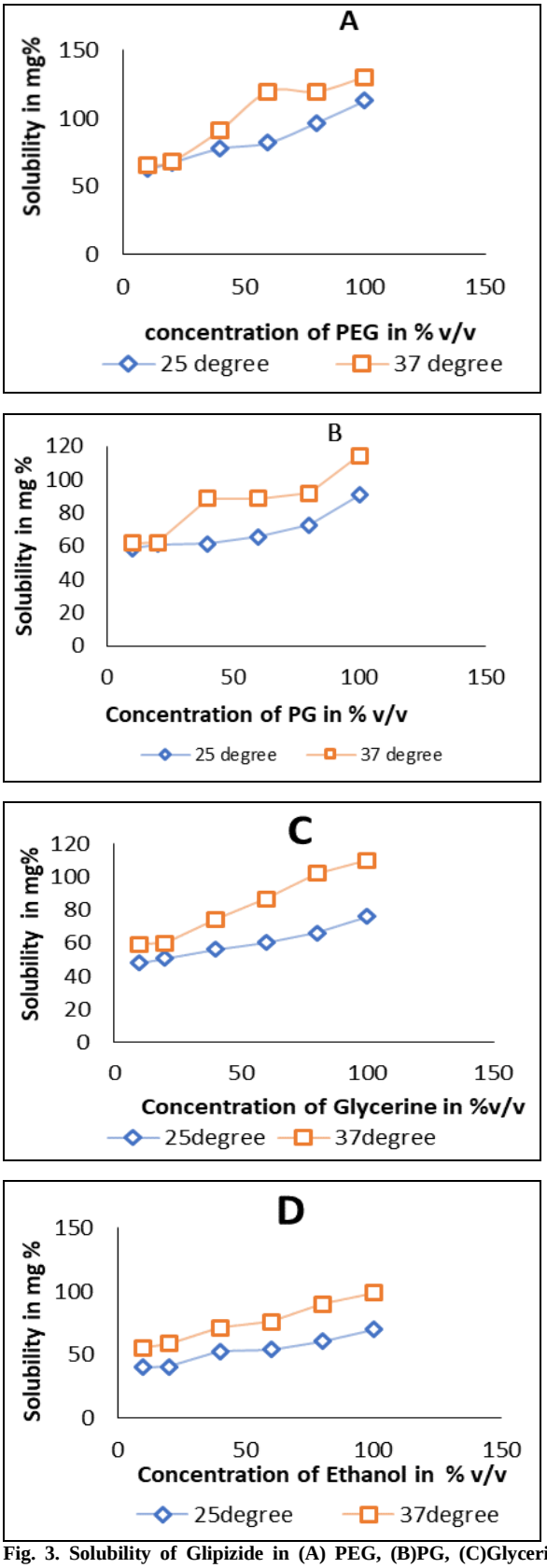


Fig. 3. Solubility of Glipizide in (A) PEG, (B)PG, (C)Glycerine, (D)Ethanol.

Table .1. Solubility enhancement ratio of Glipizide in cosolvent at different temperature

Cosolvent/ water ratio	10:90		20:80		40:60		60:40		80:20		100:0	
Temperature	25^{0}C	37^{0}C	25^{0}C	37^{0}C	25^{0}C	37^{0}C	25^{0}C	37^{0}C	25^{0}C	37^{0}C	25^{0}C	37^{0}C
PEG/Water	17.90857	18.71429	19.24857	19.48	22.22571	25.94029	23.40857	34.08286	27.59114	34.08286	32.191430	37.14286
PG/Water	16.68657	17.66	17.41257	17.738	17.54743	25.29743	18.67457	25.29743	20.71429	26.21429	25.857140	32.61086
Glycerin/ Water	13.69429	16.91657	14.46429	17.1625	16.05543	21.25371	17.23	24.738	18.88486	29.15057	21.71429	31.38857
Ethanol/ Water	11.452	15.82514	11.62286	16.76	15.03171	20.37686	15.48	21.742	17.36886	25.64571	20	28.15057

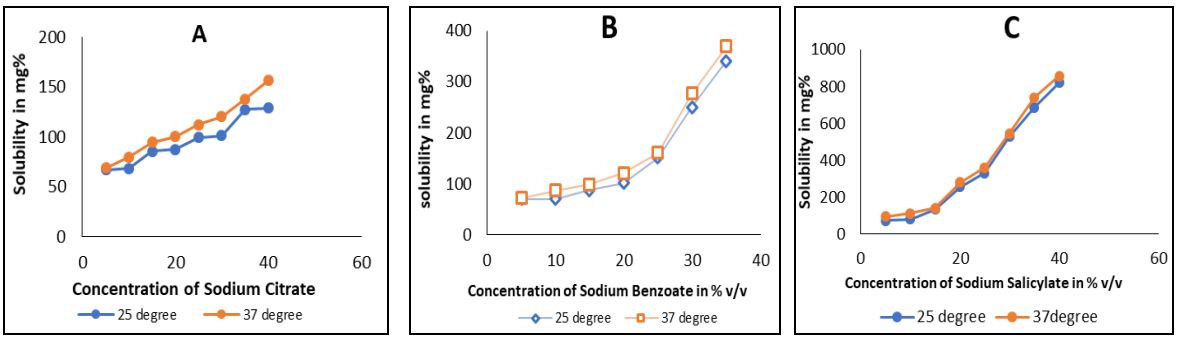


Fig.4. Concentration of hydrotropes in (A) Sodium Citrate, (B) Sodium Benzoate and (C) Sodium Salicylate.

Table .2. Solubility enhancement ratio of Glipizide in solution containing 40 % PEG as cosolvent and hydrotrope mixture at different concentration % w/v at different temperature:

Concentration of hydrotropes	5		10		15		20	
Temperature	25^{0}C	37^{0}C	25^{0}C	37^{0}C	25^{0}C	37^{0}C	25^{0}C	37^{0}C
Sodium Citrate	19.10314	19.67457	19.57143	22.73143	24.37686	27.00571	24.90457	28.69429
Sodium Benzoate	19.93143	20.60714	20.03571	24.70486	24.976	28.07143	25.87429	28.64571
Sodium Salicylate	20.76771	26.7	23.04286	31.58857	37.90571	40.19143	72.78857	79.55714

Table .2. continued

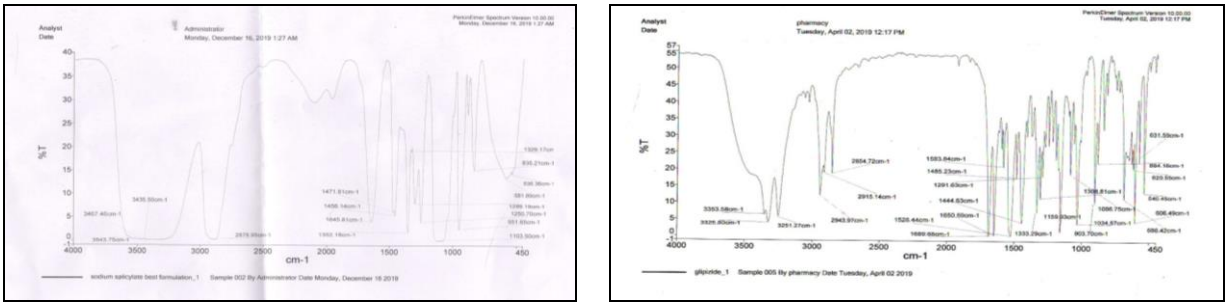
Concentration of hydrotropes	25		30		35		40	
Temperature	25^{0}C	37^{0}C	25^{0}C	37^{0}C	25^{0}C	37^{0}C	25^{0}C	37^{0}C
Sodium Citrate	28.37286	32.09714	28.924	34.38286	36.40057	39.14286	36.727430	44.57143
Sodium Benzoate	28.88086	34.53143	43.11143	45.84857	71.55714	79.04	97.39143	105.5886
Sodium Salicylate	94.44	103.1114	151.0486	155.9114	196.3943	211.5457	234.60860	244.7886

Table.3. Evaluation parameters of different formulations using Sodium Salicylate.

Sodium Salicylate	pH	Viscosity (centipoises)	Specific gravity (g/cm$^{3)}$	Surface tension(dyne/cm)
F1	6.2	0.82	1.06	71.13
F2	6.4	0.87	1.08	67.56
F3	6.6	0.95	1.06	66.34
F4	6.8	1.06	1.09	65.78
F5	6.7	1.35	1.10	64.57
F6	6.5	1.46	1.18	62.54
F7	6.7	1.57	1.27	60.43
F8	7.0	2.10	1.48	60.55

Table.4. Evaluation parameters of formulation.

Formulation of Glipizide	Specific (gravity g/cm$^{3)}$	pH	Viscosity (centipoises)	Absorbance
A1	1.170	7.7	3.9404	0.1711



A. Drug –excipient Compatibility study. **B. FTIR of Glipizide.**

Fig.5. Compatibility Study (A)Drug–Excipient and (B)FTIR of Glipizide.

The physicochemical properties like specific gravity, pH, viscosity and surface tension of different concentration solution of sodium salicylate were determined. The specific gravity was found that it slightly decreased with increase in concentration of hydrotropes it may indicate that the partial molar volume increases with aggregation due to expansion of hydrocarbon part of the molecule. The positive deviation in the viscosity indicates that aggregate formulation is associated with an increase in viscosity of hytrotrope concentration due to self-association of phenolic compounds. The surface tension very little decreases with increase in the hydrotrope concentration.

The syrup of glipizide was formulated taking 40% w/v of sodium salicylate, 40% v/v of Polyethylene glycol (PEG) and sucrose 40g and drug 1g to prepare 100ml of glipizide syrup. The properties of syrups given in table no.4.

Compatibility studies were carried to find out the interaction between the drug in formulation with the other excipient. From the FTIR studies (at wavelength 4000 cm-1 to 400 cm-1) comparing the formulation of syrup with pure drug disclosed that there were no major shifts, no loss of functional peaks.

It was found from the FTIR studies of glipizide that 3353.58 cm^{-1} (N-H stretching),3325.80cm^{-1} to 3251.27 cm^{-1} (O-H), 2943.97cm^{-1}(aromatic C-H), 1444.63 cm^{-1} (O-H bending), 1528.44(C-H), 1650.59(C=N), 1689.68 cm^{-1} (COOH) peaks were found. But in formulation the peak were found to shift to higher wavenumbers for OH group stretching represented by 3435.50 cm^{-1} and the CO group stretches toward the lower wave number. The intermolecular hydrogen bonding and the Vander Waals interaction which may occur between the pure drug and mixed solvent system may influence and cause the shifting of the peaks.

CONCLUSION:

Mixed solvent system technique can be utilized to formulate poorly water-soluble drugs belonging to BCS class II and IV drugs. The solubility of glipizide was found to increase in mixed solvent system compared to that in purified water. This method opens a new path for tackling the solubility problem associated with glipizide. Using this method syrup of glipizide was formulated and evaluated. Success of these formulation would increase the bioavailability and improve patient compliance of geriatric patients suffering from diabetes mellitus. The solubility of glipizide has improved drastically by mixed solvents.

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RESEARCH ARTICLE

Formulation and Evaluation of Mouth dissolving tablet containing Non-Steroidal Anti-inflammatory Drug

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ABSTRACT:

Oral drug delivery system is one of the most widely used systems for treatment of various types of diseases. Conventional dosage forms like tablets, capsules have various disadvantages. They cannot be easily swallowed by young and elderly patients. They require water for swallowing which makes it difficult to suit patient choice during travelling. Another disadvantage of conventional dosage form is that their therapeutic response is slow and patient has to take tablets around two to three times a day to get proper relief. Nowadays in order to give rapid relief to patients mouth dissolving or fast orally disintegrating tablets are formulated. Mouth dissolving tablets are a type of tablets which disintegrates in mouth within seconds and they give rapid relief to patients within minutes without use of water. The main objective of present study was to develop, formulate and evaluate mouth dissolving tablet of nonsteroidal anti-inflammatory drug. Meloxicam basically belongs to COX-1 inhibitor. The drug Meloxicam is used to provide rapid relief from chronic pain by inhibiting production of prostaglandin hormone. The mouth dissolving tablet of Meloxicam was prepared by direct compression method with an aim to check fast disintegration of tablet by using different concentrations of super disintegrants. The formulated mouth dissolving tablets were evaluated for weight variation, friability, hardness, in vitro disintegration time, in vitro dissolution time etc. Different concentration of super disintegrants like sodium starch glycolate (SSG) and maltodextrin (MDx) were used to find out which tablets batch disintegrates fast and will provide quick patient relief.

KEYWORDS: Mouth dissolving tablet, NSAIDS, Meloxicam, Super disintegrant.

INTRODUCTION:

Mouth dissolving drug delivery systems (MDDDS) are a type of new generation of formulations. They provide the convenience of a tablet formulation and also allow the ease of swallowing provided by a liquid formulation. MDDDS offer the advantage of much more accurate dosing than the conventional oral liquids. This segment of formulation is especially designed for dysphasia, geriatric, pediatric, bed-ridden, travelling and psychotic patients who are unable to swallow or refuse to swallow conventional oral formulations[1]. The tablet is the most widely used dosage form because of its convenience in terms of self- administration, compactness, and ease in manufacturing.

For the past one decade, there has been an enhanced demand for more patient- friendly and compliant dosage forms. As a result, the demand for developing new technologies has been increasing annually. Since the development cost of a new drug molecule is very high, efforts are now being made by pharmaceutical companies to focus on the development of new drug dosage forms for existing drugs with improved safety and efficacy together with reduced dosing frequency, and the production of more cost- effective dosage forms.

However, geriatric and pediatric patients experience difficulty in swallowing conventional tablets, which leads to poor patient compliance. To overcome this weakness, scientists have developed innovative drug delivery systems like "melt in mouth" or" mouth dissolve (MD)" or “dispersible” tablets. These are novel types of tablets that disintegrate/dissolve/disperse in saliva.

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The addition of superdisintegrant in many orally disintegrating tablet technologies based on direct compression affects the rate of disintegration and dissolution. The other ingredients in the formulation such as water-soluble excipients and effervescent agents further increase the process of disintegration. By addition of super disintegrants, the tablet having quick dissolving property which is needed for fast dissolving tablets. Some examples of super disintegrants are crospovidone, croscarmellose, spray dried lactose, microcrystalline cellulose etc[2].

The non-steroidal anti- inflammatory drugs (NSAIDs) are widely used for the treatment of minor pain and for the management of edema and tissue damage resulting from inflammatory joint disease (arthritis). A number of these drugs possess antipyretic activity in addition to having analgesic and anti-inflammatory actions, and thus have utility in the treatment of fever.

Meloxicam is a non-steroidal anti-inflammatory drug having poor solubility and low bioavailability. So this drug can be formulated as mouth dissolving tablet by addition of superdisintegrant.

MATERIALS AND METHODS:

Meloxicam drug was obtained as a gift sample from Zydus Cadila (Ahmadabad, India), Sodium starch glycolate, Talc, Microcrystalline cellulose and Calcium carbonate was obtained from Oxford laboratory (Mumbai, India), Maltodextrin was obtained from Hi Media Laboratories Pvt. Ltd, Aspartame was obtained from Zydus wellness Pvt. Ltd, Magnesium stearate was obtained from Loba chemicals (Gujarat) and Vanilla flavor was obtained from Asian chem. works Pvt Ltd.

Preformulation studies:

Preformulation studies are the first step in development of dosage form of a particular drug substance. It can be defined as investigation of physical and chemical properties of drug substances.

1. Colour and Odour:

Weigh accurately a small amount of drug on butter paper and visualize it physically for colour and odour.

2. Melting Point:

The melting point was determined by the capillary method using melting point apparatus. In this method one end of capillary tube was fused by heating it on Bunsen burner. Then capillary tube was filled by pressing the open end gently into meloxicam (pure drug) sample by tapping the bottom of the capillary on a hard surface so that the drug pack down into the bottom of the tube. When the drug was packed into the bottom of the tube, the tube was placed into the slot behind the eye-piece in the melting point apparatus. In other slot of melting point apparatus place a thermometer. Make sure the unit is plugged into the set to zero and then turn it on.

3. Solubility analysis:

Solubility may be defined as spontaneous interaction between two or more substances to form a homogeneous mixture. Qualitative determination of solubility of drug was calculated in different solvent like water, methanol, ethanol, 0.1 N HCl, Phosphate buffer (pH 6.8).

Table no 1: Solubility Profile according to BP

Descriptive term	Parts of solvent required for part of solute
Very soluble	Less than 1ml
Freely soluble	From1ml to 10ml
Soluble	From 10ml to 30ml
Sparingly soluble	From 30ml to 100ml
Slightly soluble	From 100ml to 1000ml
Very slightly soluble	From 1000ml to 10000ml
Practically insoluble	From 10000ml or more

4. Estimation of Absorption Maxima of Meloxicam in phosphate buffer (pH 6.8):

4.2 Calibration curve of Meloxicam in phosphate buffer pH-6.8:

The drug (Meloxicam) was accurately weighed 20mg using the digital weighing balance and the drug was transferred to the 100ml volumetric flask. This was dissolved by adding a 20ml of phosphate buffer pH 6.8 to get a stock solution- From above stock solution pipette out 1ml of the solution and dilute it up to 20 ml by adding phosphate buffer pH6.8 to obtain sub stock solution. From this sub stock solution pipette out 0.2ml, 0.4ml, 0.6ml, 0.8ml and 1ml and dilute it up to 10ml by adding phosphate buffer pH6.8 to obtain required concentrations of 2µg/ml, 4µg/ml, 6µg/ml, 8µg/ml, 10µg/ml, respectively.

5. Drug excipients compatibility:

FTIR spectra of the selected formulations were taken and compared with the spectrum of pure drug. The characteristic peaks of the drug were obtained by scanning in the range of 400-4000 cm-1 by using the spectrometer (Bruker-α-T, Germany)

6. Flow Properties of Drug:

6.1 Angle of Repose:

The angle of repose is measure of extent of inter particle forces or index of flow. The angle of repose was determined by fixed funnel method. Weigh accurately 5g of pure drug. Prepare a setup of funnel and tripod stand on a white paper. Then try to flow the weighed amount of drug through funnel on white paper. A heap of pure drug is obtained. With the help of pencil draw a circle round the heap (Diameter of circle). Then with the help of scale measure height of heap (Height). Then with the help of formula calculate angle of repose.

The radius of the base of the cone was measured. The angle of repose was measured using the following equation.

Angle of repose tan "Ɵ" = h/ r

Where, H = Distance between tip of funnel and the base,
R = Radius of the base of the cone.

6.2 Bulk Density (Db):

It is the ratio of total mass of powder to the bulk volume of powder. An accurately weighed quantity of powder, which was previously passed through sieve # 40 [USP] and carefully poured into graduated cylinder. Then after pouring the powder into the graduated cylinder the powder bed was made uniform without disturbing. Then the volume was measured directly from the graduation marks on the cylinder as ml. The volume measure was called as the bulk volume and the bulk density is calculated by following formula; [3]

$$Bulk\ Density = \frac{Volume\ of\ powder}{Mass\ of\ Powder}$$

Where, M is the mass of powder
V_b is the bulk volume of the powder.

6.3 Tapped Density:

Weight 5 g. of powder and placed in a measuring cylinder. Measuring cylinder containing known mass (5 gm) of powder was tapped for 100 times or fixed time. The minimum volume (Vt) occupied was measured. The tapped density was calculated using following formula.

$\rho t = M / Vt$

6.4 Compressibility Index

The simplest way for measurement of free flow of powder is compressibility, a indication of the ease with which a material can be induced to flow is given by Compressibility Index. The value below 15% indicates a powder with give rice to good flow properties, whereas above 25% indicate poor flowability. Which is calculated as followed. [4]

$\%\ C.I. = \rho t - \rho b\ \rho t \times 100$

6.5 Hausner' Ratio

The ratio between bulk density and tapped density of powder is known as Hausner's ratio.

$$Hausner'\ Ratio = \frac{Bulk\ Density}{Tapped\ Density}$$

Method of Preparation of Mouth Dissolving Tablet:

Mouth dissolving tablets of Meloxicam drug were prepared by direct compression method. In this method Meloxicam drug, sodium starch glycolate, maltodextrin, magnesium stearate, calcium carbonate, aspartame, talc and HPMC (E-15) were accurately weighed. All the ingredients were mixed with the help of pestle mortar. The formulation mixture were passed through sieve no # 20. Add a drop of vanilla essence to the formulation mixture. This mixture was converted to mouth dissolving tablet by punching with Shakti ten stations tablet punching machine. Formulations batches of tablet are shown in table no 1[5].

Table no. 2 Formulation batches of tablet

S. No.	Ingredients (mg)	F1	F2	F3	F4	F5	F6
1	Meloxicam	7.5	7.5	7.5	7.5	7.5	7.5
2	Sodium starch glycolate (SSG)	6	7	8	0	0	0
3	Maltodextrin (MD_X)	0	0	0	6	7	8
4	Magnesium stearate	1.5	1.5	1.5	1.5	1.5	1.5
5	$CaCO_3$	100	99	98	100	99	98
6	Aspartame	3	3	3	3	3	3
7	Talc	2	2	2	2	2	2
8	HPMC	30	30	30	30	30	30
9	Vanilla essence	qs	qs	Qs	qs	qs	Qs
	Total weight	150	150	150	150	150	150

Preformulation studies of meloxicam:

Meloxicam is a pale yellow coloured and odourless powder. It is freely soluble in phosphate buffer pH 6.8, slightly soluble in acetone and very slightly soluble in alcohol. The pure drug has melting point 240°C.

Evaluation parameters of tablet:

1. Weight variation:

Weigh accurately 20 tablets individually. Then average weight was calculated. Then comparison of weight of individual tablet with average weight of tablet is done. The percentage of weight variation is calculated by using formula:

$$Percentage\ weight\ variation = \frac{Individual\ weight - Average\ weight}{Average\ weight} \times 100$$

2. Thickness:

Tablet thickness is an important characteristic in reproducing appearance and also in counting by using filling equipment. Some filling equipment utilizes the uniform thickness of the tablets as a counting mechanism. Ten tablets were taken and their thickness was recorded using Vernier calliper[6]

Table no 3. Flow Properties of Drug

Drug	Bulk density (g/cm^3)	Tapped density (g/cm^3)	Angle of repose	Carr s index (%)	Hausner' s ratio
Meloxicam	0.467±0.09	0.521± 0.12	24.84± 0.53	14.82±0.39	0.896± 0.06

Table no 4: Weight variation limits for Tablets according to I.P.

S. No	Average weight of tablet (mg)	Maximum percentage difference allowed
1	130 or less	±10.0
2	130-324	±7.50
3	More than 324	±5.0

Table no: 5. various evaluation parameters of formulation batches

Formulation	Weight variation(mg)	Thickness (mm)	Hardness (kg/cm^2)	Friability (%)	Drug content (%)
F1	151.4±0.79	2.25±0.04	3.20±0.08	Pass	99.40±0.09
F2	148.2±0.14	2.20±0.12	3.43±0.10	Pass	99.01±0.07
F3	152.3±0.16	2.27±0.09	3.32±0.13	Pass	99.25±0.12
F4	150.4±0.64	2.21±0.17	3.12±0.06	Pass	99.3±0.15
F5	148.5±0.22	2.19±0.13	3.25±0.09	Pass	99.61±0.12
F6	149.8±0.30	2.22±0.07	3.31±0.12	Pass	99.34±0.07

3. Hardness:

Tablet hardness is measured with the help of hardness testers like Monsanto hardness tester and Pfizer hardness tester. A tablet is placed in the hardness tester and force required to crush the tablet is measured. The hardness of MDTs is generally kept lower than conventional tablets as increased hardness delays the disintegration of the tablet. A good compromise between mechanical strength and disintegration time is achieved for a satisfactory mouth dissolving formulation.

4. Friability:

Friability is a measure of mechanical strength of the tablet. If a tablet has more friability it may not remain intact during packaging, transport or handling. Roche friabilator is used to determine the friability by following procedure. Pre weighed tablets are placed in the friabilator.

Friabilator consist of a plastic chamber that revolves at 25 rpm, dropping those tablets at a distance of 6 inches with each revolution. The tablets are rotated in the friabilator for at least 4 minutes. At the end of test tablets are dusted and reweighed; the loss in the weight of tablet is the measure of friability and is expressed in percentage as:

$$\% \text{ Friability} = \frac{\text{Tablet weight before friability-tablet weight after friability}}{\text{Tablet weight after friability}} \times 100$$

5. Disintegration Time:

Six tablets were placed in each tube of disintegration apparatus. Phosphate buffer solution of pH 6.8 was placed in the basket and temperature was maintained at 37°C ± 2°C. The pH of the solution was checked with the help of pH meter. The time taken by tablets for complete disintegration was recorded.

6. In Vitro Dissolution Time:

In vitro dissolution study of mouth dissolving tablets of drug meloxicam was performed using phosphate buffer (pH 6.8) maintained at a temperature of 37±0.5oC in USP II dissolution test (Electro Lab Ltd., Mumbai, India. paddle type) apparatus and at rotation speed of 50rpm. At a predetermined time interval, samples were withdrawn and filtered through Whatman filter paper. Absorbance of suitably diluted samples was analysed by UV spectrophotometer at 360nm and the percentage of drug release was calculated. The dissolution experiments were conducted for about 20 to 25 minutes.[7]

RESULTS AND DISCUSSION:

Hardness:

The hardness of prepared formulation is shown in Table: 3. The hardness for mouth dissolving tablets of each formulation of Meloxicam was in the range of 3.12±0.06 to 3.43±0.10kg/cm2. This ensures good handling characteristics of tablets. (Monsanto hardness tester Rolex India)

Thickness:

The thickness of prepared formulation is shown in Table: 9. the thickness of mouth dissolving tablets of each formulation of Meloxicam was in the range of 2.20± 0.12 to 2.27±0.09mm. This ensures good handling characteristics of tablets.

Weight variation:

The weight variation of the prepared formulation is shown in Table: 9. The weight variation of mouth dissolving tablets of each formulation of Meloxicam was found in the range of 148.2±0.14 to 152.3±0.16mg; indicating that the weight variation of prepared formulations is within the acceptable limits. (Jyoti scientific industries Gwalior India)

Friability:

The friability of prepared formulation is shown in The percentage friability of mouth dissolving tablets of each formulation of Meloxicam was found in the range PASS; indicating that the friability of prepared formulations is within the acceptable limits and tablets are mechanically stable. (Friability test apparatus, Khera Instrument Pvt. Ltd. India.)

In vitro disintegration time:

The mouth dissolving tablets of Meloxicam got disintegrated rapidly within 15 seconds to 3minutes. Due to use of superdisintegrant there was fast disintegration. As a result patients will get relief from pain as soon as possible.[8]

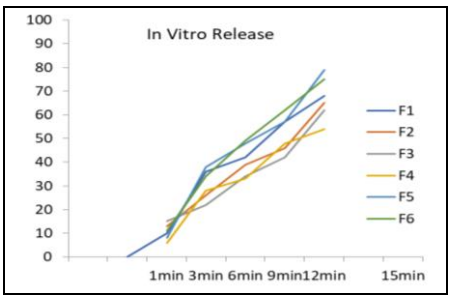

Figure No 1 In vitro drug releases of different batches

In vitro drug release:
The drug release study shows that as the concentration of super disintegrants increases the drug release rapidly. Also it was found that formulation batches of maltodextrin release drug faster as compared to sodium starch glycolate.

SUMMARY AND CONCLUSION:

The oral route of drug administration is the prominent route of drug administration because of ease of access and patient compliance. However, the route is disadvantageous for drugs which undergo significant first-pass metabolism and acid hydrolysis. Therefore, it is necessary to deliver drugs by other routes. Mouth dissolving drug delivery system is one of the easiest and convenient routes for the drug delivery.

Meloxicam is a non-steroidal anti-inflammatory drug (NSAID) with analgesic and antipyretic activity. It is an oxicam closely related to piroxicam, and fall in the enolic acid group of NSAIDs. It was developed by Boehringer-Ingelheim. Meloxicam starts to relieve pain about 30 to 60 minute after administration. It is mainly used in the treatment of rheumatoid arthritis, osteoarthritis and ankylosing spondylitis. Meloxicam is having a plasma half-life of 2-6 hrs with a dose of 7.5 to 15mg. The lowest effective dose should be used for each patient. Meloxicam therapy usually is started at 7.5mg daily. Some patients require a dose of 15mg daily, but this larger dose should be taken only under the direction of a physician. Juvenile rheumatoid arthritis is treated with 0.125mg/kg daily up to 7.5mg per day. Meloxicam may be taken with or without food. Since it is having low molecular weight and shorter half-life, it was selected as a model drug to develop mouth dissolving dosage form.

Mouth dissolving tablet of meloxicam was prepared by using HPMC E15 as a sustain release polymer and sodium starch glycolate and Maltodextrin as super disintegrating agent. Magnesium stearate and talc as lubricating agent. Mouth dissolving tablet prepared by direct compression method FTIR studies indicated that there was no drug-polymer and polymer-polymer interaction. The hardness for mouth dissolving tablets of formulation F1 to F6 was in the range of 3.12± to 3.43± kg/cm2. The thickness of mouth dissolving tablets of formulation F1 to F6 was in the range of 2.20±0.12 to 2.27±0.09 mm. The weight variation of mouth dissolving formulations from F1 to F6 was found in the range of 148.2±0.14 to 152.3±10.16 mg. The percentage friability of mouth dissolving formulations was found in the range of PASS and the percentage.

All prepared mouth dissolving tablets showed an acceptable range of weight variation, hardness, thickness, friability and drug content as per pharmacopoeia specification.

The increase in the concentration of polymer significantly increases the drug disintegration.
The in-vitro cumulative drug release profile of formulation F1, F2, F3, F4, F5 and F6 owed in the range of 49.08% to 99.61%, respectively in 12 hrs. Among these, the formulation F5 was found to release the highest percentage of drug (99.61±0.12%).

The dissolution profile of all factorial batches were fitted to various models such as kinetic of drug.
The stability studies of optimize formulation F5 had shown a good stability throughout the duration of studies in terms of drug content and in-vitro dissolution. In – vitro drug release was (99.61±0.12%).λ

Difficulty in the swallowing of conventional tablets by geriatric and pediatric patients may lead to poor patient compliance and ineffective therapy. To overcome such problems, a new dosage form has been introduced, known as fast-dissolving tablets.

This new dosage form provided the benefits of enhanced patient compliance, rapid onset of action, and increased bioavailability.

The novelty in this study is demonstrated in formulating meloxicam into fast-dissolving tablets. It is likely that a superdisintegrating medium, break, and dissolve quickly, resulting in fast release and rapid dissolution of the drug, and hence improved absorption and bioavailability

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RESEARCH ARTICLE

Assessment of Pharmacognostic Parameters and Antioxidant Potential of Bitter melon or Karela (*Momordica charantia* L.) fruits by DPPH Method

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ABSTRACT:

The aim of the present study was to investigate the different pharmacognostic parameters and to evaluate the antioxidant activity of the fruits of *Momordica charantia* L. by the DPPH method. Aqueous, ethanol, petroleum ether and chloroform extracts of the *Momordica charantia* L. fruits were prepared and subjected to determine physical constants and phytochemical screening (Qualitative Chemical Tests), which revealed the details of loss on drying, ash values, extractive values, fluorescence analysis and presence of phytoconstituents such as carbohydrates, proteins, steroids, terpenoids, glycosides, flavonoids, and lipids. The antioxidant activity of the *Momordica charantia* L. fruit extracts was also determined by the DPPH method using ascorbic acid as standard. The results obtained in this study support the use of *Momordica charantia* L. in herbal medicine and it can be used as a potent antioxidant in the treatment of many diseases resulting from more reactive oxygen species (ROS) presence.

KEYWORDS: Herbal medicine, *Momordica charantia* L., Antioxidant, Ascorbic acid DPPH, ROS.

INTRODUCTION:

In recent times natural products are becoming an integral part of human health care system, because there is a now popular concern over toxicity and side effects of modern drugs. There is also a realization that natural medicines are safer and allopathic drugs are often ineffective in several aliments. Medicinal plants existed even before human being made their appearance on the earth. Man's existence on this earth has been made possible only because of the vital role played by plant kingdom in sustaining his life. Since the down of civilization, in addition to food crops, man cultivated herbs for his medicinal needs[1].

Over the most recent couple of decades, there has been an exponential development in the field of homegrown medication. It is getting popularized in developing and developed countries owing to its natural origin and lesser side effects.

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In excess of 700 mono and polyherbal arrangements as decoction, tincture, tablets, and containers from in excess of 100 plants are in clinical use[2].

Momordica charantia L. is a medicinal plant belonging to the family cucurbitaceae, found in tropical and subtropical regions of the world such as India, Asia, South America, and widely used as food and medicine[3]. It is commonly known as Bitter gourd in English, Paakharkaai in Tamil, Karela in Hindi and Bengali, kakarakaya in Telugu and hagalakayi in Kannada[4].

Different arrangements of *Momordica charantia* L. extricate from organic product juice to dried natural product bits have been utilized generally around the world, especially for glucose bringing down effects[4,5]furthermore, it has been accounted for to show differing organic exercises, for example, cell reinforcement, antimicrobial, antiviral, antihepatotoxic and antiulcerogenic action.

Product of *Momordica charantia* L. contains terpenoids, steroids, heart glycosides, anthraquinine glycosides, saponins, flavonoids, tannins and phenolic compound, alkaloids. Free radicals play an important role in

development of tissue role and pathological events in living organisms[6,7]. There are evidences that explain that increased uptake of fruits and vegetables reduce the risk of cancer[8,9]. This is attributed by antioxidants presents in fruits and vegetables[10-14]. The present study was carried out to evaluate the antioxidant efficacy of aqueous, ethanol, pet. ether and chloroform extract of *Momordica charantia* L. fruit which helps in the development of new, novel drugs.

Figure 1: ***Momordica charantia*** **L. fruits**

MATERIAL AND METHODS:

Chemicals and reagents:

Solvents, chemicals and reagents of analytical grade or best possible grade supplied by Himedia Laboratories Pvt, Ltd., S.D. Fine Chemicals Ltd. India.

Collection of plant sample:

The Fruits of *Momordica charantia* L. were procured from local market Jaipur. Sample was shade dried at room temperature and powdered mechanically and passed through a sieve # 40.

Preliminary phytochemical investigation:

Extraction:

The air-dried parts of the plants were powdered and extracted with 95% ethanol, chloroform, pet ether (400-600) and aqueous solvent systems by hot percolation method by using Soxhlet apparatus assembly at a controlled temperature. After complete extraction, marc was squeezed to gather the micelle, blended in with the substance of RBF, separated and thought to get the concentrate. The shading and consistency of the concentrate were noted. This concentrate was additionally exposed to phytochemical examination[15,16].

Determination of physical constants:

Preliminary extraction of individual plant material (*Momordica charantia* L. fruits) is carried out with 95% ethanol using soxhlet extractor and then concentrated. The extract obtained is subjected for preliminary physicochemical investigation such as loss on drying (LOD), ash values (Total ash value, Water soluble ash value, Acid insoluble ash value), extractive values (Alcohol soluble extractive value, Water soluble extractive value) and fluorescent analysis.[17-23].

Qualitative chemical tests:

Synthetic tests are led on the concentrate of the plant test and furthermore of the powdered type of the plant tests utilizing standard techniques.

Tests for Carbohydrates:

The test arrangement was set up and exposed to the Molish's test, Fehling's test, Benedict's test, Barfoed's test, Cobalt-chloride test, Tests for Non-Reducing Sugars, The tannic basic analysis for starch tests.

Tests for Proteins:

The test arrangement was set up and exposed to the Biuret test, Million's test, Xanthoprotein test (For protein-containing tyrosine or tryptophan), Precipitation tests.

Tests for Steroids:

The test arrangement was set up and exposed to the Salkowski response, Libermann-Burchard test, Libermann's tests.

Tests for Amino Acids:

The test arrangement was set up and exposed to the Ninhydrin test, Test for Tyrosine, Test for tryptophan.

Tests for Glycosides:

The test arrangement was set up and exposed to the Baljet's test, Bromine water test, Legal's test (For cardenoloids), Test for deoxysugars (Kellar Killani test), Libermann's test for Cardiac Glycosides. The Borntrager's test, Modified Borntrager's test for anthraquinone glycosides. The Grignard's test for Cyanogenetic glycosides. The Foam test, Foaming list, Haemolytic test for Saponin Glycosides.

Tests for Coumarin Glycosides:

Test arrangement when made soluble watched for blue or green fluorescence.

Tests for Alkaloids:

The test arrangement was set up and exposed to the Dragendorff's test, Mayer's test, Hager's test, Wagner's test.

Tests for Flavonoids:

The test arrangement was set up and exposed to the Shinoda test and Ferric chloride test.

Test for Vitamins:

The test arrangement was set up and exposed to the Test for Vitamin A and Vitamin D.

Test for Saponins:
The test arrangement was set up and exposed to the Foam test, Haemolysis test, Test for steroidal saponins, Test for triterpenoid and saponins.

Test for Tannins and phenol mixes:
The test arrangement was set up and exposed to the 5% $FeCl_3$ arrangement, Lead acetic acid derivation arrangement, Bromine water test, Acetic corrosive arrangement, Dilute iodine arrangement tests[24-29].

Antioxidant activity by DPPH method:
All the extracts were tested for antioxidant activity by DPPH radical scavenging method. Serial dilutions were performed with the stock solution (10mg/ml) of all extracts of the plant (*Momordica charantia* L. fruits). Diluted solutions (2ml each) were mixed with DPPH (2 ml) and allowed to react. The UV absorbance was recorded at 517nm and the RC50 value was calculated in µg/ml for each extract. Ascorbic acid was used as standard antioxidant drug.

The percentage of DPPH scavenging activity was determined by; $A= (A_0- Ae) x100/ A_0$

Where, A represents a percentage reduction of the DPPH, A_0 is an initial or blank solution absorbance and Ae is an absorbance value for sample concentration in the absence of DPPH solution.

This activity also expressed as the inhibition concentration at 50% (EC50/ IC50/ RC50). The RC50/ EC50 value, defined as the amount of the sample sufficient to elicit 50% reduction of the initial DPPH concentration, was calculated from the linear regression of plots of concentration of test compounds (µg/ml) against the mean percentage of antioxidant activity obtained from the three replicate tests. The free radical scavenging activity of ascorbic acid (Vit. C) was also measured under the same condition to serve as positive control[30-36].

RESULTS:

Table 1: Results of estimation of physical characteristics of *Momordica charantia* L.

S. No.	Parameter	Physical characteristics of *Momordica charantia* L. fruit
1.	Loss on Drying	8% w/v
2.	Ash Value Total Ash Acid insoluble ash	 16.5 % w/w 4.5 % w/w
3.	Extractive Values Aqueous Alcohol	 36.65% 11.18%
4.	Fluorescence Analysis	Blue fluorescence

Table 2: Results of estimation of solvent used for extraction and % yield.

S. No.	Drug	Weight of drug Taken	Solvent	Volume of Solvent Taken	% yields after Extraction
1.	***Momordica charantia* L.**	900 grams	Petroleum ether	2.5 lit.	06.78
2.		900 grams	Chloroform	2.5 lit.	04.44
3.		900 grams	Ethanol	2.5 lit.	06.30
4.		900 grams	Aqueous	2.5 lit.	25.00

Table 3: Results of phytochemical estimation of *Momordica charantia* L.

S. No.	Test	Pet. Ether Extract	Chloroform Extract	Alcohol Extract	Aqueous Extract
I	Test for Carbohydrate				
A	Molish Test	-	-	-	+
B	Test for reducing sugars				
	Fehling Test	-	-	-	+
	Benedict test	-	-	-	+
C	Test for Monosaccharides				
	Barfoeds Test	-	-	-	+
D	Test for HexoseSugars				
	Cobalts Chloride test	-	-	-	+
E	Test for Non- Reducing Sugars	-	-	-	+
F	Test for Non- Reducing polysaccharide				
	Iodine test	-	-	-	+
	Tannic acid test	-	-	-	+
II	Test for Proteins				
	Biuret test	-	-	-	+
	Millon's test	-	-	-	+
	Xanthoprotein	+	+	-	+
	Test for proteins containing Sulphur	-	-	-	-
	Precipitation test	+	+	+	+
III	Test for Amino Acid				
	Ninhydrin test	+	+	+	+
	Test for tyrosin	-	-	-	+
	Test for tryptophan	-	+	+	+
	Test for cysteine	-	+	+	+

IV	Test for Steroids				
	Liebermann-Buchard	+	-	+	-
	Liebermann reaction	+	+	+	-
V	Test for Terpenoids				
	Liebermann-Buchard	+	+	+	-
	Liebermann reaction	+	+	-	-
VI	Test for Glycosides				
A	Test for Cardiac Glycoside				
	Baljet test	-	+	-	-
	Legal's test	-	-	-	-
	Test for deoxy sugar (Keller killani test)	-	-	-	-
	Liebermann's test (Bufadienolides)	-	-	-	-
B	Test for Anthraquinone glycoside	-	+	-	-
C	Test for Saponin Glycoside	-	+	-	-
D	Test for Coumarin Glycoside	-	-	-	-
VII	Test For Flavonoids				
	Ferric chloride test	-	-	-	-
	Shinoda test	+	+	-	+
	Alkaline reagents	+	+	-	+
	Lead acetate test	+	+	-	+
VIII	Test for alkaloids	+	+	-	+
IX	Test for Tannins and Phenolic cpd.	-	+	-	-
X	Test For Lipids	+	-	+	-

Antioxidant Activity by DPPH Method:

Anti-oxidant activity is carried on all the fractions of plant extract to assess their efficacy in tissue healing. The cell reinforcement action of antioxidative agents has been credited to different systems, for example, the anticipation of chain commencement, an official of progress metal particle impetus, deterioration of peroxides, and avoidance of proceeded with hydrogen deterrent, reductive limit, and radical rummaging. The maximum absorption of a stable DPPH radical in ethanol is at 517nm. The decrease in absorbance of DPPH radical caused by anti-oxidants is due to the reaction between anti-oxidants molecules and radical. Hence, DPPH is usually used as a substance to evaluate anti-oxidant activity.

The results as summarized in Table 4 also shows all the extracts of *Momordica charantia* L. exhibited degree of antioxidant activity.

Table 4: Results of estimation of antioxidant activity by DPPH method.

S. No.	Drug	Extract	RC50 value (µg/ml)
1.	*Momordica charantia* L.	Petroleum ether	058.98
2.		Chloroform	155.97
3.		Alcohol	097.13
4.		Aqueous	102.82
5.	Ascorbic acid	-	040.12

DISCUSSION:

Momordica charantia L. is a therapeutic plant from the Family Cucurbitaceae, used as a Indian traditional therapeutic agent. In light of the Phytochemical Investigation or Qualitative examination of *Momordica charantia* L. fruits the different physical parameters were evaluated. The present examinations were led to assess the 8%w/v loss on drying, ash values (16.5% w/w total ash and 4.5%w/w acid insoluble ash), extractive values (Aqueous 36.65%, Alcohol 11.18%). The Fluorescence Analysis has given blue coloured fluorescence which was observed under the UV radiation lamp to gain more details about the *Momordica charantia* L. fruits.

Therefore, chemical tests were performed on 4 various extracts of the fruit to estimate the presence of different Phytoconstituents as aqueous extract shows, it contains carbohydrates (Reducing sugars, Monosaccharides, Non-Reducing Sugars), proteins (proteins containing sulphur), amino acids (tyrosine, tryptophan and cysteine). Alcoholic, chloroform and pet. ether extract of fruit proved the presence of sterols, triterpenes, glycosides (cardiac glycoside, saponins glycoside, anthraquinones glycoside), flavonoids alkaloids, tannins, phenolic compounds and lipids.

The antioxidant potential was determined the DPPH method taking ascorbic acid as standard. All the four extracts of the *Momordica charantia* L. fruit have shown antioxidant efficacy in comparison to the standard drug (Ascorbic acid). The standard ascorbic acid has given the RC50 value for the DPPH method was 040.12µg/ml. The RC50 estimation of the prescription expels was viewed as chloroform 155.97µg/ml, watery 102.82 µg/ml, alcohol 097.13 µg/ml, and Petroleum ether 058.98 µg/ml, which shows the basic ability of *Momordica charantia* L. as a cell fortification pro.

CONCLUSION:

The extracts of *Momordica charantia* L. after concentration is first subjected for preliminary physical and phytochemical investigation to assess the quality of plant material and understand the nature of active constituent's present. After preliminary studies all the 4

extracts were subjected for antioxidant activity by DPPH method to guide us in the selection of extract fraction which will probably has the desired activity. Therefore *Momordica charantia* L. fruit can be used as a potential source for the development of an antioxidant agent.

Results of phytochemical investigation revealed the presence of various Phytoconstituents like glycosides, carbohydrates, proteins, amino acids, sterols, triterpenes, cardiac glycosides, flavonoids and lipids.

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We wish to affirm that there are no known irreconcilable circumstances related to this production and there has been no huge budgetary help for this work could have impacted its result.

CONFLICT OF INTEREST:

The authors declare no conflict of interest.

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RESEARCH ARTICLE

Hepatitis B and C among blood donors in Tartous city, Syria. A Retrospective study

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ABSTRACT:

Aim of study: This study is the first done in the blood bank of Tartus to study the percentage of infected donors with hepatitis B and C. The aim was to determine the serological prevalence of Hepatitis B and C infection among Syrians, and factors that increase the risk of infection. **Method of study**: The subjects were blood donors at the Blood Transfusion Center in Tartus city from 02/01/2017 to 31/12/2017, all personal data of donors relating to age, gender, professional status and medical. history were recorded. After collecting blood units, a blood sample of 5 ml was taken in the EDTA tube for testing, using Enzyme -Linked Immunosorbent Assay (Biokit®, Spain), we calculated the value of CUT-OFF according to the manufacturer's instructions. at any positive result, the tube was retested in duplicate before final interpretation. The statistical comparison was conducted and the p value of <0.05 was considered statistically significant. **Results**: The study showed a significant decrease in the number of donors with hepatitis B and C in Tartus governorate, which makes this percentage the lowest in Syria and developing countries, and reduce state spending on patient's treatment. **Conclusion**: The study emphasizes the importance of spreading the culture of blood donation because, in addition to the fact that blood donation is a humanitarian duty, tests performed free in blood banks, may alert us early to infection with these diseases, and the results are reported to the Ministry of health, which may be remedied in the premiere stages.

KEYWORDS: Hepatitis B Virus (HBV),: Hepatitis C Virus (HCV), Hepatitis B Surface Antigen (HbsAg), blood donors

INTRODUCTION:

Hepatitis B and C continue to be an important cause of morbidity and mortality around the world. Several local and international studies have been conducted involving population groups in major cities, leaving knowledge gaps in relation to relatively smaller cities, such as Tartous city. Numerous studies have shown that higher prevalence of hepatitis B virus positive factor in blood donors reflects an increase in infection rates in patients receiving recurrent blood.[1,2] There are 2 billion people living with hepatitis B, with an estimated 360 million people having chronic hepatitis B and around 600,000 deaths each year from either HBV-related liver disease or hepatocellular carcinoma worldwide.[3]

13 million people are infected with chronic hepatitis B virus in the European Union and about 15 million others are chronically infected with hepatitis C[4] The World Health Organization's 2017 World Hepatitis Report suggests that the vast majority of these people are unable to access life-saving tests and treatment, and as a result, millions of people are at risk of slow progression of chronic liver disease, cancer and mortality.[5]

The hepatitis B vaccine was included in the national vaccination program in Syria during the early 1990s based on a three-dose regimen given after birth, in the third and in the seventh month.[6] Surface antigen antibodies that protect against the surface antigen in 90% of immunocompetent individuals appear after the three doses.

MATERIAL AND METHODS:

The is a retrospective cohort study that included all 24,166 blood donors in the blood transfusion center of

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Tartous city during the year of 2017. Personal data were recorded for all donors related to age, gender, professional status and their medical history. After a clinical examination to ensure the ability to donate, donor was directed to the blood-picking room where a blood unit was harvested, and a blood sample of 5 ml was taken in an EDTA tube for laboratory tests, using the ELISA method, and kits from BIOKIT company, Spain. The cut-off value was calculated based on the manufacturer's instructions, and at any positive result, the tube was re-examined doubly, before the final interpretation of the results.

Blood donors were considered a random sample representing the entire community of Tartous city, because the donation includes all population groups from the age of 18 to 60, and most of them donate their blood voluntarily, in addition to other categories (blood donors for a specific person, youth at the age of recruitment, young university graduates, and citizens who want to obtain driving licenses). We excluded phlebotomy patients, as their blood is drained and not utilized, and no studies are conducted for blood safety checks for them, we also excluded the destroyed blood units due to poor manufacturing, excess weight or hemolysis.

RESULTS:

Screening of hepatitis B virus:

Among the 24,166 donors, the serological HbsAg tests were negative for 24030 subjects, which constitutes the vast majority of the studied donors group (99.4%), while the number of patients who had positive HbsAg tests reached 136, or 0.56% of the donors.

Classification of HBV patients according to gender:

Results show that 92% of the infected donors are male, while the percentage of infected females is about 8% of the donors, and this may be due to the fact that males are more susceptible to infection than females because of the nature of life and wrong habits such as the use of non-sterile shaving tools. When comparing the numbers of infected donors, 4264 women were HbsAg negative among all 4275 female blood donors, and the number of infected women was 11, while the number of infected males who had HbsAg positive tests was 125 among all 19891 male blood donors. As it is demonstrated in the following table:

Table (1): Classification of HBV patients according to gender

	Male	Female
HbsAg positive	125	11
HbsAg negative	19766	4264
Total	19891	4275

Classification of HBV patients according to age:

When HBV infected subjects were divided into two age groups, the first for those born before 1993, and the second for those born later on, we observe the following table:

Table (2): Classification of HBV patients according to age

Groups according to age	Number	Percentage
Born before1993	113	%83.1
Born before1993	23	%16.9
Total	136	P<0.0001

This table is important to illustrate the effectiveness of the national vaccination program against hepatitis B that was implemented in Syria starting in 1993. 83.1% of total infected subjects were born before 1993, this high percentage is related to the fact that they did not receive the vaccination against HBV, with a significant correlation (P <0.0001). The table shows a significant decrease in the number of infections for ages 24 and below, 16.9% (23 out of 136 infected people), which may be attributed to failure to complete the vaccination program by the parents (three doses) or that this strain carry mutations on the gene of epitope regions of surface antigen[5], or have impaired immune response mainly among these people for different reasons.

Screening of hepatitis C virus:

The data of blood transfusion center in Tartous city show that among 24,166 donors, tests were negative for anti-HCV antibodies for 99.52% of total donors, while the percentage of infected individuals who had positive antibodies reached 0.48%.

Table (3): Screening of hepatitis C virus

	HCV positive	HCV negative	Total
Number	116	24050	24166
Percentage	0.48%	99.52%	P<0.0001

Classification of HCV patients according to gender:

The table shows that 92% of infected subjects are male, and this result is similar to hepatitis B data. The difference in ratios is statistically significant (P <0.05).

Table (4): Classification of HCV patients according to gender

	Male	Female
HCV positive	107	9
HCV negative	19784	4266
Total	19891	4275

DISCUSSION:

Our study included 24,166 blood donors at Tartous Blood Transfusion Center during year of 2017, to shed light on the spread of hepatitis B, C infection. Donors' blood safety tests were performed and HBsAg surface antigen was investigated, of whom 19891 (82.3%) were males and 4275 (17.7%) were females.

We found that 24030 (99.44%) citizens were HbsAg negative and 136 (0.56%) cases were HbsAg positive, divided into 125 (0.56%) infected males and 11 (8%) infected females. This result may be explained by the fact of wrong habits and life style of males such as the use of non-sterile shaving tools as well as tattoo tools in some centers and beauty salons, where these centers are not subject to health control standards.

When the donors were divided into two age groups, the first was born before 1993 (who did not receive the national vaccination against hepatitis B) and the second category was born after 1993 and they are all supposed to have undergone this program. Hepatitis B there were 113 people (83.1%) who were born before 1993, and 23 people (16.9%) were born after 1993, (P <0.0001), which may be attributed either to neglect by the family and health authorities in the Ministry Health that does not have a mandatory mechanism for imposing vaccines and punishing those left behind for the fact that infection with these diseases is an economic burden and Large community, or it may be due to missing one of the three required doses of the vaccine against the surface antigen of the virus, or that immunization was not effective.[6]

The fact that 23 infected persons born after 1993 out of 24,166 people (0.01%) are relatively small, and confirm that these cases are individual cases that may be easily explained by the responsible authorities in the Ministry of Health, which is supposed to be entrusted with the task of communicating with patients and interrogating them medically, and not only Inform them of the results of laboratory tests - as is currently done - in order to draw conclusions and take lessons and avoid them in the future.

A study carried out by the Ministry of Health in the governorates of Damascus and Aleppo in 2004 showed 3168 individuals that the prevalence of infection in Damascus is about 5.6% for hepatitis B and 2.8% for hepatitis C and there was a clear geographical difference with a greater prevalence in Aleppo, where It reached 10.14% for hepatitis C and 10.5% for hepatitis B, and Al-Jazeera by more than 10% for hepatitis B, while in our study the percentage did not exceed 0.56% for HBV and 0.48% for HCV.[7]

In a study conducted in Iran[8], the prevalence of hepatitis B was 3%, the distribution of which showed that hepatitis C virus prevalence varied in different governorates from 0.87% to 8.86%. The hepatitis B virus rate was higher in Golestan (8.86%) and lower in the Kurdistan Region (0.87%).

A European study showed that the prevalence of hepatitis B virus and hepatitis C virus is low to moderate in most of the European Union / EEA countries, but the situation is diverse and dynamic, due to the arrival of immigrants, national estimates of seroprevalence in the general population vary from 0.1% to 4.4% for HBV, and from 0.1% to 5.9% for HCV. [9]

CONCLUSION:

Applying surveillance for infection cases to direct public health policies is very important to control the spread of viral hepatitis and this requires the availability of epidemiological data, which confirms the urgent need for credible data from serological surveys on a large scale which is present in the current study.

Improvisation sterilizes medical tools used in circumcision, tattoo and ear piercing, as well as cupping in addition to the dangers of digging containers - the fact that garbage in our country is not subject to scientific standards regarding medical waste - including needles from pharmacy waste and others, to a great risk in the transmission of infection, and must In addition to avoiding these matters, it is necessary to require dentists to investigate the accuracy required to sterilize their tools, as well as hairdressers and popular cleansers.

The HBV vaccine is considered one of the best means to protect society from hepatitis B, especially in areas with medium and high epidemiology and is given in three doses. The level of response and the formation of high protective antibodies in children reach more than 95% and about 90% at the middle age, which indicates the necessity of vaccinating all the newborns with the hepatitis B vaccine in the first 24 hours of the child's age and adherence to the three doses, as well as spreading awareness in the community about prevention methods, especially among high-risk groups, and the necessity of remedial vaccinations for those who missed the vaccination program in Syria.

CONFLICT OF INTEREST:

The authors declare no conflict of interest

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RESEARCH ARTICLE

Determination of Montelukast by New Spectrophotometric Method using Bromocresol Green

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ABSTRACT:

Simple, sensitive and accurate spectrophotometric method has been developed for the determination of Montelukast Sodium in raw and tablets formulation. The method is based on the formation of yellow ion-pair complex between Montelukast Sodium and Bromocresol green in Dichloromethane medium. The absorption maximum of the complex was found to be at 405 nm. Different parameters affecting the reaction such as: effect of solvents, reagent concentration, correlation ratio, etc. were carefully studied and optimized. The formed complex was quantified spectrophotometrically at absorption maximum. Linearity range was 2 – 19 µg/mL, regression analysis showed a good correlation coefficient R^2 = 0.9998. The limit of detection (LOD) and limit of quantification (LOQ) were to be 0.50 µg/mL and 1.51 µg/mL. The developed methods could be successfully applied to pharmaceutical formulations. The proposed method is simple, direct, sensitive and do not require any extraction process. Thus, this method could be readily applicable for the quality control and routine analysis.

KEYWORDS: Montelukast Sodium, Bromocresol Green, Spectrophotometric method.

INTRODUCTION:

Montelukast Sodium (MTK) Fig. (1, a) is chemically a Sodium[1-[[[(1R)-1-[3-[(E)-2-(7-chloroquinolin-2-yl)ethenyl]phenyl]-3-[2-(1-hydroxy-1-methylethyl)phenyl]propyl]sulfanyl]methyl] cyclopropyl]acetate[1]. Which is a leukotriene receptor antagonist used as an alternative to anti-inflammatory medications in the management and chronic treatment of asthma and exercise-induced bronchospasm (EIB)[2]. It is usually administered orally. Montelukast is a CysLT1 antagonist; it blocks the action of leukotriene D4 (and secondary ligands LTC4 and LTE4) on the cysteinyl leukotriene receptor CysLT1 in the lungs and bronchial tubes by binding to it**.** This reduces the bronchoconstriction otherwise caused by the leukotriene and results in less inflammation[3].

Several methods have been reported in the literature for the analysis of Montelukast Sodium such as Liquid chromatographic (LC)[4-5], High performance liquid chromatographic (HPLC) and high-performance thin layer chromatographic (HPTLC)[6-7], Voltammetric method[8-9], Spectrophotometric method[10-11], Reverse phase high performance liquid chromatographic (RP-HPLC) method[12-13], Ultra Performance Liquid Chromatography (UPLC) method[14].

Bromocresol Green (BCG) as shown in Fig. (1, b) is a sulphonphthalein dye commonly used as indicator and spectrophotometric reagent.

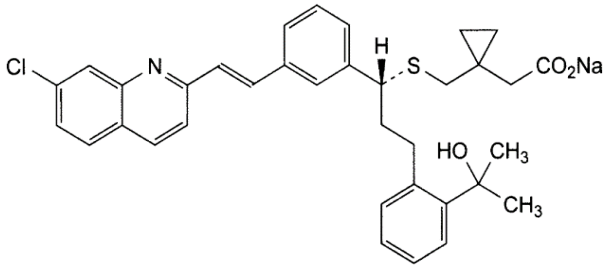

Fig. (1, a): Structural formula of Montelukast Sodium.

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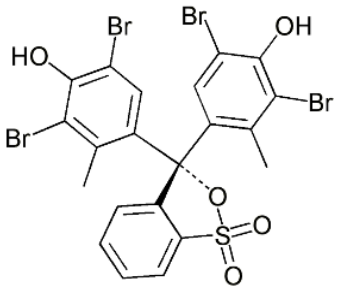

Fig. (1, b): Structural formula of Bromocresol Green.

MATERIALS AND METHODS:

Apparatus:

All spectral measurements were carried out using a Spector Scan T80+, UV/Vis spectrophotometer instrument Ltd (UK), connected to computer, quartz cells 1 cm. Ultrasonic processor (Hwashin Technology, Power sonic 405, KOREA), and analytical balance (Sartorius, model 2474, GERMANY).

Chemical regents:

Dichloromethane from Riedel-de Haën (GERMANY) was used to prepare the solutions. Pharmaceutically pure samples were Montelukast Sodium, purity 99.6 %, was obtained from (JAYCO CHEMICAL, INDIA). Bromocresol Green from (BDH, ENGLAND).

Pharmaceutical formulations:

The listed commercial products were subjected to analytical procedure:

- Asmalair® tablets, UNIPHARMA pharmaceutical company, Damascus – Syria, labeled 4,5,10 mg/tab of Montelukast.
- Lukast® tablets, ALFARES pharmaceutical company, Damascus – Syria, labeled 4,5,10 mg/tab of Montelukast.
- Monkast® Tablets, PHARMASYR pharmaceutical company, Damascus – Syria, labeled 4,5,10 mg/tab of Montelukast.

Standard preparation:

Montelukast Sodium stock solutions:

Stock solutions 1.64×10^{-3} M of Montelukast Sodium (M_W = 608.169g/mol) was prepared by dissolving 10mg of pharmaceutically pure raw material sample equivalent to 10.04mg (by taken the purity in consideration) in volumetric flask 10mL of Dichloromethane, then 1mL of the solution was taken to volumetric flask 10mL and diluted with Dichloromethane to give concentration 1.64×10^{-4} M equivalent to 100μg/mL. The working standard solutions of Montelukast Sodium samples were prepared by appropriate dilutions among (100 - 950)μL of 100μg/mL in volumetric flask 5mL and added 1mL of Bromocresol Green 2.4×10^{-3} M then completed to volume with Dichloromethane to give concentrations between (2 - 19)μg/mL of Montelukast Sodium.

Reagent stock solution:

Bromocresol Green 2.4×10^{-3} M was prepared by dissolving 42.73mg of pure Bromocresol Green (M_W = 698.014g/mol) in volumetric flask 25mL and completing to volume with Dichloromethane.

Calibration Curve:

To construct the calibration curve, five standard solutions for each concentration were prepared and the absorbance was measured of each solution five times.

Sample preparation:

Ten tablets weighed and finely powdered and an accurate weight equivalent to labeled content (4, 5 or 10 mg/tab) was weighed accurately, dissolved in volumetric flask 25ml of Dichloromethane and sonicated for 15 minutes. Then sample was filtered by using BÜCHNER funnel. After that, further dilutions were made to obtain an appropriate concentration (among the range of linearity) and then the general procedure was conducted.

RESULTS AND DISCUSSION:

Montelukast Sodium forms with Bromocresol Green at 25±5ºC yellow ion-pair complex and Stability of the complex was 24 hours. The result solution was scanned in the range of wavelengths 300 - 550nm against a blank of BCG prepared in Dichloromethane, and then measured the absorbance at maximum wavelength 405 nm. We studied all the parameters of the colored result solutions to obtain the optimal conditions. Fig. 2 shows the spectrum of complex between Montelukast Sodium and Bromocresol Green in Dichloromethane medium.

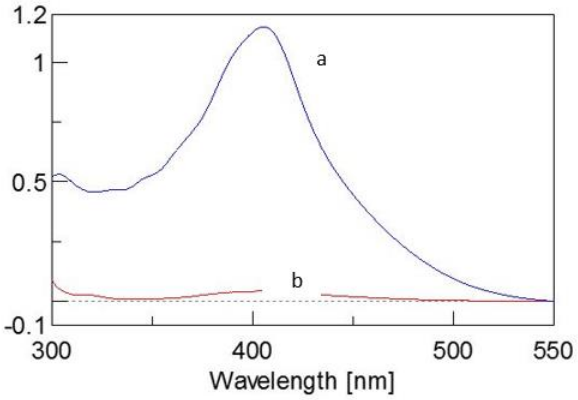

Fig. 2: a- Spectrum of complex MTK-BCG in Dichloromethane medium for MTK (2×10^{-5} M).
b- Spectrum of BCG (1×10^{-4} M) in Dichloromethane medium.

Effect the kind of solvent:

In order to select a suitable solvent for preparation of the reagent solutions used in the study, the reagents were prepared separately in different solvents such as aceton, chloroform, dichlorometane and ethyl acetate, and the reaction of MTK and BCG was followed. Dichlorometane was preferred as the most suitable

solvent because in this medium, the reagent blank gave negligible blank absorbance and the formed ion-pair complex was found to exhibit higher sensitivity and stability. In other solvents, the reagent blank yielded high absorbance values.

Effect of reagent concentration:

To study the effect of reagent concentration on the colored complex solution, we made a series of 10mL of separated volumetric flasks, by adding 1mL of Montelukast Sodium 2×10^{-4} M equivalent to 20µM and added between (0.025 – 2.5mL) of (BCG) 1×10^{-3} M, equivalent to (5 - 500µM), and completed to 10mL by Dichloromethane. The absorbance at 405nm for every added (BCG) reagent was measured against the blank of Dichloromethane. It was found that the completed colored complex formation was 1.5mL of (BCG) solution. The best concentration addition of (BCG) was 300µM with fifteen times of Montelukast concentration.

Stoichiometric Relationship:

The stoichiometric ratio between drug and dye in the complex MTK-BCG was determined by Molar ratio method and Job's method of the continuous variation method as following:

1. **Molar ratio method:**

We have prepared a series of complex solutions MTK-BCG in the medium of the Dichloromethane. The concentration of Montelukast Sodium changes within the ratio (7.83×10^{-6} – 7.83×10^{-5}) M while the concentration of the reagent was constant in each solution and equal to 3.132×10^{-5} M. We measured the absorbance values of these solutions at the wavelength of the maximum absorbance 405nm against the blank of BCG in Dichloromethane. The absorption changes of the molecular ratio of the Montelukast to the reagent permitted us to measure correlation ratio, we obtained the curve A = f([MTK]/[BCG]) shown in Fig. (3, a) where the correlation ratio is (1:1).

2. **Job's method of the continuous variation:**

We have prepared a series of complex solutions MTK-BCG in the medium of the Dichloromethane. The concentration of the reagent and the concentration of Montelukast Sodium changes in solutions between (0 - 4×10^{-5} M) where the sum of both concentrations remains constant and equal to 4×10^{-5} M. We plotted the spectral curves of each solution at the wavelength 405 nm and plotted the absorption changes of the solutions of the formed complex in terms of molecular fraction of Montelukast Sodium. We obtained the curve A = f([BCG] /{[BCG] + [MTK]}) shown in fig. (3, b) where the correlation ratio is (1:1).

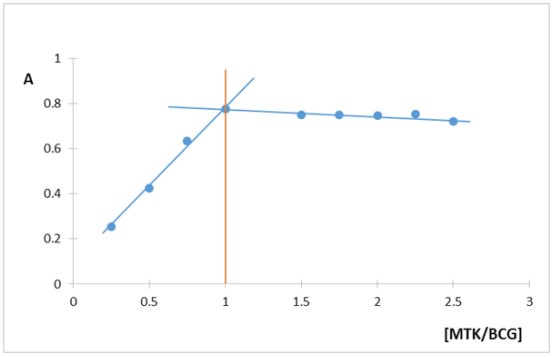

Fig. 3, a: molar ratio of MTK-BCG complex (1:1).

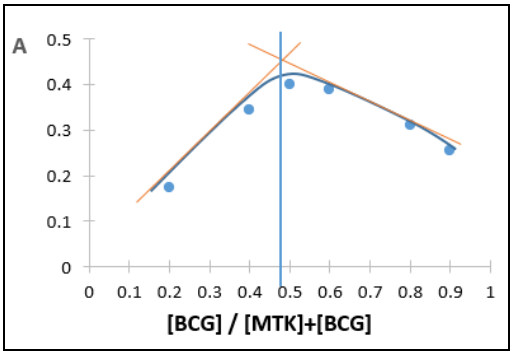

Fig. 3, b: Job's method of the continuous variation of MTK-BCG complex

Method validation:

The objective of validation of an analytical procedure is to demonstrate that it is suitable for its intended purpose. The objective of the analytical procedure should be clearly understood since this will govern the validation characteristics which need to be evaluated. Typical validation characteristics which should be considered are accuracy, precision, detection limit, quantitation limit, linearity and range.

Range and linearity of Montelukast Sodium:

We studied the linearity of Montelukast Sodium concentrations at the optimal conditions where we made a series of 5mL of separated volumetric flasks, each one contains the equivalent volume of BCG 2.4×10^{-3} M, and Montelukast Sodium stock solution 1.6×10^{-4} M, and completed to 5mL with Dichloromethane, finally we measured the absorbance at 405nm for each concentration against the blank of BCG in Dichloromethane. Fig. 4 presents the Montelukast Sodium spectra. The range of linearity was obeyed to Beer's law in concentration (2 - 19) µg/mL and the linearity curve is presented in Fig. 5.

Accuracy and precision:

To determine the precision and accuracy of the proposed method, five replicate determinations were carried out on three different concentrations of standards (MTK). The validation results are shown in table 1.

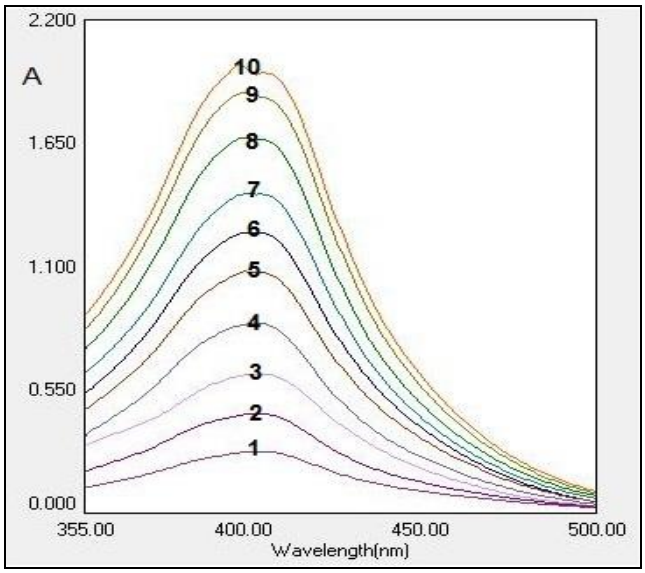

Fig. 4: spectra of (MTK-BCG):
C_1: 2 μg/mL, C_2: 4 μg/mL, C_3: 6 μg/mL
C_4:8 μg/mL, C_5:10 μg/mL, C_6: 12 μg/ mL
C_7:14 μg/mL, C_8: 16 μg/ mL, C_9: 18 μg/ mL
C_{10}: 19 μg/ mL.

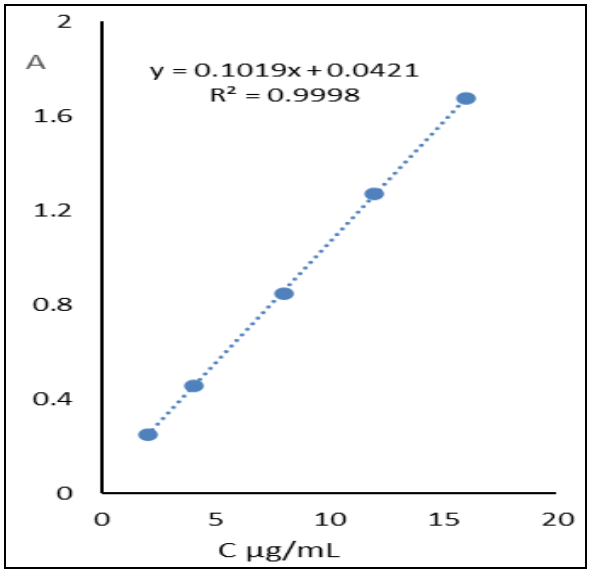

Fig. 5: Calibration curve for (MTK-BCG):
C_1: 2 μg/mL, C_2: 4 μg/mL, C_3: 8 μg/mL
C_4:12 μg/mL, C_5:16 μg/mL.
n = 5 for each concentration.

Table 1: Precision and accuracy for determination of Montelukast Sodium.

Theoretical concentration (µg/mL)	intra-day n=5				inter-day n=5			
	$\bar{x}$ found concentration (µg/mL)	SD µg/mL	Precision (RSD %)	Accuracy (%)	$\bar{x}$ found concentration (µg/mL)	SD µg/mL	Precision (RSD %)	Accuracy (%)
6	5.86	0.08	1.36	97.66	5.85	0.14	2.39	97.57
13.5	13.50	0.21	1.55	100	13.56	0.06	0.44	100.44
17	17.07	0.36	2.10	100.41	17.18	0.11	0.64	101.06

$\bar{x}$: mean of five replicated determinations, Accuracy (%) = (observed concentration/theoretical concentration) × 100,

Precision (RSD %) = (standard deviation/mean concentration) ×100.

Detection limit, Quantitation limit and Sensitivity Sandell's:

The mean molar absorptivity ε, Sandell's sensitivity, limit of detection (LOD) and limit of quantification (LOQ) were calculated from the standard deviation of the absorbance measurements obtained from Beer's law and are presented in table 2.

Table2: Analytical parameters of Montelukast Sodium determination

Parameter	Value
λ_{max} (nm)	405
Beer's law limits (µg/mL)	2 – 19
Stability of the complex	24 hours
Temperature of solution	25 ± 5 ºC
Solvent	Dichloromethane
C_{BCG}: C_{MTK} , M	≥15
Molar absorptivity, ε (L/mol.cm)	65550
Regression equation	Y = 0.1019X + 0.0421
Slope (b)	0.0421
Intercept (C)	0.1019
R^2	0.9998
LOD (µg/mL)	0.50
LOQ (µg/mL)	1.51
Sandell's sensitivity SS (µg/cm^2)	0.0092

RECOVERY:

The recovery was studied by three addition standards for every product. Table 3 presents the recoveries result for the two products (Asmalair UNIPHARMA 4mg and Lukast ALFARES 5mg).

Application of the method: Estimation of Montelukast Sodium in nine tablets Syrian products:

The developed method was applied for quantitative determination and identification of Montelukast Sodium in nine Syrian pharmaceutical products. The samples were prepared as described in the section of samples preparation and analyzed. Quantitative analysis was done by using calibration curve. The obtained results are summarized in table 4 for all products.

In general, the concentrations of the detected Montelukast Sodium compounds in the products were within in the allowed limits under BP legislation (the tablets must contain not less than 98.00 percent and not more than 102.00 percent of labeled amount). Therefore, the obtained results are conformed to BP legislation1.

Table 3: Recoveries for Asmalair UNIPHARMA 4 mg and Lukast ALFARES 5 mg Syrian products.

product name	Dosage as montelukast sodium	Sample µg/mL	Added µg/mL	Total Found $\bar{x}$ µg/mL	Recovery %	SD µg/mL	RSD%	Recovery Average %
Asmalair 4	4.2 mg/tab	**6.16**	4.8	10.96	100.00	1.28	1.28	100.05
			6	12.14	99.74	1.09	1.09	
			7.2	13.39	100.42	0.90	0.90	
Lukast 5	5.2 mg/tab	**6.212**	4.8	11.07	101.30	1.84	1.82	100.75
			6	12.24	100.50	1.40	1.39	
			7.2	13.44	100.46	1.28	1.28	

$\bar{x}$ Mean for five separate determinations were performed and calculated the mean.

Table 4: Results of Montelukast Sodium in Syrian pharmaceutical products.

product name	Label claim (Montelukast Sodium)	Found Concentration $\bar{x}$ mg/tablet	SD mg/tablet	RSD %	Per %
Asmalair 4	4.2 mg/tab	4.18	0.016	0.39	99.52
Asmalair 5	5.2 mg/tab	5.22	0.085	1.63	100.38
Asmalair 10	10.4 mg/tab	10.49	0.186	1.77	100.86
Lukast 4	4.2 mg/tab	4.26	0.046	1.085	101.43
Lukast 5	5.2 mg/tab	5.23	0.081	1.56	100.58
Lukast 10	10.4 mg/tab	10.49	0.110	1.05	100.86
Monkast 4	4.2 mg/tab	4.26	0.043	1.02	101.43
Monkast 5	5.2 mg/tab	5.24	0.104	2.00	100.77
Monkast 10	10.4 mg/tab	10.61	0.103	0.975	102.02

$\bar{x}$ Mean for five replicates.

CONCLUSION:

We developed a new method was applied for the identification and quantification of Montelukast Sodium in Syrian tablets formulation. A good percentage of recovery shows that the method can be successfully used in routine analyses. The proposed method is simple, sensitive, rapid, a little cost and could be applied for quality control of Montelukast Sodium. The levels of Montelukast Sodium compounds were within the permissible limits set by the BP legislation[1].

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RESEARCH ARTICLE

In vitro Antimicrobial and Antitubercular screening of newly synthesized Mercaptobenzimidazole –clubbed chalcone derivatives

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ABSTRACT:
In this present investigation to studies of mercaptobenzimidazole-clubbed chalcone derivatives was efficiently synthesized, which is further characterized with the help of thin layer chromatography, spectroscopy techniques such as FTIR and ^{1}HNMR. The newly synthesized derivatives were examined against in vitro antimicrobial and antitubercular activities. The result revealed that the titled compounds an average antibacterial activity against gram-positive *Bacillus subtilis*, gram-negative *Escherichia coli* as compared to standard ciprofloxacin. Compounds DPK4B2d2, DPK4B2d3, DPK4B2d4 and DPK4B2d6 shown potent antifungal activity against *Aspergillus niger* as compared to standard fluconazole. Antitubercular activity of the synthesized compounds examined against *Mycobacterium tuberculosis*, compounds DPK4B2d1 and DPK4B2d2 shown potent activity in the comparison of standard such as Pyrazinamide, Ciprofloxacin, and Streptomycin.

KEYWORDS: Mercaptobenzimidazole, Chalcone, Antimicrobial, Antitubercular.

INTRODUCTION:
In the 20th century chemotherapy has revolutionized the treatment of infective diseases since the innovation of antibacterial dyes by Paul Ehrlich, covered the way to a great victory for human health and long life. The development of resistance against currently used antimicrobial drugs led to an invigorated curiosity of the researchers in infective diseases to develop new chemical entities to battle them[1-3]. Patient morbidity, costs of treatment, rates of hospitalization, and use of broad-spectrum agents are remarkably increased by antimicrobial resistance[4-6].

Tuberculosis is a deadly disease usually caused by *Mycobacterium tuberculosis*. It has killed an estimated one billion people beyond the preceding two decade and even remains the top ten reasons of death in the world. According to the 2018 report of WHO, 5,58,000 people developed rifampicin-resistant (RR TB), multidrug-resistant tuberculosis (MDR-TB) or extensively drug-resistant (XDR TB) in the world.

Therefore, it is essential to develop rational chemotherapeutic agents to deferral the development of resistance and, ideally, shorten the period of therapy of this infection[7-9].

Benzimidazole is a lead molecule for the most of the biological agent use in the pharmaceutical industry. It consists of fused benzene ring with heterocyclic aromatic imidazole. The existence of imidazole creates it a resourceful heterocycles with an extensive range of biological activities such as antiulcer (Gastric H^{+}/K^{+}-ATPase inhibitors), antihypertensive, anti-inflammatory, anticonvulsant, analgesic, antiprotozoal, antitrichinellosis, antidiabetic, anti-HIV, antimicrobial, antitubercular, anticancer, antihistaminic, antioxidant, antiviral**,** antiparasitic agents, diuretic, and DNA binding activities[10-23].

Encouraged by the upstairs findings and in the persistence of our work on synthesis and in vitro antimicrobial and antitubercular screening of newly synthesized mercaptobenzimidazole –clubbed chalcone derivatives.

MATERIALS AND METHODS:
The chemicals of analytical grade required for the synthesis of mercaptobenzimidazole–clubbed chalcone derivatives were purchased from Sigma-Aldrich and SD fine chemicals (India). The procured novel synthesized

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compounds having purity and homogeneity preliminarily checked by determining melting points and were uncorrected. Progress of chemical reaction was authenticated by the thin layer chromatography study and spots were visualized in UV chamber or iodine chamber. FTIR spectra of intermediate and derivative compounds were recorded with help of pressed pellet technique on Jasco FTIT-460 plus spectrophotometer and vibrational frequencies expressed in cm^{-1}. Also, skeleton structure of synthesized derivatives confirmed with help of ^{1}HNMR study by using BRUCKER 400 MHz spectrometer in deuterated DMSO, TMS as internal standard and chemical shifts were recorded as δ (parts per million).

General procedure for synthesis of Mercaptobenzimidazole:

O-phenylenediamine (10.8g, 0.1 moles) treated with carbon disulfide (7.67g, 0.1 moles) in the presence of potassium hydroxide (5.65g, 0.1 moles), 100ml of 95% ethanol and 15 ml of water used as solvent in a round bottom flask was refluxed on water bath for three hours. After the completion of reaction, reaction mixture was cooled and filtered. After that, 1-1.5g of activated charcoal was added carefully in the filtrate. Further, filtrate refluxed for 10 minutes; the activated charcoal was removed by filtration. Filtrate was treated with 100ml of warm water at 60-70ºC for 10 minutes. Dilute acetic acid was poured into the reaction mixture for acidification with gentle agitation to yield shiny crystals as product, which is further kept in a refrigerator for three hours to allow the complete crystallization process. The obtained solid product was separated through Buchner funnel and dried at 40ºC overnight and recrystallized from the ethanol.

General procedure for Synthesis of N-Acetylmercaptobenzimidazole:

Mercaptobenzimidazole (5.5g, 0.1 moles) was treated with acetic anhydride (5ml, 0.1 moles) in the presence of glacial acetic acid (5ml, 0.1 moles) and 5ml of pyridine used as acetylating agent. Prepared solution mixture refluxed on sand bath for 10-15 minutes. After the completion of reaction, reaction mixture was cooled and filtered. Filtrate pours it slowly in 100ml of ice-cold water and stirring with help of glass rod. The obtained solid product was separated through Buchner funnel and recrystallized from the ethanol. The completion of reaction was ascertained by TLC (Benzene: Methanol/ 5:1).

General procedure for Synthesis of mercaptobenzimidazole–clubbed chalcone derivatives (DPK4B2d1-DPK4B2d1):

N-Acetylmercaptobenzimidazole (2g, 0.01 moles) treated with aromatic aldehyde (1.5g, 0.01 moles), 10ml of 95% ethanol used as solvent in a round bottom flask equipped with a magnetic stirrer. Then, 10ml of 0.1N sodium hydroxide was added drop wise to the reaction mixture on vigorous stirring for 30 minutes at 20-25ºC. The completion of reaction was ascertained by thin layer chromatography. After that, reaction mixture was neutralized by 0.1 N Hydrochloric acid whereby the precipitation occurred. The obtained crude chalcone was separated through Buchner funnel and were dried in air and recrystallized from the ethanol.

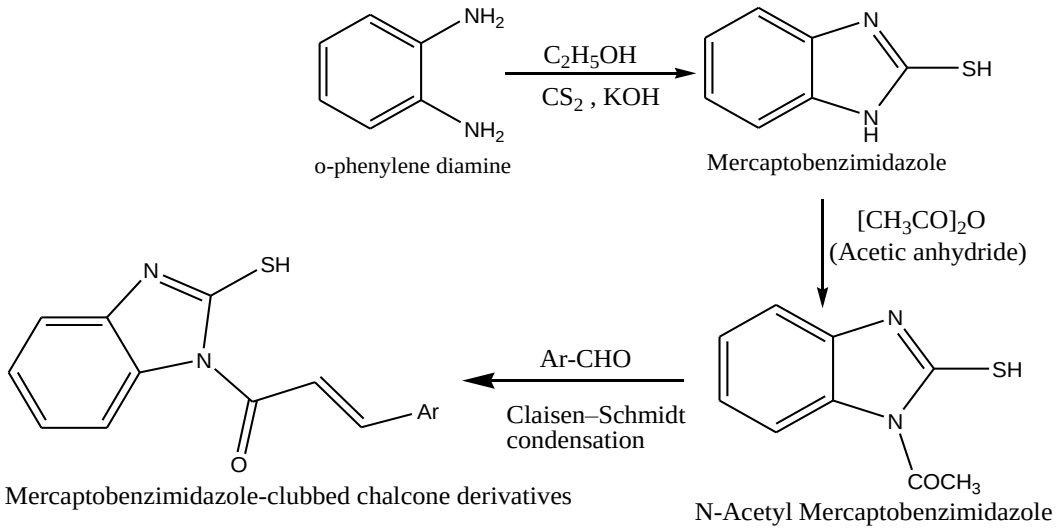


Figure 1: Scheme for synthesis of mercaptobenzimidazole–clubbed chalcone derivatives.

Table 1: Attachment of different aromatic aldehyde.

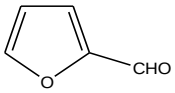
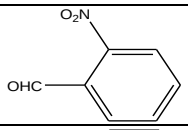
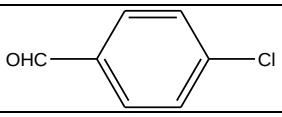
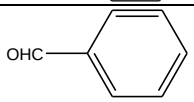
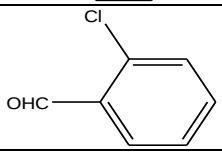
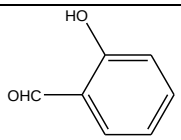
Compound Code	Ar-CHO	Compound Code	Ar-CHO
DPK4B2d1		DPK4B2d4	
DPK4B2d2		DPK4B2d5	
DPK4B2d3		DPK4B2d6	

Table 2: Characterization of mercaptobenzimidazole–clubbed chalcone derivatives.

Compound code	Molecular Formula	M.P.(ºC)	R_fValue	% Yield	FTIR (KBr cm-1)	NMR) (δ ppm)
DPK4B2d1	$C_{14}H_{10}N_2O_2S$	320-325ºC	0.56	68	3047, 2908, 2337, 1257, 1666, 1566, 1512, 1188, 696	6.4-8.5, 3.3- 6.3, 2.3-2.6
DPK4B2d2	$C_{16}H_{11}ClN_2OS$	346-351ºC	0.60	72	3147, 2965, 2299, 1319, 1689, 1620, 1512, 709, 696	7.114-10.016, 3.386-4.492, 2.377-2.652
DPK4B2d3	$C_{16}H_{11}ClN_2OS$	348-353ºC	0.65	65	3055, 2965, 2453, 1365, 1689, 1627, 1512, 709, 648	7.114-10.352, 3.364, 2.511-2.645
DPK4B2d4	$C_{16}H_{11}N_3O_3S$	355-360ºC	0.67	55	3063, 2931, 2360, 1257, 1674, 1625, 1519, 1565, 1442, 678	7.514-8.046, 3.340-5.556, 2.503
DPK4B2d5	$C_{16}H_{12}N_2OS$	300-305ºC	0.68	62	3063, 2965, 2453, 1265 ,1697, 1620, 1512, 655	7.222-8.529, 3.350-4.489, 2.360-2.641
DPK4B2d6	$C_{16}H_{12}N_2O_2S$	321-326ºC	0.58	63	3680, 3115, 2993, 2453, 1357, 1712, 1645, 1512, 655	7.108-8.018, 3.012-3.386, 2.369-2.642

RESULT AND DISCUSSION:

From the literature survey, it was revealed that benzimidazole has been reported to develop number of molecules have exposed various potent pharmacological activities. In this research study, we have reported synthesized mercaptobenzimidazole–clubbed chalcone derivatives. These newly synthesized derivatives were screened against in vitro antimicrobial and antitubercular activities. Synthesized derivatives having purity and homogeneity preliminarily checked by their physical constants and spectral studies such as FTIR, ^{1}HNMR for structural elucidation and studies showed satisfactory results.

In vitro antimicrobial activity:

In vitro antimicrobial activity of synthesized mercaptobenzimidazole-clubbed chalcone derivatives were screened by the tube dilution method against *Escherichia Coli* (Gram-negative bacteria/ATCC 25922), *Bacillus Subtilis* (Gram-positive bacteria/ATCC 6051), and *Aspergillus Niger* (fungal strain/ATCC 6275). Synthesized compounds having observed MIC values are showed in Table 3. Some of the mercaptobenzimidazole-clubbed chalcone derivatives were found to be highly efficient as antimicrobial agents. All the synthesized compounds showed average antibacterial activity as compared to standard ciprofloxacin. Compounds DPK4B2d2, DPK4B2d3, DPK4B2d4 and DPK4B2d6 shown potent antifungal activity with MIC value of 0.8µg/ml in the comparison of standard fluconazole.

Table 3: Antimicrobial activity, MIC values of synthesized compounds.

Sr. No.	Compound Code	MIC in µg/ml		
		Antibacterial activity		Antifungal activity
		B. subtilis	*E. Coli*	*A. niger*
1	DPK4B2d1	100	100	1.6
2	DPK4B2d2	100	50	0.8
3	DPK4B2d3	100	50	0.8
4	DPK4B2d4	100	50	0.8
5	DPK4B2d5	100	100	1.6
6	DPK4B2d6	100	6.25	0.8
7	Ciprofloxacin	2	2	-
8	Fluconazole	-	-	8

In vitro antitubercular activity:

In vitro antitubercular activity of synthesized mercaptobenzimidazole-clubbed chalcone derivatives were screened by the Microplate Alamar Blue Assay (MABA) against *Mycobacterium tuberculosis* (H37Rv strain, ATCC 27294). Synthesized compounds having observed MIC values are showed in Table 4.

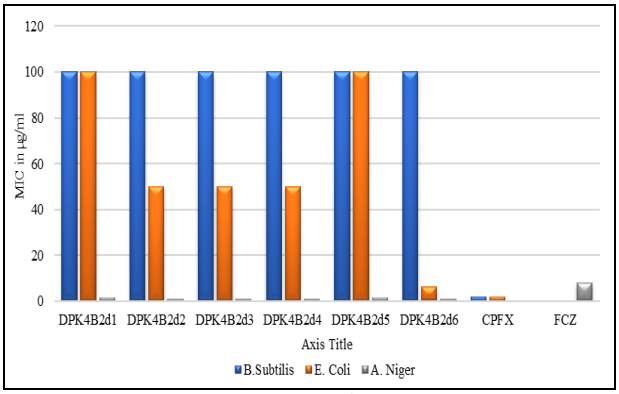


Figure 2: Graphical representation of antimicrobial activity, MIC values of synthesized compounds

Compounds DPK4B2d1 and DPK4B2d2 showed potent activity and also, rest of the compounds shown moderate activities in the comparison of standard antitubercular drugs such as Pyrazinamide, Ciprofloxacin, and Streptomycin.

Table 4: Antitubercular activity, MIC values of synthesized compounds.

Sr. No.	Compound Code	MIC in µg/ml	Sr. No.	Compound Code	MIC in µg/ml
1	DPK4B2d1	0.8	6	DPK4B2d6	12.5
2	DPK4B2d2	0.8	7	Pyrazinamide	3.12
3	DPK4B2d3	1.6	8	Ciprofloxacin	3.12
4	DPK4B2d4	3.12		Streptomycin	6.25
5	DPK4B2d5	6.25			

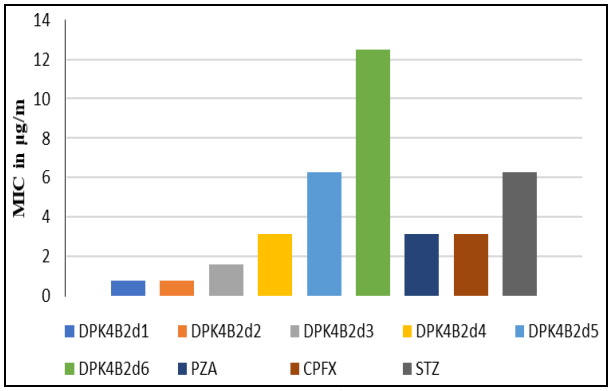


Figure 3: Graphical representation of antitubercular activity, MIC values of synthesized compounds.

Structure - activity relationship of mercaptobenzimidazole–clubbed chalcone derivatives:

From the comparison of antimicrobial and antitubercular activities of synthesized mercaptobenzimidazole–clubbed chalcone derivatives, the following SAR may be assumed:

1. From the results of antimicrobial activities of the mercaptobenzimidazole–clubbed chalcone derivatives compared to the standard drug ciprofloxacin and fluconazole conclude that, there should be slight structural modifications to develop affinity of drug to the binding of a molecule to the target site.
2. From the results of antitubercular activities of the mercaptobenzimidazole–clubbed chalcone derivatives compared to the standard drugs such as Pyrazinamide, Ciprofloxacin, and Streptomycin may draw attention that the synthesized compounds have a very good interaction with target sites and has need of supplementary in vivo studies to confirm the antitubercular activity.
3. The above results also indicated a fact that different structural requirements are essential for a compound to show different activities. The structure-activity relationship amongst the mercaptobenzimidazole–clubbed chalcone derivatives outcomes are summarized as follows:

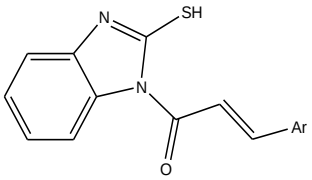


Figure 4: Basic nucleus of mercaptobenzimidazole–clubbed chalcone derivatives.

- Presence of mercaptobenzimidazole ring essential for antimicrobial and antitubercular activities.
- Presence of–SH (Mercapto group), C=O (Carbonyl group) and ethylene side chain are essential for antimicrobial and antitubercular activities.

At position Ar:

- Presence of aromatic ring such as benzene as well as furan ring important for antimicrobial and antitubercular activities.
- Substitution of -Cl (Chloro group) in the benzene ring at para as well ortho position shows potent to moderate antimicrobial and antitubercular activities.
- Substitution of $–NO_2$ (Nitro group) and –OH (Hydroxy group) in the benzene ring at para as well ortho position shows moderate antimicrobial and potent antitubercular activities.

CONCLUSION:

Novel series of mercaptobenzimidazole-clubbed chalcone derivatives were efficiently synthesized. These newly synthesized derivatives were examined against in vitro antimicrobial and antitubercular activities. Amongst the synthesized compounds DPK4B2d2, DPK4B2d3, DPK4B2d4 and DPK4B2d6 shown significant and potent activity against *A. Niger* as compared with the standard fluconazole. Compounds DPK4B2d1 and DPK4B2d2 showed potent antitubercular activity in the comparison of standard drugs such as Pyrazinamide, Ciprofloxacin, and Streptomycin.

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CONFLICTS OF INTEREST:

No.

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RESEARCH ARTICLE

Antiretroviral Effect Simulation from Black Tea (*Camellia sinensis*) via Dual Inhibitors Mechanism in HIV-1 and its Social Perspective in Indonesia

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ABSTRACT:

This study aims to investigate the general social perspective related to black tea (*Camellia sinensis*) as a traditional treatment in Indonesia, the potency of bioactive compounds in black tea as a dual inhibitor of the replication of HIV-1 as it inhibits the chemical interactions between ligands and target protein domain. The samples is prepared from specific databases that is about HIV-1 target protein, black tea chemical compounds, and HIV-1 drug compounds as positive control. After that the virtual screening, protein-ligand interaction, and molecular visualization tests were performed. Thirteen black tea compounds obtained from the database and 2 control compounds consisting of neverapine and darunavir. In addition, the 3D structure of the target protein from HIV-1 consisting of reverse transcriptase (RT) (ID 3LP1) and protease (PR) (ID 4HLA) was obtained from a protein database. Interestingly, the social perspective regarding HIV-1 treatment is predicted to be more likely to choose traditional medicine compared to the use of antiretroviral (ARV) drugs. Traditional medicine with black tea may be very popular among many people. In conclusion, almost all chemical compounds contained in black tea can inhibit the HIV-1 replication. Furthermore, there are two compounds that play an important role in the process because it is predicted to inhibit the biological activity of PR and RT in HIV-1, (-)-epicatechin-gallate and epigallocatechin-gallate called dual inhibitors.

KEYWORDS: Antiretroviral, *Camellia sinensis*, Dual Inhibitor, HIV-1, Social Perspective.

INTRODUCTION:

Indonesian people were living with Human immunodeficiency virus type 1 (HIV-1) approximately 640,000 in 2018. Based on UNAIDS data, 46,000 people were newly infectedwith HIV-1 within the same year and there were 38,000 deaths from an AIDS-related illness. The number of AIDS-related deaths has increased by 60% since 2010, from 24,000 to 38,000 deaths. The new of HIV infections has decreased,

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however, from 63,000 to 46,000 in the same period[1]. HIV-1 infections are major serious health in Indonesia. Highly active antiretroviral therapy (HAART), combination antiretroviral therapy (cART), and improvement antiviral therapy have been developed as therapeutic strategies to minimize attack and prevent spread of HIV infection. However, control of the disease has not yet been entirely implemented[2].

A retrovirus is a type of RNA virus that inserts a copy of its genome into the DNA of a host cell that it invades, thus changing the genome of that cell. HIV is a member of the genus Lentivirus, part of the family Retroviridae[3]. This virus has several coding genes such as group antigens (gag) and polymerase gene (pol) which function as structural protein coding genes and viral replication[4]. Pol gene consists of reverse transcriptase (RT), protease (PR), and integrase (INT)[5]. However, this study only discuss RT and PR, RT HIV-1 has the role of converting RNA to DNA so that it can be recognized by the enzyme ligase in the host cell, while PR cuts the polypeptide from the translational resulting into smaller pieces undergoing the process of modification into a part of the virus HIV-1[6,7]. This prompted some previous researchers to design HIV-1 drugs, because they already knew the important role of the target protein, RT and PR, on the mechanism of viral replication[8].

Previously, a cure for HIV-1 infection called ARV was found, this drug is a type of non-nucleoside inhibitors (NNs) because it uses chemical compounds with simple structures[9]. NNs are called nevirapine on RT and darunavir on PR, both drugs are taken together by HIV-1 patients, which have a performance by binding to the target protein by producing weak binding interactions to produce biological activity in the form of inhibiting the activity of the target protein[10,11]. The molecular interaction is the presence of hydrogen and hydrophobic bonding types which are often used as parameters in the *in silico* drug design study[12,13].

However, several studies in Indonesia revealed lack of recognition of the existence of the drug, because around 46% of the role of health workers in rural area did not conduct socialization even though there were still other factors such as awareness of PLWHA and environmental factors[14]. Therefore, there are people reluctant to take ARV because of the lack of recognition of these ARV drugs, but according to Jennifer and Saptutyningsih (2015) people in Indonesia prefer traditional medicine, for example black tea. This study used traditional medicine based on the chemical compounds are contained in black tea to generate theoretical predictions related to the use of black tea in HIV-1 infection cases[15]. Thus, this study aims to determine the general social perspective related to black tea as a traditional medicine in Indonesia, to investigate the potency of bioactive compounds in black tea as a dual inhibitor, and to predict the chemical interaction between ligands and target protein domains which might produce an inhibitory effect of HIV-1 replication.

MATERIAL AND METHODS:

Social Literature Study:

This study used the method of observation through several theories or data obtained from scientific articles in the social scope from national journals in Indonesia.

Samples:

Chemical compounds derived from black tea from previous studies[16]. Sample preparation of ligands or black tea compounds and controls was obtained on the PubChem database (pubchem.ncbi.nlm.nih.gov) by obtaining 3D structures in structure data format (*.sdf*). In addition, the preparation of the target protein is carried out by obtaining the 3D structure of the target protein in the format of the protein databank (*.pdb*) at PDB (rcsb.org). The target protein in this study consisted of PR and RT from HIV-1. The protein samples were first sterilized in PyMol software before the next analysis phase was carried out.

Virtual Screening:

In this study, the prediction of the activity of black tea compounds was performed using virtual screening methods via molecular docking with the VinaWizard plug-in on PyRx 0.8, but before the process was carried out, the first ligand went through a minimization process in OpenBabel plug-in so that it can get the format of the databank protein (*pdb*).

Protein-Ligand Interactions:

Complex molecules with scores of binding energy higher than control that were found in two protein targets and then analyzed for their interaction position and chemical bond formation type, using the webserver PoseView (proteins.plus). Output of this analysis were interaction position between atoms forming ligands and the type of chemical bonds formed during the interaction between ligands and protein.

Molecular Visualization:

In the final stage, molecular complexes were visualized in a representative form based on the selection of coloration and type of structure, molecular visualization in this study was carried out in PyMol.

RESULTS AND DISCUSSION:

Social Perspective of Black Tea in Indonesia:

Traditions are included in a particular belief or trait which is derived from a group of people with symbolic meaning from ancestors from ancient times, traditions can vary as forms of beliefs or habits such as animism, dynamism, and some activities in life[17]. One tradition

that can be found in general is drinking tea, which has been carried on for a long time through parts of Asia, as is the case in Japan[18]. Although there are not many definite explanations of he existence of this tradition in Indonesia since ancient times, it is a common tradition and regularly practiced by Indonesians today. Tea can be classified as herbal medicine because it is one of the natural ingredients consisting of a number of chemical compounds, tea is usually taken for several purposes namely as a sedative agent and holistic (herbal) medicine[15,19].

The synthetic drugs used in computational simulations in this study are nevirapine and darunavir, both of which include ARV drugs that are often used to treat people with HIV-1 infection[20]. However, a study concerning general awareness of these ARV drugs in Indonesia shows a devastatingly low number of people, especially HIV-1 sufferers, have any awareness of the existence of such drugs. This is speculated due to many factors, such as the lack of socialization by health workers in different regions (46%), the lack of awareness of ODHA (47,9%), and environmental factors as well (77%)[14]. Therefore, there are some people who are reluctant to take ARVs, and around 338,363 thousand people from 2005 to 2019 in Indonesia were infected with HIV-1 and in AIDS[20]. However, according to Jennifer and Saptutyningsih (2015), most people in Indonesia believe more in herbal-based products such as herbal medicine to produce better efficacy than of synthetic drugs. This is also due to the increasing demand and use of natural-based medicines which are high as "back to nature" and “holistic” endorsements that raised because of the more available and cost-effective ingredients found in Indonesia[21,22,23], such as black tea.

The results of previous studies indicate that people in Indonesia often use black tea for antioxidant, antibacterial, and antifungal use, some also believe it can be used to treat obesity[24,25,26]. However, research related to the potency of black tea for therapeutic treatment of viral infections such as HIV-1 in Indonesia is still unknown. Therefore, this study provides a deeper explanation, related to the potency of chemical compounds contained in black tea that can produce a therapeutic effect by inhibiting HIV-1 virus infection, so that many Indonesian people can find out the potency of black tea and researchers can conduct further research in this topic.

The Potency of Biochemical Compound from Black Tea as Antiretroviral Candidate via Dual Inhibitors Mechanism:

A total of 13 chemical compounds contained in black tea were obtained from the database, these compounds consisted of (-) epicatechin gallate (ID 107905), (-)-epigallocatechin gallate (ID 65064), chlorogenic acid (ID 1794427), (-)-epigallocatechin (ID 72277), (-)epicatechin (ID 72276), caffein (ID 2519), pectin (ID 441476), theobromine (ID 5429), gallic acid (ID 370), and theanine (ID 228398). In addition, two control compounds were also obtained consisting of darunavir (ID 213039) (PR control) and nevirapine (RT control) (ID 213039). 3D structure of the target protein from HIV-1 consisting of RT (ID 3LP1) and PR (ID 4HLA) obtained from a protein database. Total sample of chemical compounds obtained from the database is 15 samples, which samples are in the .sdf format and must experience energy minimization in the OpenBabel plugin, so that the .pdb format is obtained and can be used in the next analysis (molecular docking). Samples of target proteins are displayed in PyMol software, with 3D visualization in the form of cartoon to find out the types of secondary protein constituents, transparent and rigid surfaces, and color selection based on the purpose of visualizing the structure comparison and differences in the chain making up a protein. In addition, sterilization of the two target proteins was performed to eliminate contaminant molecules, which consisted of undesired water, ligands, and peptides. After the sterilization process was successful, the target protein was stored in the form of a .pdb file without contaminants when re-visualizing. The results of the visualization of the target protein structure (Figure 1) showed that everything was composed of secondary protein structures in the form of cartoons, namely alpha helix (red), coil (green), and beta-sheet (yellow). But for RT only has one chain, namely chain A which is marked by visualization of the rigid surfaces structure in green, while PR consists of two chains, A and B.

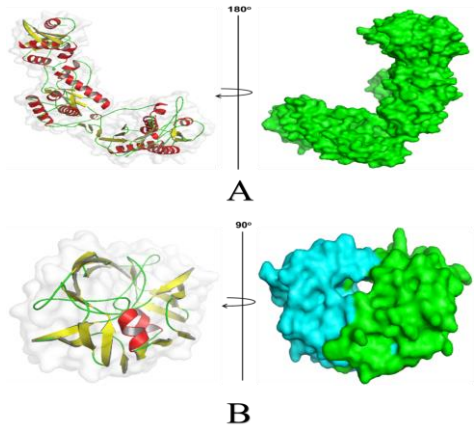

Figure 1. Molecular visualization of comparison of target protein structures. (A) Reverse transcriptase; and (B) Protease. Structure displayed (left) with white transparent surfaces with cartoon structure colored based on the secondary protein structure and composition of rigid surface (right) with domain selection based on chains.

After the preparation of the ligand and target, the molecular docking process was carried out, this analysis aimed to determine the effect of ligand binding with the

target protein that leads to a biological response. Docking is a simulation of the interaction between molecules with one another to determine the interaction pattern model and the amount of binding energy, with the help of bioinformatics software[16]. The amount of binding energy resulting from docking refers to Gibbs' law, if a ligand has a negative binding energy value, the effect is greater on the target protein[27]. One docking method is blind, its aimed at screening the potential compounds with a functional side of an unknown protein[28]. This research uses blind docking method because it aims to screen the potency of black tea chemical compounds that produce a biological response in the form of inhibition of activation of target proteins. The grid position in this study is in all parts of the target protein, thus allowing ligand binding to all parts (Table 1). The grid is a cube that directs the ligand binding to the position of target protein domain, when docking analysis has performed.

Based on the results of the study showed the docking on RT (Table 2) has about 13 chemical compounds from black tea with lower binding energy compared to controls consisting of -8.5 kcal/mol (-)epicatechin (ID 72276), -8.4 kcal/mol (-)-epigallocatechin (ID 72277), -8.2 kcal/mol (-)epicatechin gallate (ID 107905), -7.8 kcal/mol (-)-epigallocatechin gallate (ID 65064), -7.1 kcal/mol chlorogenic acid (ID 1794427), -6.4 kcal/mol gallic acid (ID 370), -6.2 kcal/mol theobromine (ID 5429), and -6.1 kcal/mol nevirapine (Control) (ID 213039). However, in PR (Table 3) only 2 compounds consisted of -9.4 kcal/mol (-)Epicatechin gallate (ID 107905), -9.3 kcal/mol (-)-epigallocatechin gallate (ID 65064), and -8.7 darunavir (Control) (ID 213039). Interestingly, there are two compounds having lower binding energy under control drug that can bind to both target proteins, namely (-)epicatechin gallate (ID 107905) and (-)-epigallocatechin gallate (ID 65064).

Control compounds under normal conditions work to inhibit the biological activity of the target protein, by binding and forming molecular complexes. Nevirapine is a drug used as an inhibitor of RT HIV-1. The mechanism of binding to the active side of the target protein uses the interaction of hydrophobic bonds, thus blocking RT activation[29]. Darunavir plays a role in inhibiting the performance of PR HIV-1 through the interaction of hydrogen bonds on the active side of PR called the catalytic site[30]. The docking results show that some binding energy scores produced by the ligand against the two target proteins thus influencing the biological activity of the protein. Ligands, which have a greater binding energy than controls, have a greater affect on the target protein[16].

Table 1. Docking Grid Position.

Target Protein	Center (Å)			Dimension(Å)		
	x	y	z	x	y	z
RT	5.525	32.077	28.188	78.343	95.421	69.505
PR	11.568	-20,014	0.294	43.557	39.935	58.914

*Note: RT: Reverse Transcriptase; PR: Protease.

Table 2. The Docking Results of Black Tea Compounds with RT HIV-1.

Ligand	PubChem ID	Target Protein	*Binding Energy* (kcal/mol)
(-) Epicatechin	72276	HIV-1 RT	-8.5
(-)-Epigallocatechin	72277	HIV-1 RT	-8.4
(-) Epicatechin gallate	**107905**	**HIV-1 RT**	**-8.2**
(-)-Epigallocatechin gallate	**65064**	**HIV-1 RT**	**-7.8**
Chlorogenic Acid	1794427	HIV-1 RT	-7.1
Gallic Acid	370	HIV-1 RT	-6.4
Theobromine	5429	HIV-1 RT	-6.2
Nevirapine (Control)	213039	HIV-1 RT	-6.1
Theanine	228398	HIV-1 RT	-5.9
Pectin	441476	HIV-1 RT	-5.8
Caffein	2519	HIV-1 RT	-5.2

Table 3. The Docking Results of Black Tea Compounds with PR HIV-1.

Ligand	Pub. Chem ID	Target Protein	*Binding Energy* (kcal/mol)
(-) Epicatechin gallate	**107905**	**HIV-1 PR**	**-9.4**
(-)-Epigallocatechin gallate	**65064**	**HIV-1 PR**	**-9.3**
Darunavir (Control)	213039	HIV-1 PR	-8.7
Chlorogenic Acid	1794427	HIV-1 PR	-8.2
(-)-Epigallocatechin	72277	HIV-1 PR	-8.1
(-) Epicatechin	72276	HIV-1 PR	-8.1
Caffein	2519	HIV-1 PR	-5.8
Pectin	441476	HIV-1 PR	-5.6
Theobromine	5429	HIV-1 PR	-5.6
Gallic Acid	370	HIV-1 PR	-5.5
Theanine	228398	HIV-1 PR	-5.3

Thus, black tea chemical compounds can potentially inhibit the activity of the target protein, which must have a more negative binding energy value than the control. However, there are two compounds that can potentially inhibit and therefore work as dual inhibitors to the two

target proteins, namely (-) epicatechin gallate and (-)-epigallocatechin gallate. The compounds from docking analysis, that act as dual inhibitors are displayed on PyMol software with cartoon structures and transparent surfaces.

The Molecular Interaction between Ligand and Protein:

When a ligand is bound to a protein it forms complex molecules, which produces molecular interactions in the form of chemical bonds. The chemical bonds in the form of weak bond interactions consist of hydrophobics and hydrogen in the ligand-making atoms with amino acid residues in the target protein[12,13]. Potential ligands as dual inhibitors are then identified by the type of chemical bond interaction and the position of amino acid residues in the target protein using the Pose View webserver. The black tea compound (-) epicatechin gallate binds to PR with a total of seven molecular interactions consisting of six hydrogen bonds (Asp29A, Gly48A, 2 Arg8B, Gly27A, and Gly27B) and one hydrophobic (Ile50B), while the compound (-)-epigallocatechin gallate binds to PR and has a total number of molecular interactions of seven, all in the form of seven hydrogen bonds (2 Arg8B, Gly27A, Gly27B, Asp29A, and 2 Gly48A). The darunavir has nine molecular interactions, four hydrogen bonds (Arg8A, Asp25A, Gly48A, and Asp29B) and five hydrophobic (Asp30B, Ile50A, Ile84B, Ile47B, and Gly49A) (Figure 2). The compound (-) epicatechin gallate bounded to RT has a molecular interaction of seven in the form of four hydrogen bonds (Asn103A, Lys101A, Ile180A, and Gly99A) and three hydrophobic (Val179, Leu100A, and Tyr181A). While the compound (-)-epigallocatechin gallate bounded to RT has a molecular interaction of six with four hydrogen bonds (Ile180A, Gly99A, Lys101A, and Asn103A) and two hydrophobics (Leu100A and Val179). The nevirapine control has two molecular interactions, one hydrogen bond (Thr107A) and one hydrophobic (Pro225) (Figure 3).

Molecular interactions identified based on the results of this study are hydrogen and hydrophobic bonds. Hydrogen bonding is a chemical bond produced by the interaction of H atoms with N, O, F and as a parameter of ligand tendency can play a role in influencing the biological response of target proteins in drug design strategies, in addition to hydrophobic bonds[31,32]. Hydrophobic bonds take form of molecular complexes and play a role in the interaction of drug molecules, thereby inducing a significant change in biological response to the protein target[33]. The existence of the hydrogen interaction and hydrophobic that are formed in molecular complexes, from the results of this study indicates that ligands consisting of black tea compounds and controls interact with the target protein and affect the biological response of the protein. Returning back to the purpose of this study which is to reveal compounds that are dual inhibitors of HIV-1, we obtain of two compounds in the form of -(-)epicatechin gallate and -(-)epigallocatechin gallate have strong interactions with two target virus proteins with a binding energy value greater than control and inhibiton of the types of chemical bonds of molecular interactions that support their affect.

Thus, black tea is predicted to be able to inhibit HIV-1 infection through the binding mechanism of chemical compounds bind to RT and PR, which protein has an important role in the process of viral replication. All chemical compounds that have lower binding energy value compared to control, and affect the biological response for target. In this study, we revealed the two compounds, -(-)epicatechin gallate and -(-)epigallocatechin gallate, have the lowest binding energy and act as inhibit the responses of biological activity of the target protein.

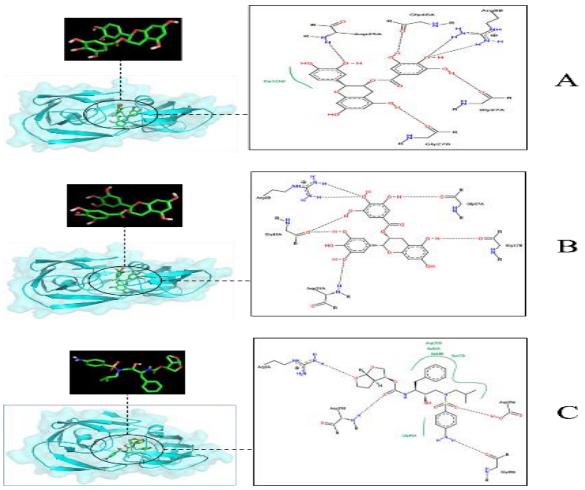

Figure 2. Molecular visualization of docking results of black tea compounds with PR. A: (-)-epicatechin-gallate-PR; B: (-)-epigallocatechin-gallate-PR; and C: darunavir-PR.

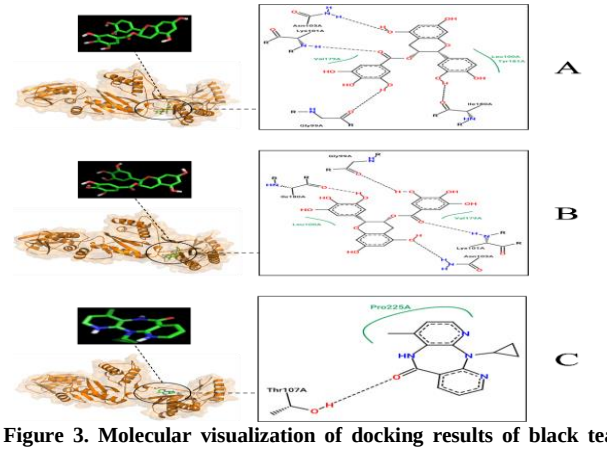

Figure 3. Molecular visualization of docking results of black tea compounds with RT. A: (-)-epicatechin-gallate-RT; B: (-)-epigallocatechin-gallate-RT; and C: nevirapine-RT.

CONCLUSION:

In sum, the social preference regarding HIV-1 treatment is herbal traditional medicine compared to ARV drugs, traditional medicine with black tea in the future might be sought after by the community. In addition, almost all of the chemical compounds contained in black tea can inhibit the replication of the HIV-1 virus, but there are two compounds that play an important role for their dual inhibition benefits. They can inhibit both the biological activity of PR and RT in HIV-1, (-)-epicatechin gallate and (-)epigallocatechin gallate.

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CONFLICT OF INTEREST:

The authors declare no conflict of interest.

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RESEARCH ARTICLE

Urinary Tract Infection and Antibiotic Resistance among Pregnant and Non-pregnant females in UAE

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ABSTRACT:

Background: Urinary tract infection (UTI) is an infection that can take place in both genders and anywhere within the urinary tract and results from certain microorganisms such as bacteria which are the most common cause of UTI. In case it was a bacterial infection the physician usually administers antibiotics depending on the nature of the UTI and in some cases antibiotic resistance might occur. **Objectives:** To determine the prevalence of urinary tract infection in pregnant and non-pregnant women in UAE, distribution of bacteria which cause UTI and its sensitivity and resistance to antibiotics. **Methods:** A Cross-sectional study was carried out. A total of 300 women results were retrospectively analyzed in the study. **Results:** From those 300 study participants (39%) were pregnant and (61%) non-pregnant. The percentage of resistance in the uropathogens in pregnant patients was more (69.49%) compared to those in non-pregnant patients (56.59 %). The pathogens distribution shows that the most common pathogen is Escherichia Coli (58%). This study shows that E. coli has high resistance to Amoxicillin. **Conclusion:** Pregnant women are more susceptible to UTI in comparison to non-pregnant women and the main cause of UTI is the E.coli bacteria. The highest uropathogen resistance was against amoxicillin, while the antibiotic of choice for UTI treatment is ciprofloxacin due to its high sensitivity. UTI needs to be detected and treated promptly since treatment has shown its efficiency in preventing pregnancy related complications.

KEYWORDS: Urinary, Infection, Pregnant, *E.coli*, Resistance, Antibiotic Leftover.

INTRODUCTION:

The urinary tract is usually made of the kidneys, ureters, urine bladder and urethra. Urinary tract infection (UTI) is an infection that can take place anywhere within the urinary tract and mostly involve the bladder and the urethra, which are in the lower part of the urinary tract. Even though UTIs in the upper part of the urinary tract are experienced, they are usually rare, but they are more severe[1]. UTI is an infection occurring for both genders and results from certain microorganisms such as bacteria which are the most common cause of UTI although there have been cases caused by fungi and rare cases caused by viruses also[2].

The most common cause of urinary tract infection or cystitis is Escherichia coli (E.coli) which is a normal flora of intestines of living organisms and does not cause any complications when it is there, normal habitat, but when it finds its way to the urinary tract, it leads to infections. Infection of the urinary tract usually happens when microscopic elements of stool find their way into the urinary tract. On the other hand, cystitis is an inflammation of the urinary bladder and happens when bacteria enter the bladder and start multiplying, leading to bladder infections[2].

The treatment of UTI depends on the causative agent of the infection. In most cases that have been reported, the causative agent of UTI is bacteria and is usually treated with antibiotics while UTIs caused by the virus are treated with antiviral and the fungal UTIs are treated using antifungals[1].

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The form of antibiotics that are used in treating bacteria depends on the part of the urinary tract that has been affected. UTI infection of the lower part is usually treated using oral antibiotics while the UTIs affecting the upper part of the urinary tract are treated using intravenous antibiotics[1].

There are different antibiotics that are used in treating UTIs according to the severity of infection. For simple infections, the drugs that have been recommended include sulfamethoxazole/trimethoprim, fosfomycin, nitrofurantoin, cephalexin, and ceftriaxone. Antibiotics such as ciprofloxacin and levofloxacin have not been recommended for treatment of simple UTIs since they are aimed at treating only complicated UTI[3].

When a patient is diagnosed with UTI for the first time, the physician usually administers antibiotics depending on the nature of the UTI. However, if a UTI reoccurs after a short period of time after the clinical administration, there are a number of recommendations that the doctor might make[3]. The doctor might recommend low-dose antibiotics usually lasts for six months or longer[4]. A doctor may also recommend self-diagnosis where the patient has to stay in touch with the doctor. A physician may also recommend a single antibiotic dose after any sexual contact especially if the infections are caused by sexual intercourse. For post-menopausal patients with recurrent UTIs, the physician usually recommends vaginal estrogens[5].

To our knowledge there is a lack of studies in UAE addressing the prevalence of urinary tract infections and antibiotic resistant, therefore the study aims to assess the prevalence of urinary tract infections among pregnant and non-pregnant women and to estimate the bacterial population and antibiotic sensitivity among these women with urinary tract infections.

MATERIAL AND METHODS:

The study design was a cross-sectional retrospective study which was conducted in the clinical laboratories in UAE at emirate of Sharjah and Ajman. All culture and antibiotic susceptibility tests of patients already diagnosed with UTI were collected from the period of January to December 2017.

The sample size for this study was n=300. In order to control any confounding and external validity, both pregnant and non-pregnant women's laboratory results were studied. The lab test results were used to obtain the most reliable data samples in which the Urine Culture & Sensitivity (C&S Urine) testing was done on various antibiotics to determine which of the bacteria spp. are resistant or sensitive to the list of the antibiotics that are commonly prescribed to women in UAE to treat UTI. In order to assess individual patient's response and sensitivity to the brand of antibiotic used for the treatment of UTI, the tests were repeated for each patient for all the antibiotics prescribed for the treatment, and the response under each treatment levels was measured and recorded in addition to finding the most suitable and effective antibiotics in the treatment of urinary tract infection in women.

Sample collection involved laboratory diagnostic methods and the records showed mid-stream ("clean catch") urine specimen collected from each patient. Bacterial isolation and identification was performed according to standard procedures of Feingold and Martin. The study involved different age groups with a UTI infection in both pregnant and non-pregnant women in which urine culture and sensitivity analysis was performed.

Antibiotic Sensitivity Assay:

The cultures from urine were used for antimicrobial sensitivity testing after their identification by gram staining and other biochemical procedures that determined the organisms. The urine sample is cultured on blood agar and MacConkey's agar for the growth of bacteria. The culture is labeled and then incubated for 24 hours at 37°C.

Ethical Considerations:

An ethical approval from the ethical committee was obtained for the current study.

The study involved patient's data that were evaluated and analyzed using the Statistical Package for the Social Sciences (SPSS) and Microsoft Excel (Version 2003).

RESULTS:

The data analysis for the raw data obtained from the stored records of the clinical laboratories in UAE at the emirate of Sharjah and Ajman, were processed. Descriptive statistics of the ages of the studied samples included in the study analyzed. The distribution of the their age skewed to the left and the most frequent patients sample age group was between 19 and 35 years old (Fig. 1).

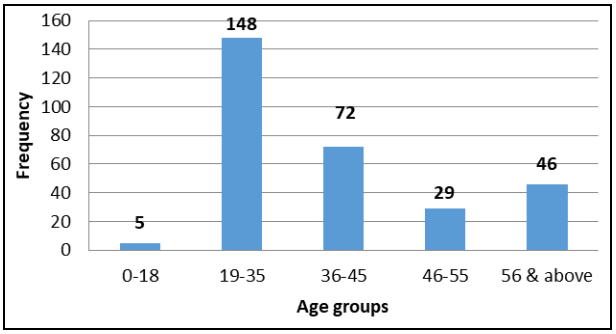

Fig. 1: The majority of patients (n=148) were in the age group between 19 and 35 years old.

The distribution of the diagnosed patients by the pregnancy tilted towards the non-pregnancy with approximately twice the proportion of the pregnant (39%) as those reporting no pregnancy (61%) during the study (Fig. 2). The results also show that the majority of the isolates in pregnant and non-pregnant women showed resistant to the administered antibiotics, but the percentage of the resistant bacterial isolates was more in the pregnant patients (69.49%) compared to those in non-pregnant patients (56.59 %) (Fig. 3).

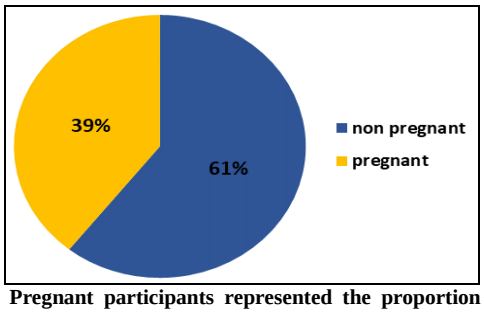

Fig. 2: Pregnant participants represented the proportion of the pregnant 118 (39%) and the majority were non- pregnant 182 (61%) during the study.

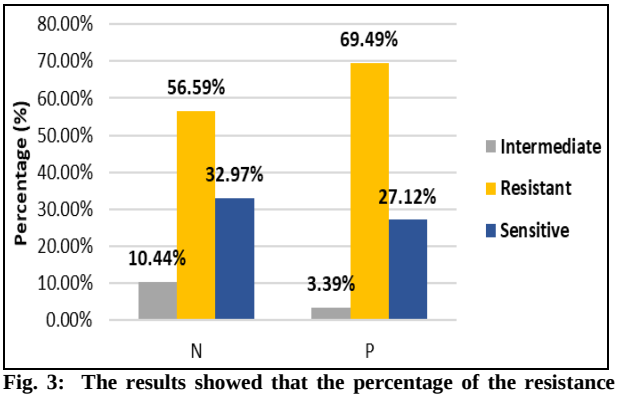

Fig. 3: The results showed that the percentage of the resistance was more in the pregnant patients (69.49%) compared to non-pregnant patients (56.59 %).

The results in Sharjah and Ajman in this study showed that the most commonly used antibiotic, is Co-Amoxiclav (6.99%) and table 1 represent the drugs that were involved in the process of sensitivity testing such as Ampicillin, Vancomycin, Augmentin, penicillin, gentamycin, Streptomycin, and Erythromycin amongst others (Table 1).

The most frequent causative pathogen of UTI is *Escherichia coli* (58%) followed by *Klebsiella*, *Streptococcus*: (*Streptococcus* group B, *Streptococcus agalactiae* and group D, *Streptococcus faecalis*), *Staphylococcus*: (*Staphylococcus aureus*, *Staphylococcus* spp.) 22%, 8% and 4% respectively (Fig. 4). The results show that the *E.coli* was the most commonly isolated pathogen (Fig. 5) whether the woman is pregnant or not (for pregnant women n=74), (for non-pregnant women n=100), followed by the *Klebsiella* (for pregnant women, n=25), (for non-pregnant, n=40) then the other pathogens.

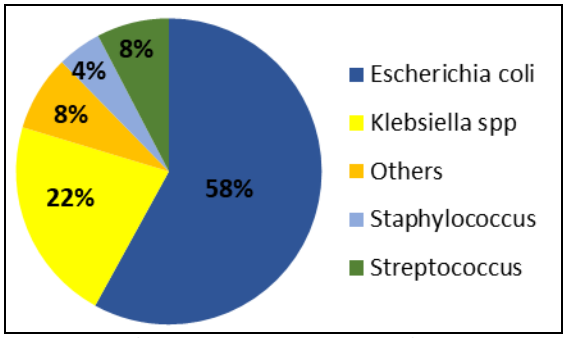

Fig. 4: The most frequent causative pathogen of UTI is *Escherichia coli* (58%) followed by *Klebsiella, Streptococcus*: (*Streptococcus* group B and group D, *Streptococcus agalactiae*), *Staphylococcus*: (*Staphylococcus aureus, Staphylococcus* spp.) 22%, 8% and 4% respectively.

Table 1: The distribution of Antibiotics used

Antibiotic Generic	Percentage (%)	Antibiotic Generic	Percentage (%)
CO-AMOXICLAV (AMC)	6.99%	CEFPODOXIME (CPD)	4.94%
AMPICILLIN (AM)	6.55%	CO-TRIMOXAZOLE (SXT)	4.57%
CEFAZOLIN (CZ)	5.73%	LEVOFLOXACIN (LE)	4.32%
CIPROFLOXACIN (CIP)	5.67%	CEFIXIME (CFM)	4.20%
CEFUROXIME (CXM)	5.38%	AMIKACIN (AK)	3.29%
CEFACLOR (CF)	5.07%	TETRACYCLINE (TE)	3.12%
CEFTRIAXONE (CRO)	5.04%	OFLOXACIN (OFX)	2.81%
CEFDINIR (CDR)	5.00%	GENTAMYCIN (CN)	2.77%
CHLORAMPHENICOL (C)	2.63%	LINEZOLID (LNZ)	2.03%
NITROFURANTON (F)	2.38%	ERYTHROMYCIN (E)	1.98%
PENICILLIN (P)	2.05%	NORFLOXACIN (NOR)	1.96%
CLINDAMYCIN (DA)	1.90%	PIPERACILLIN/ TAZOBACTAM	1.49%
IMIPENEM (IPM)	1.90%	PIPERACILLIN	1.10%
CEFTAZIDIME (CAZ)	1.76%	CEFEPIME (FEP)	0.85%
CEFOXITIN (FOX)	1.61%	AMPICILLIN/SULBACTAM (A/S)	0.60%
VANCOMYCIN (VA)	0.17%	TOBROMYCIN (TOB)	0.02%
AMPICILLIN/SULBACTAM	0.06%	MOXIFLOXACIN (MO)	0.02%
		DOXYCYCLINE	0.02%

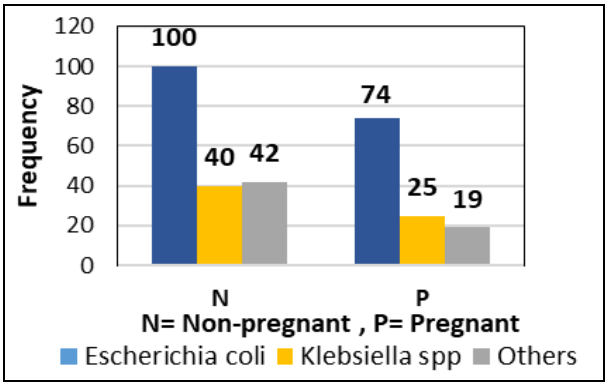

Fig. 5: The results show *that E.coli* was the most commonly isolated pathogen in both pregnant and non- pregnant women.

The E. coli resistance to antibiotic test shows that the E. coli has high resistance to amoxicillin with 39% and also high resistance to ampicillin with 31% followed by Nitrofurantoin, Tetracycline and SXT (15%, 10% and 5%) respectively. The ciprofloxacin showed the highest effect on the pathogens on urine culture so it is one of the best treatments for UTI, followed by Nitrofurantoin. It was noticed that the amoxicillin did not show any effect on the pathogens, which means it has the higher resistance and no sensitivity (Fig. 6 and Fig. 7).

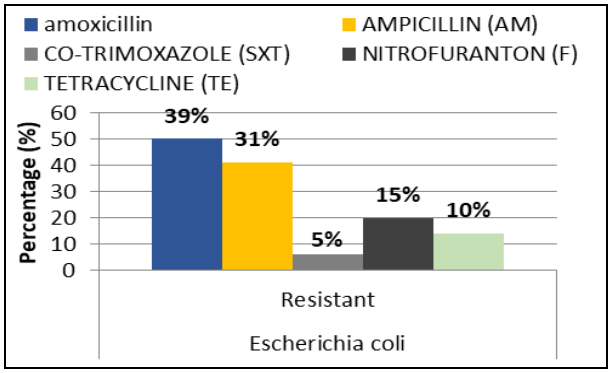

Fig. 6: The *E. coli* resistance to antibiotic test shows that the *E. coli* has high resistance to amoxicillin with 39% and also high resistance to ampicillin with 31% followed by Nitrofurantoin, Tetracycline and SXT (15%, 10% and 5%) respectively.

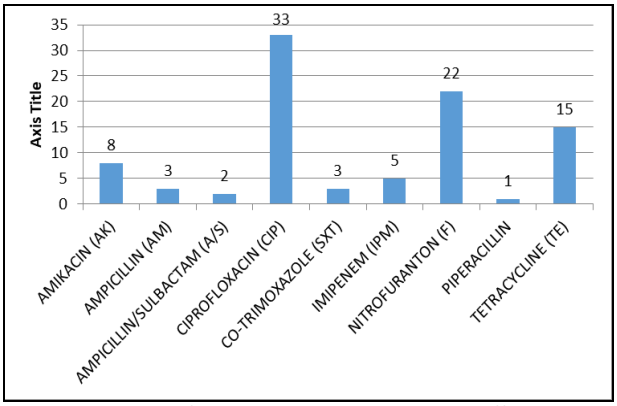

Fig. 7: The ciprofloxacin showed the highest effect on the pathogens on urine culture so it is one of the best treatments for UTI, followed by Nitrofurantoin.

DISCUSSION:

The study involved 300 females both pregnant and non-pregnant who visited the clinic for help regarding UTI infection. The results indicate that 39% of pregnant women were susceptible to UTI infection which can either come as pathogenic, invasive microbes or the risk increased because of the pressure on the bladder which lead to blockage in the urine draining. The bacteria are of different species and strains and they include normal flora such as Escherichia coli, Klebsiellas and others like streptococcus group B, streptococcus group D, while the invasive and pathogenic kinds are also present and they include staphylococcus aureus and other staphylococcus spp.

In a study conducted by researchers in 1998, aiming at studying the antibiotics that were most effective in treating UTI. However, there are major drawbacks from the results, which affect the contribution of the study to understanding recurrent UTI and antibiotic resistance[6]. One of the major drawbacks is that the subjects that were studied only had uncomplicated UTI and it was difficult to be able to understand the relation between the use of specific antibiotic and the chances of the UTI recurring within the subject. However, the studies provided limelight on some of the antibiotics that are no longer effective in treating UTI among women. Additionally, the studies conducted did not provide any relation between the anatomical and physiological structures and the failure of some of the antibiotics to be effective on some of the patients. Different UTI patients have different anatomical and physiological structures, which provide a different environment for the effectiveness of an antibiotic.

Most of the drugs that were used in the study were very efficient in the treatment of the UTIs. However, it is emerging from the study that even normal flora are becoming uncontrollable and resisting the antibiotics available in the market. From our results it is evident that drugs such as Ciprofloxacin, Nitrofurantoin and tetracycline are efficient in controlling most of the bacterial infections of the UTI. However, Ciprofloxacin is the best at the moment as it inhibits the growth of most of the urinary tract infections. The resistance pattern was high for Amoxicillin and Ampicillin and these results were similar to a study held in 2011 in Ethiopia[7].

From the study, it was evident that the prevalence of antibiotic resistance was more manifested in the uropathogens of pregnant women as compared to non-pregnant women. The high resistance among the uropathogens of pregnant women can be explained by the physiological change that results from the pregnancy, like the increase in plasma and reduction in the concentration of urine that lead to development

glycosuria in up to more than two-thirds of women, a factor that eventually leads to bacterial growth in urine. Another explanation to the difference between the antibiotic resistance in the uropathogens of pregnant and non-pregnant can be attributed to the fact that during the non-pregnancy state, the uterus is located far above as compared to during pregnancy when the uterus becomes enlarged, and it descends affecting the urinary tract. The implication of the situation of the bacteriuria is that such high occurrence of UTI among the pregnant cases increases the risk factors for both the unborn child and the mother.

The most common cause of the simple UTI is *Escherichia coli* which account for nearly 74 to 99 percent of all infections. This longitudinal study of 300 UTI patients found that *E. coli* was the only causative pathogen in 58% of cases followed by *Klebsiella*, *Streptococcus* and *Staphylococcus* (22%, 8% and 4%) respectively which is similar to a study on pregnant women attending the antenatal clinic in Kanpur, India[8].The results were also similar to a recent study held in 2019 in Atlanta stating that the most frequent isolates were *Escherichia coli* (52%) and *Klebsiella pneumoniae* (15.6%)[9]. Similarly it was observed in a recent study in 2018 held in Germany that the main pathogens were Escherichia coli (64.2%) followed by *Klebsiella pneumoniae* (12.4%)[10].

Authors should discuss the results and how they can be interpreted in perspective of previous studies and of the working hypotheses. The findings and their implications should be discussed in the broadest context possible. Future research directions may also be highlighted.

CONCLUSION:

The study shows that the pregnant women with UTI have higher resistance uropathogens to the antibiotic and they are more susceptible to UTI than the non-pregnant. E.coli is the main bacteria to cause UTI and the best antibiotic to treat UTI is ciprofloxacin while amoxicillin showed no effect.

CONFLICT OF INTEREST:

The authors declare no conflict of interest.

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RESEARCH ARTICLE

Design, Synthesis, Characterization of Antimicrobial activity of Schiff bases of novel Quinazolin-4-one Derivatives

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ABSTRACT:
Some novel Schiff bases of quinazolin-4-(3H)-one were synthesized in good yields and evaluated for their possible anti-bacterial, anti-fungal activities. The structures of the newly synthesized compounds were confirmed by their spectrum of IR, NMR, and MASS. Their antibacterial, fungal activities were evaluated by Plate hole diffusion method against gram positive, gram negative bacteria and Aspergillus Niger, candida albicans were used. The newly synthesized compounds showed moderate to significant anti-bacterial and fungal activities when compared to standard drug. The obtained results showed that the most active compounds could be useful as a template for future design and modification, investigation to produce more active pharmacophores.

KEYWORDS: Quinazolin-4-one, Schiff base, para amino phenol, anti-microbial activity, Aromatic aldehydes.

INTRODUCTION:
To ever-increasing microbial antibiotic resistance leads to ongoing testing of new biologically efficient compounds of either natural or synthetic origin for infectious diseases[1]. Quinazoline nucleus is an exciting molecule with two nitrogen atoms in its structure among the most significant classes of aromatic bicyclic compounds which is one of the most widespread scaffolds among natural and synthetic bioactive compounds. This heterocyclic compound resembles both the purine nucleus and the pteridine one[2,3]. These nucleuses are an interesting class of organic compounds being studied over the years and reported to possess a wide spectrum of biological activities such as antibacterial[4], antifungal[5], Antitubercular[6], antioxidant[7] and antihypertensive[8], anticonvulsant[9], anti-inflammatory[10].

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The pharmacodynamic versatility of quinazolin-4-one moiety has been documented not only in many of its synthetic derivatives but also in several naturally occurring alkaloids isolated from animals, families of plant kingdoms and from microorganism[11].The quinazolinone is a versatile lead molecule for designing potential bioactive agents. Imines (Schiff bases) and their reaction products are an interesting class of organic compounds being studied over the years and reported to possess a wide spectrum of biological activities such as antimicrobial, antioxidant, cytotoxic, and anticonvulsant. Moreover, the Schiff bases formed by simple condensation of amine and aldehydes in alcoholic medium to form an azomethine linkage[12].

EXPERIMENTAL:
The present work, synthetic grade chemicals were utilized and purchased from sigma-aldrich. The purity of the compounds was confirmed by thin layer chromatography plates coated with siliga gel G, benzene: chloroform as mobile phase, iodine vapour as the detection method and the pure compounds were recrystallized from ethanol. The melting points were taken in open capillary tubes in concentrated in melting point apparatus and therefore the values reported are uncorrected. UV spectra were recorded on Shimadzu 1700, UV-Vis spectrophotometer and spectral grade,

ethanol was used as the solvent. The IR spectra of the compounds were recorded in the region, 4000-400 cm-1 using KBr discs on JASCO 4100 FTIR and the NMR spectral study was done using DMSO as the solvent on JOEL FX90Q, Fourier transform NMR spectrometer.

General procedure for the synthesis of quinazolin 4 one derivatives[13,14]

The intermediate compound of benzoxacine 4 one prepared by mixture of ortho amino benzoic acid (0.1mol) and acetic anhydride (0.2mol) in pyridine. The reaction mixture was stirred refluxed at 60-80^{0}C for 1hr followed by cooled with poured into cursed ice. The progress of reaction is monitored by thin layer chromatography (TLC), the solid mass was obtained and recrystallized by ethanol.

Synthesis of quinazolin 4 one derivatives:

Synthesis of 3-(4-hydroxyphenyl)-2-methyl quinazolin-4-one[15]

The intermediate compound of benzoxacine 4 one (0.1mol) and p-amino phenol (0.1mol) suspended in glacial acetic acid. The mixture was dissolved in ethanol (30ml) and stirred reflux for 4 hours. The completion of the reaction was monitored by thin layer chromatography. The content of the flask poured into the cold water and the solid mass is obtained, the mixture was filtered and dried followed by recrystallized by methanol.

Synthesis of 3-(4-hydrazinylphenyl)-2-methyl quinazolin-4-one[16]

Compound of 3-(4-hydroxyphenyl)-2-methyl quinazolin-4-one and 0.01M Hydrazine hydride dissolved in a sufficient quantity of Ethanol, 2-3 drops conc. sulphuric acid (H2SO4) in a RBF and stirred well. The reaction mixture refluxed for 6 hours. After the completion of reaction, pour into the beaker containing cold water and the precipitate is formed. Filtered, dried and recrystallized by diethyl ether.

Synthesis of 3-{4-[2-benzylidenehydrazinyl] phenyl}-2-methylquinazolinon-4-one derivative[17]

Compound of 3-(4-hydrazinylphenyl)-2-methyl quinazolin-4-one which is acidified with glacial acetic acid and add substituted Benzaldehydes derivative (0.01mol) in a RBF. The reaction mixture was stirred well and refluxed for 8 hours. The reaction was monitored by TLC. After the completion of the reaction the reaction mixture is poured into a beaker containing cursed ice and the precipitate of benzaldehydes derivative was formed. Filter the precipitate and dried well followed by recrystallized with Ethanol.

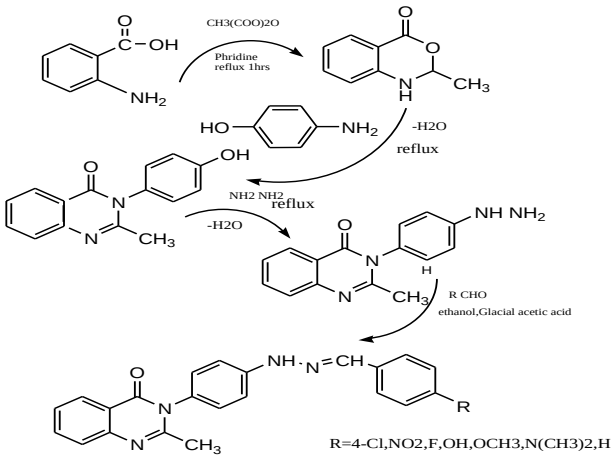

Fig: 1, Scheme of the newly synthesized compounds

Table.1: Physiochemical properties of synthesized compounds

Compound code	Molecular formula	Molecular weight	Melting point (°C)	RF value	yield
QD-1	$C_{22}H_{17}ClN_4O$	399	175	0.84	83
QD-2	$C_{22}H_{17}N_5O_3$	389	165	0.90	75
QD-3	$C_{22}H_{17}FN_4O$	372	172	0.75	83
QD-4	$C_{22}H_{18}N_4O_2$	370	160	0.92	81
QD-5	$C_{23}H_{20}N_4O_2$	384	158	0.80	72
QD-6	$C_{24}H_{23}N_5O$	397	169	0.76	85
QD-7	$C_{22}H_{18}N_4O$	354	148	0.86	81

Spectral data:

QD-1: IR (KBr, v_{max}, cm^{-1}):3042(Ar-H) 1616(C=O):1456.26(C=N) 1530(C-N), 1031.92(CO-C), 686.66 (-Cl)

H NMR, (δppm):7.4(CH, Ar), 7.9(Ar-C=O (N), 7.62 (benzylidenimine), 4.0(amine), 8.1(Ar-C-NH), 0.9 (CH3), 6.44 (Ar-N-C=O), 7.2(-Cl). MS m/z: 388.

13C NMR (500 MHz, DMSO-d6, δppm): 151.7(imine), 22.4 (-CH3), 147(C=O), 147.74(C-CH3), 160.9(O=C-NH), 138.7(Cphenyl ring), 127.4(2CH), 122.4(2CH),

QD-2: IR (KBr, v_{max}, cm^{-1}):3431.36(Ar-CH), 1720 (C=O):1521.84(C=N), 1068.56(C-N), 1319.81(C-NO2)

H NMR, (δppm):7.4(CH, Ar), 7.9(Ar-C=O (N), 7.62 (benzylidenimine), 4.0(amine), 8.1(Ar-C-NH), 0.9 (CH3), 6.44 (Ar-N-C=O), 8.2(Ar-NO2).MS m/z: 399.
13C NMR (500 MHz, DMSO-d6, δppm): 151.7(imine), 22.4 (-CH3), 147(C=O), 147.74(C-CH3), 160.9(O=C-NH), 138.7 (Cphenyl ring), 127.4(2CH), 122.4(2CH), 150.7(Ar-NO2).

QD-3: IR (KBr, v_{max}, cm^{-1}):3042(Ar-H) 1616 (C=O): 1456.26(C=N) 1530(C-N), 1031.92(CO-C), 410(-F)
H NMR, (δppm):7.4(CH, Ar), 7.9(Ar-C=O (N), 7.62(benzylidenimine), 4.0(amine), 8.1(Ar-C-NH), 0.9 (CH3), 6.44 (Ar-N-C=O), 7.0(-F). MS m/z: 372.

13C NMR (500 MHz, DMSO-d6, δppm): 151.7(imine), 22.4 (-CH3), 147(C=O), 147.74(C-CH3), 160.9(O=C-NH), 138.7(Cphenyl ring), 127.4(2CH), 122.4(2CH), 165.2(-F).

QD-4: IR (KBr, v_{max}, cm^{-1}):3042(Ar-H) 1616(C=O): 1456.26(C=N) 1530(C-N), 1031.92(CO-C), 3017 (C-OH)

H NMR, (δppm):7.4(CH, Ar), 7.9(Ar-C=O (N), 7.62(benzylidenimine), 4.0(amine), 8.1(Ar-C-NH), 0.9 (CH3), 6.44 (Ar-N-C=O), 5.0(C-OH). MS m/z: 370

13C NMR (500 MHz, DMSO-d6, δppm): 151.7(imine), 22.4 (-CH3), 147(C=O), 147.74(C-CH3), 160.9(O=C-NH), 138.7(Cphenyl ring), 127.4(2CH), 122.4(2CH), 16O.8, (C=N), 128.8 (C=O-N).

QD-5: IR (KBr, v_{max}, cm^{-1}):3042(Ar-H) 1616(C=O): 1456.26(C=N) 1530(C-N), 1031.92(CO-C), 2723 (-OCH3)

H NMR, (δppm):7.4(CH, Ar), 7.9(Ar-C=O (N), 7.62(benzylidenimine), 4.0(amine), 8.1(Ar-C-NH), 0.9 (CH3), 6.44 (Ar-N-C=O), 3.73 (O-CH3. MS m/z: 384

13C NMR (500 MHz, DMSO-d6, δppm): 151.7(imine), 22.4 (-CH3), 147(C=O), 147.74(C-CH3), 160.9(O=C-NH), 138.7(Cphenyl ring), 127.4(2CH), 122.4(2CH),163,114.9-(C=N),55.9 (OCH3).

QD-6:IR (KBr, v_{max}, cm^{-1}):3042(Ar-H) 1616(C=O): 1456.26(C=N) 1530(C-N), 1031.92(CO-C), 1568 (-N(CH3)2)

H NMR, (δppm):7.4(CH, Ar), 7.9(Ar-C=O (N), 8.1(benzylidenimine), 4.0(amine), 6.6(Ar-C-NH), 0.9 (CH3), 6.44(Ar-N-C=O), 2.85 (N (CH3)2). MS m/z: 397

13C NMR (500 MHz, DMSO-d6, δppm): 151.7(imine), 22.4 (-CH3), 147(C=O), 147.74(C-CH3), 160.9(O=C-NH), 138.7(Cphenyl ring), 127.4(2CH), 122.4(2CH), 163,114.9-(C=N), 40.3 (N (H3)2)).

Antibacterial Study (Plate Hole Diffusion Method)[18]:
The ingredients were dissolved in water, adjust the Ph is 7.4±0.2 and autoclave at 120°C for 20 mints.250µg/ml concentration of the test compounds are prepared and Dimethyl Sulphoxide was used as vehicle and gentamycin was used as the standard.

Nutrient agar plates were prepared aseptically to get a thickness of 5-6mm.The plates were allowed to solidify and inverted to prevent condensate falling on the agar surface. The plates were dried at 37°C just before inoculation.

The standard inoculums are inoculated in the plates prepared earlier aseptically by sterile swab in the inoculums, excess of inoculums removed by pressing and rotating the swab in the inoculums. Excess of inoculums removed by the level of the liquid and finally streaking the swab all over the surface of 60 after each application. Finally, press the swab round the edge of the agar surface. The sterilized discs for the test drugs were placed in the pertiplates aseptically. Incubate the Petri plate at 37±0.2 for 18-24 hrs, after placing them in the refrigerator for 1 hr to facilitate uniform diffusion. The average zone diameter of the plates was measured and recorded. All compounds synthesized were tested for antibacterial activity against for gram (+ve) and gram (ve) bacteria.

Antifungal activity[18,]
Two fungal strains, *Candida albicans* and *Aspergillus niger* were employed to test the antifungal activity of the title compounds in the concentration of 100 µg/mL using the above protocols. The experiments were done in triplicates on *A. niger* and *C. albicans*. Standard Fluconazole and DMSO were served as positive and negative control, respectively. The zone of inhibition measured in millimeter was taken for the evaluation of an antifungal activity. Nutrient agar were added slowly to the filtered solution of peeled potatoes, which were pre-boiled in 200 mL of water for 30 min. Fluconazole in 10 µg/mL concentration and DMSO was taken as standard and control, respectively. The diameter of the zones of inhibition was recorded.

Table 2: Antibacterial activity of the compounds against Gram-positive and Gram -negative bacteria

Bacteria	zone of inhibition of the compounds(mm)								
	QD-1	QD-2	QD-3	QD-4	QD-5	QD-6	QD-7	Control	Standard-(10µg/ml) Ciprofloxacin
S.aureus	24	23	25	20	26	20	18	3	35
B.Subtilis	27	25	25	23	24	19	20	4	32
P.aurginosa	15	07	24	20	23	17	17	2	30
E.coli	24	10	23	24	24	21	20	4	35

Table 3: Antifungal activity of the compounds

Bacteria	zone of inhibition of the compounds(mm)								
	QD-1	QD-2	QD-3	QD-4	QD-5	QD-6	QD-7	Control	Standard-(10µg/ml) Fluconazole
C.albicans	23	25	25	23	24	18	15	3	28
A. niger	25	23	24	22	24	17	14	2	27

Antibacterial screening of the compounds (QD1-QD7) was screened against two Gram-positive, namely, *S. aureus* and *B. Subtilis*, two Gram-negative, namely, *Pseudomonas aeruginosa* and *E. coli* using agar plate method. All the synthesized compounds display considerable antibacterial properties. Interestingly, compounds QD-QD-5, showing comparatively higher inhibition to both Gram-positive and Gram-negative bacteria. The results are tabulated in Table 2. The results of antifungal screening performed against C. albicans and A. Niger are depicted in Table 3. Results display the antimicrobial potential of the title compounds against selected bacterial and fungal strains. The phenyl ring substituted with both electron-donating and electron-withdrawing groups produced considerable antimicrobial activity. From the above results, the substituted derivatives of quinazolinone and QD1, QD2 and QD3, QD5 displayed good antimicrobial potential. The above findings can clear that these quinazolinone derivatives can serve as lead molecules to the development of potent antimicrobial agents.

Determination of minimum inhibitory concentration (MIC)

The minimum inhibitory concentration was found for all the test compounds against bacterial and fungal organism shown in table no: 3 Plate hole diffusion method

This method based on the inhibition of growth of microbial culture in a uniform solution of the test sample in a fluid medium with the aim of its rapid growth. The test sample was dissolved in dimethyl Sulphoxide (DMSO), diluted to highest concentration 1000µg/ml to 15.6µg/ml in sterile test tubes containing standard inoculums. All the tubes were incubated at 37^0C for 24hr (bacteria),24^0C for 48hr (fungi). Later minimum inhibitory concentration values were determined. The highest dilution of extract which shows no turbidity was observed and recorded. This dilution was measured to have the concentration of the drug equivalent to MIC.

Table: 4. MIC of synthesized compounds

Micro organism	Compound code	1000µg/ml	500µg/ml	250µg/ml	125µg/ml	62.5µg/ml	31.25µg/ml	15.625µg/ml	blank
Staphylococcus aureus	QD-1	-	-	-	+	+	+	+	+
Bacillus Subtilis	QD-2	-	-	-	+	+	+	+	+
Candida albicans	QD-2	-	-	-	+	+	+	+	+
Candida albicans	QD-3	-	-	-	-	+	+	+	+
Aspergillus Niger	QD-5	-	-	-	+	+	+	+	+
Aspergillus niger	QD-3	-	-	-	-	+	+	+	+

-: inhibition, +: No inhibition

RESULTS AND DISCUSSION:

The titled compounds were synthesized in four step process. The first step was synthesis of 2 substituted benzoxacine 4 one by the acetylation of ortho amino benzoic acid and acetic anhydride in the presence of pyridine commonly known as Niementowski reaction. The advantage is that which is useful intermediate to afford various medicinally important heterocyclic compounds. The intermediate compounds were further condensed with Para amino phenol to afford 2,3 substituted quinazolin-4- one derivatives which is further condensed with hydrazine hydrate and finally condensed with various 4^{th} position aromatic aldehydes to afford to titled compounds which is shown in fig:1.

The melting points of all the newly synthesized compounds were reported and determined by open capillary tubes with electrical heating melting point apparatus and are uncorrected. The solubility of all the titled compounds was done by using the following solvents: benzene, chloroform, ethanol, methanol, water, DMSO. The purity of the all the newly synthesized compounds were done by thin layer chromatography using silica gel as stationary phase, employed benzene: chloroform (7:3) as mobile phase, spots were visualized using iodine vapours. The rf values of the synthesized compounds were also reported in table: 1.

The infrared spectra of all the synthesized compounds was elucidated and expressed as wave number in cm^{-1}. The presence of N=CH bond stretch confirmed the formation of titled compounds. The nuclear magnetic resonance spectra of synthesized compounds were elucidated. The presence of N=CH proton confirmed the formation of Schiff bases, spectral data were in correlation with the expected structure. The maximum

frequency of the synthesized compounds were determined and also wavelength at which each compound has shown maximum absorbance has specific absorptivity.

the newly synthesized compounds were tested for antibacterial activity against gram positive such as *Staphylococcus aureus*, *Bacillus Subtilis* and gram negative organism such as *Pseudomonas aeruginosa*, *Escherichia coli* which shown in table 2 and 3. The zone of inhibition, MIC were measured and compared against a standard. The newly synthesized compounds were tested for antifungal activity against the organism of *Candida albicans*, *Aspergillus niger*. The zone of inhibition of minimum inhibitory concentration (MIC) were measured which is shown in table:4. The antibacterial and fungal evaluation of all the new compounds showed a significant zone of inhibition than standard ciprofloxacin, Fluconazole.

All the newly synthesized compounds exhibited better activity against almost all the bacterial and fungal organism. Among the quinazolin-4-one derivatives of synthesized compounds QD-1, QD-2, QD-3 and QD-5 exhibited potential inhibitory action in comparison with standard drugs. Furthermore bacterial organism of *S. aureus*, *B. subtilis* and *E. coli* and fungal organism *C. albicans*, *A. niger* were found to be better inhibition and significant activity by quinazolin-4-one derivatives .it was found to be that the quinazolin-4-one derivatives exhibited a significant anti-microbial activity.

CONCLUSION:

Quinazolinone were therapeutically essential class of compounds. The allowed work describes the synthesis of series of 2,3 substituted quinazolinone derivatives via Niementowski reaction. The purity of the compounds were established by thin layer chromatography. The structures were established by detailed spectral analysis. Antimicrobial studies were performed on the synthesized compounds against selected microbial strains and the results revealed the antibacterial, antifungal properties.

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REVIEW ARTICLE

Herbosomes: Impressive tool for better Bioavailability and Bioactivity of plant extracts and Botanicals

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ABSTRACT:
Herbosomes are the minute structures in which standardized herbal extract are incorporated into a phospholipid, resulting to a lipid friendly complex which is also called phytosomes. The potent phytoconstituents are incorporated within a phospholipid complex which is well absorbed by the lipid rich cell membrane system, thus bioavailability of the poorly absorbed polar phytoconstituents are enhanced. These herbosomes may be prepared by various methods like lyophilization technique, salting out process, solvent evaporation etc. The Visualization, vesical size, zeta potential, entrapment efficiency, transition temperature, surface tension measurement, vesicle stability and drug content are used for evaluation of Herbosomes. Herbosome are becoming more popular day by day because of their specificity selectivity and improved bioavailability. Laying down the absorption problem with traditional herbal extracts and other dosage form herbosomes are the complexes with enhanced bioavailabilty and potency. The herbosome of the silymarin, curcumin, sinigrindiomisin are successfully prepared and are available to the market. Thus it is clear that the herbosomes are the novel preparation for the complex natured herbal constituents for the better bioavailability and selectivity and maintaining the potency. This paper highlighted about advanced technology, preparation, properties, advantages, merits, demerits, characterization techniques, i*n vivo* evaluation, application and marketed formulation of Herbosomes.

KEYWORDS: Herbosomes, phospholipid complex, characterization techniques, marketed formulation.

INTRODUCTION:
The term Herbo is considered as plant and the some means cell like. Most of the compounds of plat origin are polar or water soluble molecules. Because of the cellular lipid structure and the large molecular size of the polar soluble phytoconstituents (viz. flavonoids, tannins, glycosides etc) these cannot absorb by passive diffusion or due to their poor lipid solubility, severely limiting their ability to pass across the lipid rich biological membrane which results the poor bioavailability of the active constituents. Since ancient time phytomedicines have been used for the treatment of various ailments.

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Various plant material have been exhibited a variety of biological activity such as hepatoprotective activity, antilipidemicactivity, anticancer activity, hypoglycemic activity etc. Phytomedicines complex chemical mixtures prepared from plants have been used for health maintenance since ancient's times. But many phytomedicines are limited in their effectiveness because they are poorly absorbed when taken by mouth. Currently, as many as one-third to approximately one-half of all the drugs available are derived from plants or other natural sources [1].

The chemical complexity of the crude or partially purified extract appears to be crucial for the bioavailability of the active constituents; hence standardization of herbal extracts has become imperative. Although having excellent bioactivity in vitro, plant extracts often exhibit poor effectiveness in vivo or in animal models. The basic reasons for the low bioavailability of herbal extracts are that the bioactive components of these herbs possess multi- ring molecular

structures which cannot be absorbed into the blood by simple passive diffusion and the bioactive phytoconstituents are mostly water soluble, hence, their poor lipid solubility limits their ability to pass across lipid biomembranes. This has restricted the use of pharmacologically effective polyphenolic plant actives for treating different disorders. Moreover, when taken orally, bioactive phytoconstituents are destroyed by or lost to the gastric environment or they may be rendered less effective by interaction with other drugs or nutraceuticals [2].

Phytomedicines, complex chemical mixtures prepared from plants have been used for health maintenance since ancient's times. But many phytomedicines are limited in their effectiveness because they are poorly absorbed when taken orally. The phytosome technologies, developed by Indena S.P.A. Italy, markedly enhance the bioavailability of selected phytomedicines, by incorporating phospholipids into standardized extracts and vastly improve their absorption and utilization. [3].

These herbosomes have proven to be highly efficient in the protection of pharmaceutically active herbal extracts against gastric secretions and gut bacteria. The herbosomes of herbal extracts of ginseng, milk thistle, grape seed, green tea and hawthorn are available in the market [4]. Hence the details of Herbosomes preparation, evaluation, merits, demerits and advantages are illustrated momentarily and it assist the researchers working for herbosomes to acquire better formulation.

2. Herbosome technology:

Prerequisite for herbosome formation

1. Standardized extract or an active phytoconstituent
2. Carrier Phospholipid
3. Solvent

Standardized extract or an active phytoconstituents selection [5, 6, 7 8]

1. Basically, either active constituents or standardized extract were selected for phospholipid complex formulation. However, natural products after isolation and purification may lead to a limited or total loss of specific biological activity so, in such cases whole plant extracts are selected. Usually, phospholipid complex formulations are prepared according to weight basis for standardized extract, whereas molar ratios for active constituent.

2. Selection of plant extract depends on its phytochemical (such as polyphenols, triterpenoids, tannins, alkaloids and saponins) and pharmacokinetic profile. Usually they have multiple ring molecules which are too large to be absorbed by simple diffusion and have low permeability across the cellular lines of the intestine.

3. A drug which contains an active hydrogen atom like – COOH, -OH, $-NH_2$, -NH etc., which have the ability to form hydrogen bond between the drug and N-(CH_3) of PC molecules.

4. Any drugs which possess π electrons can be formulated into different complexes with phospholipid molecules.

5. Both hydrophilic and lipophilic actives can be complexed to improve bioavailability.

Phospholipids and their importance. [5, 9].

In general, fats, phospholipids, and steroids are different types of lipids present in the body and perform various functions. Among them, phospholipids which are major components of cell membranes also serve as a vehicle, thus making the design of drug delivery systems more flexible, and are suitable for the body needs. Phospholipids are bio friendly and offer various advantages such as formulation flexibility and the choice of different NDDS based on the intended use. Phospholipids are lipids containing phosphorus, a polar portion and non-polar portion in their structures.

A human biological membrane constitutes different classes of phospholipids, like phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylcholine (PC), phosphatidic acid (PA), and phosphatidylserine (PS). PC possess two neutral tail groups and a positive head group which contains an oxygen atom in the phosphate group that has a strong tendency to gain electrons, while nitrogen to lose electrons, a rare molecular characteristic that makes PC miscible in both water and lipid environments. Earlier "Lecithin" is a word which created perplexity in researchers for identification but later on it was clearly discussed by Wendel. In commercial perspective, lecithin refers to PC, PE, PS, PI and other phospholipids. But in historical point of view lecithin includes lipids which contains phosphorous obtained from brain and egg. However, scientifically lecithin refers to PC.

Phospholipid source and its additional benefits as adjuvant. [5, 9].

Phospholipids are obtained from both natural and synthetic source. Phospholipids are widely found in plants and animals, and the main sources are vegetable oils soya bean, sunflower seed, rapeseed, and cotton and animal tissues include e.g. egg yolk and bovine brain. Most of the literature suggests the use of soya bean phosphatidylcholine while few others have used egg lecithin, in the preparation of phytophospholipid complex. In fact, phospholipids are one of the most abundantly present lipid fractions in biological membranes and can form bilayers and act as amphipathic

molecules. After oral administration of phospholipids, they are absorbed to a great extent and reach the peak plasma concentration within 6 hours. FDA and German Cancer Research Centre, Heidelberg stated that Soy phosphatidylcholine has no carcinogenicity and no risk in formation of tumour. Additionally, PC is said to have varied advanced beneficial properties like hepatoprotective activity, nutritional supplement to support brain health, role in membrane fluidity, shows superior host defences (like enhancing NK cell activity and phagocytosis), excellent emulsifying activity, major component of the gastric mucosa lining of the stomach protecting from ulcer, precursor for acetylcholine, reducing serum cholesterol, improving the perception of taste and smell, recuperate fatigue and even in nourishing skin.

3. Preparation of Herbosomes:

Phytosomes are prepared by different methods by interacting 3-2 moles natural or synthetic phospholipid, mainly phosphotidylcholine with one mole of phytoconstituent. The most preferable ratio for complexes formation between these two moieties is in the range from 0.5 to 2.0 mole [10].

Solvent evaporation method:

A natural or synthetic phospholipid phosphotidylcholine and phytoconstituent is suspended in an appropriate solvent, further refluxed for few hours. The resultant clear mixture is being evaporated under vacuum [11].

Salting out method:

The phytoconstituent or standardized extract and phosphotidylcholine is dissolved in an aprotic solvent, such as dioxane or acetone where the solution is being stirred overnight then the formed complex is isolated from by precipitation from non-solvent like n-hexane[12].

Lyophilization technique:

Both natural or synthetic phospholipid and phytoconstituent is dissolved in different solvent and further solution containing phytoconstituent were added to a solution containing phospholipid followed by stirring till complex formation takes place. The formed complex is isolated by lyophilization[13].

The phospholipid which are used in preparation of phytosome consist of acyl group which may be same or different in phosphatidylcholine, phosphatidylserine, phosphatidyl ethanolamine and mostly derived from palmitic, stearic, oleic, and linoleic acid[14,15]. In phytosome active principle becomes an integral part of the membrane as the active principle is anchored to the polar head of phospholipid [16].

Role of phosphatidylcholine in Herbosome preparation:

Phospholipid serves as a major moiety in composition of cellular and subcellular membrane. They are basic substance to maintain life activity. The human body uses phosphpolipid as emulsifiers and also enhances the absorption of fat-soluble substances. Furthermore, it act as surface active agent in the pleura and alveoli of lung, joints, pericardium, etc [17]. They can be extracted from egg yolk or soybeans through mechanical or chemical methods with the aid of hexane. Phosphatidylcholine has two groups mainly lipophilic, phosphatidyl group, and the choline group which is a hydrophilic moiety. Choline moiety improves memory function and aids muscle control. The choline portion binds to the herbal extract while thephosphatidyl group covers the phytoconstituents like a cell form which further protects the active constituent from destruction from the digestive juices. Due to drugphospholipid complex formation the bioavailability of the active constituent is increased, along with prolonged duration of action [18].

4. Properties of Herbosomes:

Physical Properties

- Herbosome has lipophilic substances with a clear melting point.
- Average size of herbosome range is 50 nm to a few hundred μm.
- They are easily soluble in non-polar solvents, insoluble in water and moderately soluble in fats.
- Liposomal like structures of miscellar shape are formed when herbosome are treated with water [19].

Chemical properties:

On the basis of their physicochemical and spectroscopic data, it has been shown that, the phospholipids-substrate interaction is due to the formation of hydrogen bond between the polar heads of phospholipids (i.e. phosphate and ammonium groups) and the polar functional groups of substrate. In herbosomes the active principle is anchored to the polar head of phospholipids, becoming an integral part of the membrane [19, 20, 21].

5. Advantages:

- These systems show enhanced permeation of drug through skin for transdermaland dermal delivery.
- These are platform for the delivery of large and diverse group of drugs (peptides, protein molecules).
- The vesicular system is passive, non-invasive and is available for immediate commercialization.
- Their composition is safe and the components are approved for pharmaceutical and cosmetic use.
- High market attractiveness for products with proprietary technology.

6. Merits of Herbosomes:

- Herbosomes show better stability as chemical bond is formed between phospholipid molecule and phytoconstituent (s).
- Dose of phytoconstituents is reduced due to more bioavailability of phytoconstituents in the complex form.
- Duration of action is increased.
- Herbosomes are simple to manufacture.
- Phytoconstituents complex with phospholipids are more stable in gastric secretion and resist the action of gut bacteria. 6. Enhanced permeability of phytoconstituents across the biological membranes[22].

7. Demerits of herbosomes:

- In herbosomes, phytoconstituents are rapidly eliminated.
- It has short half-life.
- Hydrolysis, fusion, leakage and oxidation is undergone by the phospholipids.
- It has a high cost of production and sometimes occurrence of allergic reactions to the herbosomal constituents may be observed.
- Because of their larger size problems can occur while trying to target to the various tissues [23].

8. Characterization Techniques:

Visualization

Transmission electron microscopy and scanning electron microscopy are used for visualization of phytosomes[24].

Vesical size and zeta potential:

Dynamic light scattering (DLS) using computerized inspection system and photon correlation spectroscopy (PCS) used to determined vesical size and zeta potential [25].

Entrapment efficiency:

Ultracentrifugation technique is used to determined entrapment efficiency [26].

Transition temperature:

Differential scanning calorimetry is used to determine transition temperature of vesicular lipid system [26].

Surface tension measurement:

Surface tension activity can be measured by ring method in a Du Nouy ring tensiometerof the drug in aqueous solution [27].

Vesicle stability:

Assessing the size and the structure of vesicles overtime gives the idea about stability of vesicles. Structural changes are monitored by TEM and mean size is measured by DLS [28].

Drug content:

Modified high-performance liquid chromatographic method or suitable spectroscopic method used to quantify the amount of drug present. [29].

9. *In vivo* evaluation:

Experimental models are chosen on the basis of anticipated therapeutic activity of the plant constituent in phytosome for in vivo and in vitro examination. For example, examination of antihepatotoxic activity can be assessed by antioxidant or free radical scavenging property of phytosome. The in vivo anti-hepatotoxic studies on animals through the effect of phytosome on alcohol induced or paracetamol-induced hepatotoxicity.[30]

10. Application of Herbosome

- Herbosome are used in the treatment of liver diseases including alcoholic hepatic steatosis, drug induced liver damage and hepatitis.
- Herbosomes are used in anti-inflammatory activity as well as in pharmaceutical and cosmetic composition.
- Herbsomes are used to treat acute and chronic liver diseases of toxic metabolic or infective origin or of degenerative nature.
- Herbosomes are used as brain tonic, immunomodulatory, skin improver, antiwrinkle, anti-aging etc.
- Herbosomes are used as anticancer and antioxidant, eg- grape seed.
- Herbosomes are used in hyperlipidemia, vein and skin disorder.
- Herbosomes are used as cancer chemo preventive agent and used to treat benign prostate hyperplasia.
- It also used to treat hypertension.

11. Commercially Marketed Formulations:

There are various Herbosomes formulation available in the market, and important formulations are illustrated in table 1.

Table 1: Herbosomes available in the market.

Trade name	Phytoconstituents complex	Daily dose	Indications
Silybinphytosome	Silybin from Silibiummarianum	120 mg	Hepatoprotective, Antioxidant
Silyphos milk thistle	Silybin from Silibiummarianum	150 mg	Antioxidant, Hepatoprotective
Grape seed (Leucoselect) Phytosomes	Procyanidins from vitisvinifera	50-300 mg	Antioxidant, Anticancer
Ginseng phytosome	Ginsenosides from panax ginseng	150 mg	Immunomodulator
Hawthorn phytosome	Flavonoids from crataegus species	100 mg	Antihypertensive, Cardioprotective

Sericosidephytosome	Sericoside from Terminalia sericea	-	Skin improver, Anti-Wrinkles
Ginko select phytosome	Flavonoids from Ginkobiloba	120 mg	Anti-aging, Protects Brain & Vascular lining
Olea select phytosome	Polyphenols from Oleaeuropea	120 mg	Anti–hyperlipidemic, Anti-inflammatory
Green select phytosome	Epigallocatechin from Thea sinensis	50-300mg	Anti-cancer, Antioxidant
Echinacea phytosome	Echinacosides from Echinacea angustifolia	-	Immunomodulatory, Nutraceuticals
Bilberry (Mertoselet) phytosome	Anthocyanosides from Vaccinium myritillus	-	Antioxidant, Improvement of Capillary Tone
Palmetto (sabalselect) phytosome	Fattyacids, alcohols & sterols from Serenoarepens	-	Anti-oxidant, Benign, Prostatic hyperplasia
Centellaphytosome	Trepans' from Centellaasitica	-	Brain tonic, Vein and Skin Disorder

12. CONCLUSION:

Herbosomes are novel formulations which offer improved bioavailability of hydrophilic flavonoids and other similar compounds through the skin or gastrointestinal tract. It has many distinctive advantages over other conventional formulations. As far as the potential of herbosome technology is concerned, it has a great future for use in formulation technology and applications of hydrophilic plant compounds.

Standardized plant extracts or mainly polar phytoconstituents like flavonoids, terpenoids, tannins, xanthones when complexed with phospholipids like phosphatidylcholine give rise to a new drug delivery technology called herbosome showing much better absorption profile following oral administration owing to improved lipid solubility which enables them to cross the biological membrane, resulting enhanced bioavailability i.e. more amount of active principle in the systemic circulation. This means more amount of active constituent becomes present at the site of action (liver, brain, heart, kidney etc) at similar or less dose as compared to the conventional plant extract. Hence, the therapeutic action becomes enhanced more detectable and prolonged. Several excellent phytoconstituents have been successfully delivered in this way exhibiting remarkable therapeutic efficacy in animal as well as in human models.

Recently Mukherjee and co associates have regarded herbosomes as a value added drug delivery system. Thorough study of literature reveals that several plant extracts (crude, partially purified or fractionated) are reported to possess different significant pharmacological or health promoting properties. These extracts can be standardized accordingly and may be formulated as Herbosomes for systematic investigation for any improved potential to be used rationally. In this way after screening and selection of potential extracts or constituents from plants, herbosomes can be developed for different therapeutic purposes like cardiovascular, anti-inflammatory, immunomodulator, anticancer, antidiabetic etc or for prophylactic and health purposes as nutraceuticals, induecourse.

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REVIEW ARTICLE

Innovative nano approaches in cutaneous delivery of drugs

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ABSTRACT:
Nanotechnology deals with exceptionally minute structures, size ranging from 0.1 to 100 nm. The field of nanomedicine and nanotechnology is currently undergoing explosive developments in various fields of science and other new areas. Properties related to the particle surface and quantum mechanics are the two main aspects that render the nanomedicine functionally different from other bulk formulations. Dermal diseases can be controlled by using the nano approaches which allows an enormous opportunity in designing the novel low dose and effective treatment systems. Therefore, they have received meticulous attention as topical drug delivery systems. The present review considers the anatomy of skin, skin diseases, novel nano-based formulation approaches with evaluation techniques and its applications in various aspects.

KEYWORDS: Cutaneous drug delivery, Nanoparticles, Nano approaches.

INTRODUCTION:
The present review is based on the potential use of nanoparticles over conventional therapy for dermal infections[1]. The cutaneous drug delivery approach became a priority area in research and also for the development process in pharmaceutical companies as it shows the larger therapeutic window when compared to other conventional delivery of drugs[2]. Penetrating the dermal barrier of the skin i.e., the stratum corneum is one of the majors challenges to deliver the large hydrophilic drugs cutaneously. Therefore, the delivery of the drug in a nanocarrier system can afford to penetrate the deeper tissues of the skin to provide a systemic action or a targeted action for therapeutic, diagnostic and prophylactic purposes[3].

As per WHO, skin diseases are responsible for much disability, disfigurement, and distress. Skin diseases mostly prevail in rural communities in developing countries and low economic class. The serious fatal deaths in the hospital might be due to infections on the localized surface because of injuries, surgeries, abrasion and burn injuries[4,5].

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Nanoparticles are target specific with high penetration power through the skin[6,7]. Multi-drug loading efficiency is enhanced. It also provides a versatile platform for the delivery of drugs and nucleic acid[8]. It also shows the potential advantages of photothermal therapy and radiotherapy[9]. Therefore, as nanotechnology is advancing in its various aspects its products are also getting extremely authoritative[10]. Therefore, it can be successfully used for cutaneous delivery of drugs without any invasive procedures.

Physiology of skin:
The skin is a complex organ presenting a total area of approximately $2m^2$ and known to be the largest organ exposed to the external environment in the human body. The properties of the skin vary from one part of the human body to the other part, also varies from one individual to another with respect to the color, thickness, texture and also the adnexal structures. Some of the vital roles of the skin include-

i. Acts as a physical barrier from the external environment from pathogens, chemicals, thermal, mechanical and radiation damages.
ii. Acts as a sensory detector and regulates interaction with the external environment
iii. Acts as the thermoregulatory system[11].
iv. Secrets hormones, cytokines, and growth factors and act as the endocrine and metabolic functioning system.

v. Known as the excretory system, where the excretory products are released out through the skin pores and glands present on the layers of the skin[12,13]

Human skin is comprised of three layers- stratified, cellular epithelium, dermis layer of connective tissue and hypodermis[14,15,16]. The presence of ridges on the epidermis (rete ridges) is projected into the dermis through the dermal-epidermal junction. The undulating section of dermal-epidermal junction not only provides mechanical support but also acts as a partial barrier. Panniculus adiposus, a sheet of a fatty layer is present just below the dermis layer of the skin known as a subcutaneous layer which is separated by panniculus carnosus, a vestigial layer of striated muscle[16].

The human skin is also susceptible to certain diseases as like other organs of the body, as listed in table-1

Table 1: Certain diseases of human skin

Sl. No	Diseases	Causes
1.	**Atopic dermatitis or eczema**	An inflammatory skin disease characterized by dry, red, itchy patches of skin.
2.	**Acne**	It occurs when hair follicles become clogged with dead skin cells and oil.
3.	**Melanoma**	A type of skin cancer caused by exposure to excess sunlight.
4.	**Psoriasis**	It causes red, flaky patches to appear on the skin.
5.	**Scabies**	An itchy skin condition caused by the human scabies mite.

Selection of drugs for dermal delivery:

The human skin serves as a selective barrier for a huge number of drugs[17]. For effective penetration, through the skin, the drug should have specific physicochemical and biological properties[18]. The drug should not develop tolerance under the slow release profile from the dosage form.

The physicochemical properties of an ideal drug for cutaneous drug delivery include:

- The drugs with molecular weight less than approximately 1000 Daltons are often considered as ideal systems for dermal delivery.
- The drug with high affinity for both lipophilic and hydrophilic phases is susceptible to cutaneous drug delivery. Extreme partitioning characteristics are not ideal for cutaneous drug delivery.
- Drugs should have a low melting point.
- Drugs should be potent in nature, with a short half-life and be non-irritating.

Therefore, molecules like insulin, human growth, hormone cyclosporine, do not fulfill these criteria. These categories of drugs are very challenging from the drug delivery point of view.

Dermal pathway for drug penetration:

There are two pathways for the drug to penetrate through the skin. They are

- Transepidermal pathway (diffusion across skin layers)
- Appendageal pathway (diffusion via follicles of hair or sweat ducts)

Transepidermal pathway:

In the trans epidermal pathway, the permeant crosses the intracellular and/or extracellular spaces through the various layers of skin. While passing the permeant crosses a series of separating and interspersing hydrophilic and lipophilic domains, through the process of diffusion and partition[18,19]. In the extracellular matrix, permeant maneuvers through the convoluted path, without passing through cells. Hydrophilic small molecules favor the transcellular route over the intercellular route and vice versa in the case of lipophilic molecules.

Appendageal pathway:

The appendageal pathway or shunt pathway is bound to permeation via follicles of hair (trans follicular route) or sweat ducts[20]. The skin penetration pathway is depicted in fig-1.

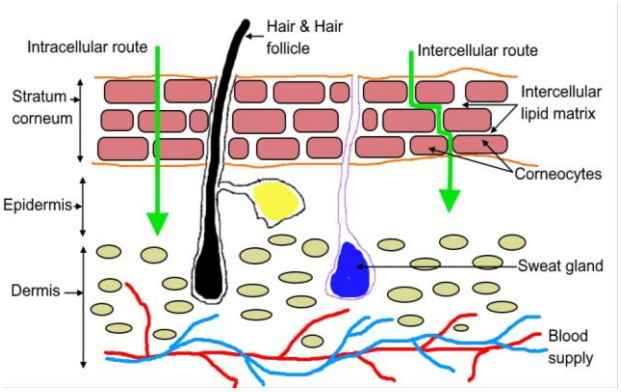

Fig-1: Skin penetration pathway

Diffusion of nanoparticles through the skin:

Passive diffusion: the drugs which are lipophilic in nature pass across a lipid membrane, through the process of passive diffusion. The drug embedded in the nano-lipid carriers also helps to penetrate a hydrophilic drug in the same manner through passive diffusion. The charge on the surface of the nanoparticles influences the diffusion process. A 3 to 5 fold increase in penetration is observed in the uptake of nanoparticles across lipid bilayer containing cholesterol[20].

Factors affecting nano delivery systems through the skin:

1. Particle size distribution and zeta potential:

Particle size and shape often influences the pharmacokinetics of the drug. The drug release, stability

and apical uptake of the nanoparticle systems are greatly dependent on its size and shape[21,22]. The critical process parameters such as homogenization speed, temperature, viscosity, and the phase volume ratio of the organic and the aqueous phase affect the yield and size distribution of each system[22,23].

2. Surface properties:

The presence of negatively charged sulphated proteoglycans on the cellular surfaces -influences cellular motility migration and proliferation[22]. Surface charge is the main functioning property of nanoparticles for its attachment to the cell membrane. The surface charge can influence the tissue binding of the direct nanoparticles to the cellular components both in vivo and in vitro.

3. Hindrance through stratum corneum:

The five layers of stratum corneum and their unique structure with a series of layers of dead cells and flattened corneocytes create a lipid envelope and provide a hindrance for penetration of the drug in the deeper layer. The layers of the skin comprise highly dynamic tissues that combine with the dead cells to form a water repellant layer that also provides protection against particles[22].Individual compounds show different permeability characteristics, dependent on their own particular properties of solubility and diffusion. The skin is also composed of hair follicles (0.2% of the skin surface) and the sweat ducts (0.4% of the skin surface) which allow faster penetration of some of the aqueous and organic solutions [23,24].Therefore, this route can be used in medicine for the release of nanoparticle bound drugs or vaccines, which are subsequently delivered into the systemic circulation[25].

Dermatopharmacokinetics:

Dermatopharmacokinetics describe the assessment of skin kinetics of external formulations to determine the drug bioavailability on application. As no correlations can be established between the availability of the drug in skin and drug blood levels dermatopharmacokinetics involves the estimation of the drug in the outermost layer of the skin with time to correlate the amount of drug absorbed[26]. The methodologies generally used to assess the dermatokinetcs of topical formulations are tape stripping and microdialysis[27,28]. Parameters used to estimate the bioavailability through dermal absorptions are the maximum quantity of drug molecules in the stratum corneum (Cmax), time to reach maximum concentration (Tmax). The bioequivalence of the cutaneous applications is estimated by assessing Area under the curve (AUC) in terms of drug level in stratum corneum versus time[29]. These approach works better for local topical formulations which are intended to give action on stratum corneum, in case of targeted delivery via skin with the use of nanoformulations the similar method can be used with few more considerations like (1) size of the nanoformulation to penetrate the rate-limiting barrier of stratum corneum (2) improvement of diffusivity of the drug in nanocarriers and (3) the surface charge of the nanomaterials[30,31].

Novel nano approaches for cutaneous drug delivery:

Nano approaches to deliver the drug cutaneously use different carrier systems in the form of niosomes, solid lipid nanoparticles, polymeric nanoparticles, carbon nanotubes, etc. The proposed carrier systems favor the transportation of drugs through the skin with a controlled release and optimum drug retention[32].

Niosomes:

Niosomes consists of nonionic surfactant vesicles and cholesterol, which are used in improving the solubility and stability of the pharmaceutical compounds. These also help in providing the targeted and controlled release of the drug from the natural carrier system by changing the characteristics and properties of the natural drugs inside the body after the administration of the dosage form. It can protect natural drugs from degradation and increases drug efficiency through ligand binding[33]. Niosomes can be mainly used in cancer chemotherapy, vaccine and antigen delivery, carrier for hemoglobin, etc.

Nanoemulsions:

Nanoemulsions are isotropic nanosized dispersed systems with two immiscible liquids resulting in O/W or W/O type emulsions. It can be used to entrap both hydrophobic and hydrophilic drugs. They are nonirritant and also nontoxic[34,35]. They are specially used for topical and Transdermal applications for cosmetic, drug and gene delivery. They have shown their mart applications on photodynamic therapy of skin. They are found to be beneficial for dermal products in terms of hydration of skin, hair and nail care products.

Lipid-based nanoparticles:

A lipid-based nanoparticle is preferred over polymeric nanoparticles. The advantage includes drug delivery of lipid particles show the ability to protect the drug from chemical decomposition, can modulate the release of drug and forms an adhesive lipid film providing the possible occlusive effect. The emerging development of nanotechnology in the recent decade has enlightened the use of lipid nanoparticle for dermal applications and mostly they are marketed as cosmetics in the treatment of fungal infections, psoriasis and Transdermal delivery of medicinal agents[36].

Solid nanoparticles:

These are the solid colloidal particles of size ranging from 1 to 1000nm. These are the macromolecular particles which can be therapeutically used as the carriers for various drugs. The active therapeutic

ingredient is dissolved, entrapped or encapsulated. The main theory of solid nanoparticles is enhanced permeability and retention properties[35,36]. Some of the solid nanoparticles which are used in topical drug delivery are Quantum dots, zinc oxide-based nanosystems, silver nanosystems.

Polymeric nanoparticles:

Polymeric nanoparticles are the systems that are prepared from polymers which are biocompatible and biodegradable. The size of biodegradable polymers ranges between 10-100nm. Polymeric nanoparticles can be prepared by either dissolving, entrapping, encapsulating the drug with the polymeric matrix to form a polymeric nanoparticle. The polymeric nanoparticles can modify the functionality of drugs, sustain the release of drug from the formulation and also adhesivity or the permeability of the drug can be increased with the use of a polymeric matrix. Stratum corneum is known to be the main skin layer responsible for the permeation and distribution of the drug throughout the skin layers. The nanoparticles act as good reservoirs for the lipophilic drugs thus helping the drug for its action[37].

Table 2: Marketed products of Nano formulations

Generic name	Brand name	Type of nano ointment/gel/cream	Reference
Diclofenac gel	Oxalginnano gel	Gel	38
Amorolfine cream	Livafin CP	Cream	39
Petrolatum skin protectant (ointment)	Aquaphor healing ointment	Ointment	40
Nanocrystalline silver gel 0.002%w/w	Silvercurenano gel	Gel	41
Nadifloxacin and adapelene gel	Nadoxin-A ™ gel	Gel	42
Framycetin skin cream	Soframycin skin cream	Cream	43
Precipitated sulfur and benzoyl peroxide cream	Persol Forte cream	Cream	44
Thuja ointment		Ointment	45
Sulfacetamide-Sulfur-Avobenzone	Rosac cream	Cream	46
Retapamulin	Altabax ointment	Ointment	47

Carbon nanotubes and fullerenes:

Carbon nanotubes are the carbon nanosystems that are stable. Carbon nanotubes possess the potential antioxidant property and cytoprotective ability. Carbon nanotubes are particularly small systems having the mean diameters (<100 nm). Fullerenes are 1-nm scale in size. A fullerene is a particle composed exclusively with carbon atoms, in the form of a hollow sphere, ellipsoid, tube, and many other shapes. The therapeutic opportunities of the carbon nanotubes and fullerenes are due to their small size, shape and hollow interior. Many of the cosmetic products such as sunscreens, moisturizers, makeup products with long-lasting properties propose the use of carbon nanotubes as their basic principle system.

The various marketed nano deliveries through cutaneous route are listed in table-2

CONCLUSION:

Nanotechnology and nanosystems are a promising development in the ground of pharmaceutics. Even the drug with larger molecular size can be incorporated into a nanoparticle. The decrease in particle size will enhance the absorption and bioavailability with the increased surface area. Multidrug resistance which is observed in conventional delivery can be overcome with the use of nanoparticles. With conventional therapy, normal tissues are damaged but with the use of nanoparticles only infected cells will be targeted without any further damage to the nearby tissues. In the case of conventional therapy, the drug may be degraded or may be metabolized from the enzymes present in the liver via the first-pass metabolism but nanoparticles release the drug at a particular site. Biological drugs can also be encapsulated within the nanoparticles and degradation of the drug prior to its release at a specific site is minimized. The route of administration may be parenteral or oral[48]. The stability of the nanoformulation is enhanced, the cost of the treatment is reduced, toxicity levels can be minimized and shelf-life of the proprietary drugs can be extended[49].

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AUTHOR CONTRIBUTIONS:

All the authors were equally involved in gathering information and framing the manuscript.

CONFLICT OF INTEREST:

The author confirms that this article content has no conflict of interest.

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REVIEW ARTICLE

Dentalgesian Physiology: A Review on the Neurophysiology of pain in Dentistry

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ABSTRACT:

Aim: To summarize the basic and current concepts of algesia that is present in the day-to-day dental practice by a thorough systematic literature research of databases. **Background:** Electronic databases were used to conduct a computerized search like MEDLINE, PubMed, Scopus and Evidence-based Medicine reviews using the terms were "odontogenic pain", "dental pain physiology", "pain biology" and "chemical mediators in dental pain". **Review:** The complexity of understanding pain transmission and its perception is of theoretical necessity for dental practitioners. The neurological aspects of pain, i.e. the innervation, stimulation of pain, conductance of pain and its mediators, are the basis that provides knowledge to the practitioners to provide effective pain management in their dental practice. **Conclusion:** Thorough knowledge about the neurobiology of pain is necessary to understand the pain perceived by the patients and thereby allows the practitioner to follow specific protocols of pain management.

KEYWORDS: Algesia, Dental pain, Transduction, Modulation.

INTRODUCTION:

Pain is a vital, physiologic, multidimensional sensory experience, which is a key early warning device, an alarm system that announces the presence of a potentially damaging stimulus.[1] The Subcommittee on Taxonomy of the International Association for the Study of Pain (IASP) in 1986 defines pain as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of tissue damage, or both".[2,3] Pain is manifested as the activity of sympathicus, producing fear, anxiety, pupillary dilation, tears, tachycardia, hypertension, nausea, vomiting, sound effects, and facial expressions.[4] It may vary in intensity, quality, duration, and referral.[1]

Pain is a highly personal experience that is communicated outwardly to healthcare providers, family members, and friends by verbal signals, as well as through body and facial expressions.[5] James Campbell in 1995, presented the idea of evaluating pain as the 5th vital sign.[6] Peripheral pain mechanisms associated with odontogenic painful conditions are overall similar to the mechanisms observed in all other body parts. These similarities include the type of sensory neurons involved as well as the different molecules that play a role in these processes.[7,8,9] Since algesia is a part of day-to-day clinical practice in dentistry, the aim of this article is to provide a brief overview of the various aspects of pain and its neurophysiology behind its perception.

METHODS:

Sources of information:

Several electronic databases were used to conduct a computerized search for available evidence: MEDLINE and other Non-indexed Citations, PubMed, Scopus, Google Scholar, and Evidence-based Medicine reviews

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like Cochrane Database of Systematic Reviews up to January 30, 2019.

Search strategy:
Terms used in this literature search were "odontogenic pain", "dental painphysiology", "pain biology" and "chemical mediators in dental pain". Details for each database search are available upon request. The reference section of the identified papers was also searched in order to identify additional articles.

Search and selection process:
The articles that appeared to fulfill the requirements of this literature search were selected. For abstracts that provided insufficient information to make a selection decision, the entire article was also obtained. Also articles in the databases without proper abstracts but titles suggesting that the articles could be of relevance were selected. The references from all of the selected articles were scrutinized for articles which may not have been in the databases due to their early publication date or for any other reason.

Extracting and synthesizing of data:
The articles containing data regarding the neurophysiology of dental pain were selected. The necessary data from results of the selected studies were also taken into consideration.

AETIOLOGY OF ACUTE OROFACIAL PAIN[10]

There are a wide range of causes of acute orofacial pain conditions, the most common being dental pain. Dental disease of the hard tissues (caries of enamel, dentine, and cementum), and soft tissues and supporting bone (gingivitis/periodontitis) are recognized as the most common diseases to afflict the general population. The most common forms of oral pain include pulpitis, pericoronitis and periapical periodontitis. Dentine sensitivity, dry socket and trauma or infection of the orofacial tissues for the minor etiologic aspects.

CLASSIFICATION:[11,12,13]

Orofacial pain can be classified as:

- Somatic
- Superficial [Skin & Mucous Membrane]
- Deep
- Musculoskeletal [Periodontium]&Visceral [Pulp]
- Neuropathic

Based on duration, pain can be classified as:

- Acute [Physiologic cause, Shorter duration, Etiology-Trauma, Surgery, Behavioral reaction seen]
- Chronic [Pathologic cause, Longer duration, Etiology not easily identified, Psychosocial changes seen]

Based on nature, pain can be classified as:

- Fast [Sharp/Pricking type, felt 0.1sec after stimulus, eg: Needle prick, transmitted by Aδ type fibers, Easy to localize]
- Slow [Burning/Throbbing type, felt>1sec after stimulus, eg: Tissue Destruction, transmitted by C type fibers, Difficult to localize]

Based on site, pain can be classified as:

- Primary - Pain at the site of stimulus

Secondary can be further classified as:

- Central - disturbance in CNS (pain is felt in the peripheral nerve distribution)
- Projected - disturbance in root of nerve (pain is felt in the same nerve distribution)
- Referred - disturbance in one nerve branch (pain is felt in different nerve branch)

Perception:
The perception of pain is a complex process initiated in the periphery by stimulation of free nerve endings by mechanical or thermal means, or by the release of chemical mediators following tissue damage.[14]

Pain is perceived by the following four mechanisms:[15, 16]

- Transduction is the conversion of the energy from a noxious thermal, mechanical, or chemical stimulus into electrical energy (nerve impulses) by sensory receptors called nociceptors.
- Transmission is the transmission of these neural signals from the site of transduction (periphery) to the spinal cord and brain.
- Perception is the appreciation of signals arriving in higher structures as pain.
- Modulation is the descending inhibitory and facilitatory input from the brain (from trigeminal spinal nucleus, reticular formation of brainstem) that influences (modulates) nociceptive transmission at the level of the spinal cord.

Stimulation:
Pain can be elicited by multiple types of stimuli. They are classified as mechanical, thermal, and chemical pain stimuli.[11] (Fig.1)

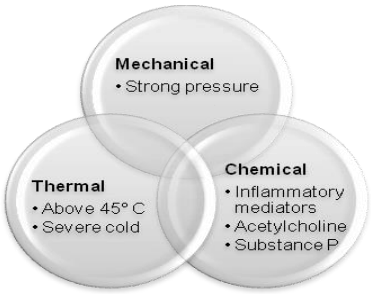


Figure1: Various stimuli of nociceptors

Following a painful stimulus, if sufficient numbers of a particular typeof nociceptor are activated, an afferent volley will be produced.[17] The transducer ion channels are nonselective cation or sodium channels that are gated not by voltage but by temperature, chemical ligands, and mechanical shearing forces.[1] Once they are activated, the channels open and sodium and calcium ions flow into the nociceptor peripheral terminal, producing an inward current that depolarizes the membrane.[1] When this depolarization is sufficiently large, it opens voltage-gated Na+ channels and triggers the generation of action potentials that are conducted to the trigeminal nucleus or the brainstem.[18] Sensory neurons express several voltage-gated sodium channels that mediate conduction of the action potentials, including 2 that are unique to nociceptors: Nav1.8 and Nav1.9.[1]Different ion channels that mediate orofacial and pulpal pain are Acid Sensing Ion Channel [ASIC3], tachykinin ion channels NK1, NK2, NK3, Transient Receptor Potential ion channels TREK1, TREK2, TRPV1, TRPV2, TRPV3, TRPV4 and TRPM3. These ion channels are concentrated in odontoblasts, capillary plexus and blood vessels in pulp, periodontal ligament and epithelial regions.[19,20,21] (Fig. 2)

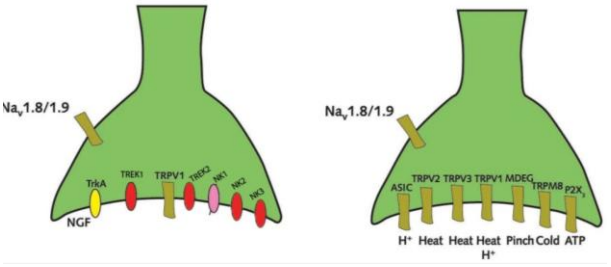


Figure 2: Ion channels involved in pain[1]

In teeth, 3 different theories are proposed which are involved in transmission of pain from the site of stimulus:[19, 22, 4, 23] (Fig. 3)

Direct nerve stimulation theory(or) Neural theory

- Changes in tooth surface temperature are conducted through enamel, dentin and finally to sensory receptors located at DEJ causing neuron excitation.

Dentinal receptor theory (or) Odontoblastic transduction theory

- External stimulus is transmitted along odontoblasts and transferred to nerves via synaptic junctions between odontoblasts and nerves.

Hydrodynamic theory:

- It is the most accepted theory. It was proposed by Gysi, and later developed by Brannstrom et al. According to this theory, pain provoked by stimuli (thermal, chemical and mechanical) is a consequence of fluidal flow in the dentinal tubules, at the speed of 2-4 mm/sec. Such circulation stimulates the mechanoreceptors and leads to the initiation of neural impulses in subodontoblastic plexus of Raschkov and interodontoblastic plexus of Bradlow in the pulp. Stimuli cause either an inward (toward the pulp chamber) or outward (away from the pulp chamber) dentinal fluid flow in dentinal microtubules. Dentinal fluid flow-induced shear stress on intradental nerve terminals may activate mechano-sensitive ion channels and cause dental pain sensation.

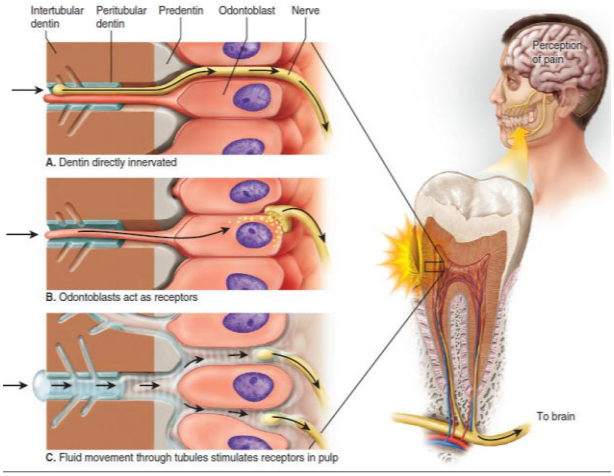
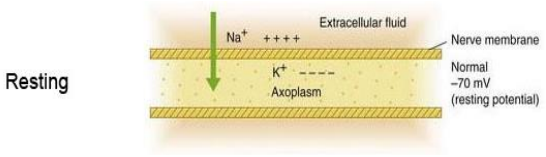


Figure 3: Theories of dentin hypersensitivity in pain perception

Impulse conduction:

Impulses are carried from the dendrites down the axon by way of an action potential. The surface of the cell membrane is slightly negatively charged. An action potential begins with a sudden change from the normal resting negative potential to a positive membrane potential and then ends with an equally rapid change back to negative potential.[13] The electrochemical gradients for sodium, calcium, and chloride are more positive than the resting membrane potential in sensory neurons, the opening of ion channels permeable to these ions will cause the membrane potential to shift in the positive direction (depolarize).[24] Since the electrochemical gradient for potassium is more negative than resting potential, closure of active potassium channels not only depolarizes the membrane potential but amplifies current-induced voltage fluctuations due to the resulting increase in membrane resistance.[24] When the membrane becomes depolarized, there is a sudden permeability to sodium ions, allowing tremendous number of ions to flow into the interior of axon. The sodium channels begin to close and potassium channels open more than normally. Potassium diffuses to the exterior and reestablishes normal negative resting membrane potential by repolarization.[13](Fig. 4)



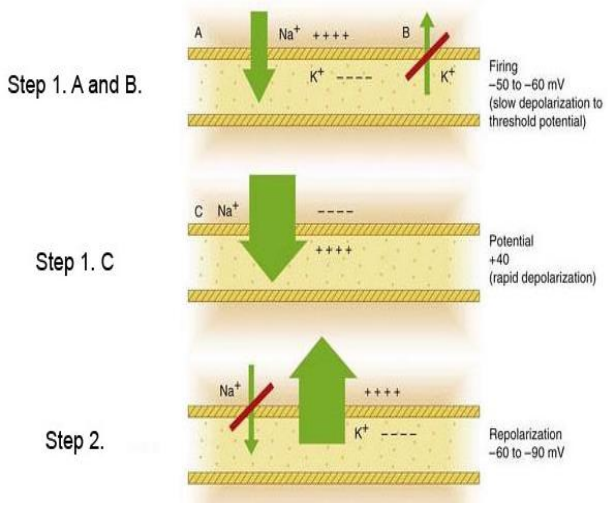


Figure 4: Nerve conduction

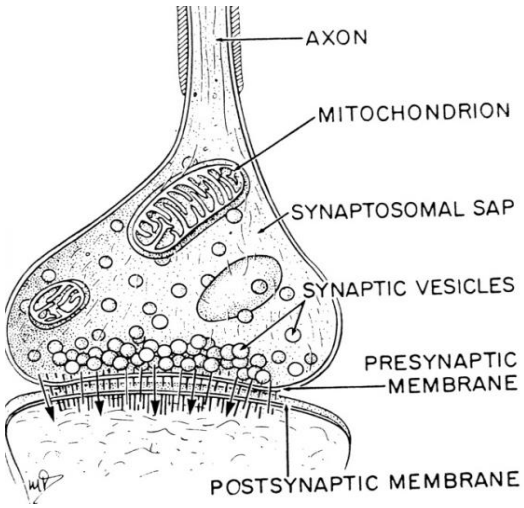


Figure 5: Synaptic terminal

Impulse conduction is faster in myelinated fibers than unmyelinated fibers because of the presence of saltatory conduction at the nodes of Ranvier along with the normal excitatory conduction.[13]

Synaptic transmission:[13]

Nerve signals are transmitted from one neuron to the next through interneuronal junctions called synapses. Each presynaptic terminal is separated from its adjacent neuron by a small distance called synaptic clefts. The synaptic vesicles are present in the presynaptic terminals, which contain transmitter substances when released into the synaptic cleft either excite or inhibit postsynaptic neuron. The postsynaptic neural membrane has both excitatory and inhibitory receptors.The mitochondria present in the presynaptic terminals provide the adenosine triphosphate required to synthesize new transmitter substances.The synaptic membrane of the presynaptic terminals contains large number of voltage-gated calcium channels. When the action potential depolarizes the terminal, large numbers of calcium ions, along with the sodium ions that cause most of the action potential, flow into the terminal. The quantity of transmitter substance that is released into the synaptic cleft is directly related to the number of calcium ions that enter the terminal.At the synapse, the membrane of the postsynaptic neuron contains large numbers of receptor proteins, which project out into the synaptic cleft as well as extend into the interior of the postsynaptic neuron. The portion that protrudes into the cleft acts as a binding area for the released neurotransmitters. The portion that extends into the neuron is the ionophore component and carries neurotransmitters into the neuron that can influence cell activity. (Fig.5)

Pathways of pain:

Direct nociceptive activation is usually gone within minutes after withdrawal of the noxious stimulus, but the resulting pain often lasts much longer.[25] Fast type of pain is transmitted by A-delta fibers (myelinated) by Glutamate and slow type of pain is transmitted by C fibers (unmyelinated) by Substance P. The impulse is transmitted from periphery to subnucleus caudalis via first order neurons where they synapse with second order neurons in different laminae for fast and slow type of pain. They are then transmitted to different nuclei in thalamus region of brain via second order neurons which are then transmitted to the cerebral cortex to perceive it as pain via third order neurons.[13]

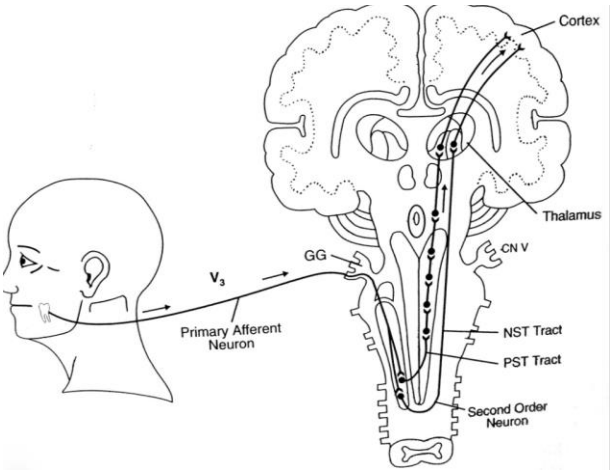


Figure 6: Pain Pathway

Nociceptive processing takes place within at least two distinct and parallel systems or tracts:[12, 26] (Fig. 6)

- Medial pain system or Paleospinothalamic tract (for transmission of slow pain).
- Lateral pain system or Neospinothalamic tract (for transmission of fast pain).

Medial pain system composed of the insular cortex, anterior cingulate, and limbic structures is held responsible for processing emotional-affective and cognitive-behavioral components of pain. Lateral pain system is attributed to sensory-discriminative components of pain and includes the lateral spinothalamic tract, the ventral posterolateral nucleus of the thalamus, and somatosensory region.[27]Impulses are relayed up the spinal cord and through the spinothalamic tract to output on the thalamus. In turn, the thalamus serves as the major "relay station" for sensory information to the cerebral cortex. Nociceptive pathways terminate in discrete subdivisions of thalamic nuclei known as the ventral posterior lateral nucleus and the ventromedial nucleus. From these nuclei, nociceptive information is relayed to various cortical and subcortical regions, including the amygdala, hypothalamus, periaqueductal grey, basal ganglia, and regions of cerebral cortex. Most notably, the insula and anterior cingulate cortex are consistently activated when nociceptors are stimulated by noxious stimuli, and activation in these brain regions is associated with the subjective experience of pain. In turn, these integrated thalamocortical and corticolimbic structures process somatosensory input and output neural impulses which influence nociception and pain perception.[28, 29, 30]

Pathway of Fast pain: Stimulus→A-delta fibers [first order neurons]→Laminae I of dorsal horns→Second order neurons→Thalamus→ Reticular areas of brain stem + Ventral Posterior Lateral Nucleus→Somatosensory Cortex

Pathway of Slow pain: Stimulus→C fibers [first order neurons]→Laminae II, III of dorsal horns→Second order neurons →Laminave V of dorsal horns→Thalamus→ Reticular nuclei of brain stem + Periaquiductal gray region + Tectal area of mesencephalon + Interlaminar & venterolateral nuclei→Somatosensory cortex

CONCLUSION:

The pulp is a tissue of high neural density. Most ofits sensory nerve terminals are distributed in the pulpdentin border zone and are thus in an ideal position torespond to external stimuli and to sense potential damage to the tooth. This literature review provides a compact information related to dental pain as necessary for the practitioner to understand and thereby provide the required medication.

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REVIEW ARTICLE

Bioactivity, Analytical Techniques and Formulative approaches for Embelin

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ABSTRACT:

Embelia ribes or black pepper contains many bioactive constituents of pharmaceutical importance. It contains alkaloids, tannins, fixed oils, and traces of volatile oils and benzoquinone derivative Embelin. Embelin (2,5-dihydroxy 3-undecyl, 1, 4-benzoquinone), a benzoquinone derivative has reported to have anticancer, anti-inflammatory, antimicrobial, anti-diabetic, wound healing, antioxidant, analgesic, antitumor and anthelmintic activities. Due to poor aqueous solubility, bioavailability its therapeutic potential is not fully utilized. This review summarises recent literature on biological, analytical and formulative aspects of EMB. Proposed mechanisms for various bioactivities of EMB are brought under limelight. UV double beam spectrophotometry, thin layer chromatography, high performance liquid chromatography (HPLC) and HPTLC techniques for EMB quantification are discussed. Types of dosage forms and its potential for controlled and targeted delivery of EMB are also discussed. This review will open the ways for development of an optimum formulation of EMB for its reported biological activities.

KEYWORDS: Embelin (EMB), bioavailability, solubility, formulations, controlled and targeted drug delivery.

INTRODUCTION:

The use of natural products gained significant popularity globally over the past few decades due to their potential health benefits. Natural products are often perceived as less toxic compared to synthetically derived products[1]. *Embelia ribes Burm. f*, as an important medicinal plant, has been used extensively in many Ayurvedic medicines for the treatment of various diseases over a long period of time.[2,3] It belongs to the family Myrsinaceae. It is medicinal woody climber commonly known as vidang or black pepper. This species is seen to be vulnerable in the Western Ghats of Tamil Nadu and Karnataka states of India and at a lower risk in Kerala state of peninsular India. E. *ribes* grows in semi-evergreen and deciduous forests at an altitude of 1,500 m, throughout India.[4]

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EMB has been reported to possess many pharmacological effects including antifertility[5], analgesic, anti-inflammatory[6], antioxidant, anti-diabetic [7,8], hepato-protective[9], anticonvulsant[10], anxiolytic[11], and antimicrobial activity[12]. The main parts of plants used include fruits (berries), roots and leaf, to cure various diseases. It has also found a valuable role in different diseases like Huntington disease, myocardial infarction, acute respiratory distress syndrome and ulcerative colitis.

Chemistry:

Chemical composition of *Embelia ribes* consist of embelin (2.3%), quercitol (1.0%), an alkaloid christembine; tannin; vilangin (methylene-bis-2-5-dihydroxy-4-undecyl 3-6-benzoquinone) from ripe fruit berries and fatty ingredients (5.2%), including resinoid, fixed oil and traces of volatile oil[13]. Phytochemical investigation resulted in three new compounds namely embelinol, embelia ribyl ester and embeliol. Along with high carbohydrates the seeds of *E.ribes* showed the presence of Cr, K, Ca, Cu, Zn and Mn[15].

Constituents of *Embelia ribes*

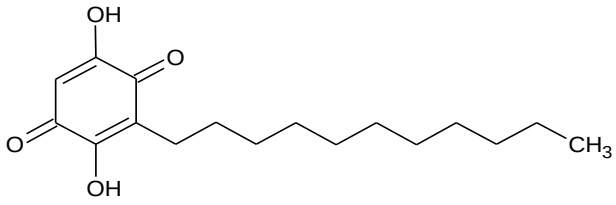

Fig 1: Embelin

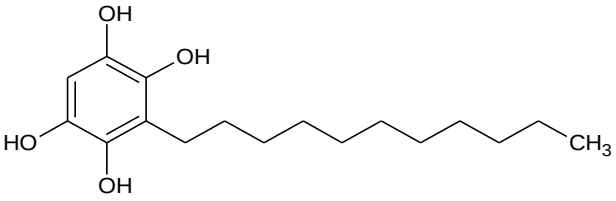

Fig 2: Embeliol

$CH_2(CH_2)_{23}CH_2OCO(CH_2)_5CH=CHCH(OH)(CH_2)_9CH_3$

EMB ($C_{17}H_{26}O_4$, molecular weight: 294.29g/mol; orange-yellow solid of melting point 142.5°C) is virtually insoluble in water (log P 4.34) but soluble in polar organic solvents such as DMSO and diethylether [14]. SAR studies revealed that anticancer activity of EMB analogues is dependent on polarity of the side chain[16]. EMB forms binary complexes with biologically important metal ions such as (Co (II), Ni(II), Cu(II) and Zn(II)[17].

Bioactivity:

EMB has an ability to affect multiple biological targets and exhibit activity against various diseases, like cancer, diabetes, neurological and autoimmune diseases. It has antimicrobial and wound healing activity. Through several mechanism like apoptosis, anti-proliferation, pro-inflammatory cytokines/mediators and cell-cycle arrest, EMB modulate several biological targets. EMB modulates tumor growth via autophagy, caspase pathways[18].

Anti-Cancer:

Proposed mechanisms of the potential anticancer activities of EMB are through apoptosis, anti-proliferation, anti-metastasis and anti- angiogenesis.[19]. EMB blocks prostate cancer cell migration and invasion. EMB induces mitochondrial-dependent apoptosis[20]. EMB enhances therapeutic efficacy by radiation therapy in human prostate cancer PC-3 both *in vitro* and *in vivo*. EMB combined with X-ray radiation, has shown to increase the tumor growth inhibition and apoptosis, which is accompanied by the EMB-induced cell cycle arrest in S-phase[21]. In another study EMB causes apoptosis in a dose-and time-dependent manner. The mitochondria and lysosomes are main targets of EMB-induced cell death response. It leads to loss of mitochondrial membrane potential[22].

Wound healing:

Phytoconstituent with wound healing activity showed more effect in diabetic patients when encapsulated as nanoparticles[23]. Study shows that the epithelialization of the incision wound was faster with a high rate of wound contraction. The tensile strength of incision wound, weight of granulation collagen content also increased.[24]

Anti-inflammatory:

EMB showed suppressed edema and mucosal damage, inhibited abnormal secretion, and m-RNA expression of inflammatory cytokines such as TN-α and IL-6. EMB showed maximum anti-inflammatory effect at colon rectal regions due to the down regulation of production and expression of inflammatory mediators[6].

Anti-Diabetic:

Meta-analysis data supports that EMB shows significant anti-diabetic activity by considering restoration of insulin, lipid profile, heamodynamic parameters, serum and oxidative stress markers[25]. Significant reduction in the serum blood glucose level and improvement in the body mass was observed in EMB treated, alloxan induced diabetic rats.[7,8].

Anti-microbial:

EMB showed bactericidal activity against Gram positive organisms, whereas against Gram negative organisms it showed bacteriostatic activity[26]. EMB at sub inhibitory concentrations possesses synergy with oxacillin and tetracycline against antibiotic resistant strains of S.*aureus*. EMB act as tetracycline efflux inhibitor in bacterial cell and hence antimicrobial resistance to tetracycline would be bypassed[27]. The highest antibacterial activity was recorded by methanolic extract against P. *aerugenosa* and the lowest activity was seen in ethyl acetate extract against E. *coli* and P. *aeruginosa*.

In antifungal activity, the highest activity was seen in methanolic extract against A. *Niger* and the lowest activity was reported by acetone extract against A. *flavus*. Acetone extract showed lowest antifungal activity. This may be due to the unavailability of fungicidal content of plant extract[28].

Central nervous system:

EMB can be used in CNS disorders like depression, anxiety, tumor, convulsions etc. It act through chloride channel by increasing the conductance. Antiepileptic activity is linked to its ability to decrease conductance of sodium ion channel. Antianxiety was mediated through the benzodiazepine receptor mediated GABA site. EMB has use in cerebral ischemia and it was through restoration of antioxidant enzyme and decrease in lipid

peroxides. EMB induce decrease in the inflammation of neurons is by inhibition of NF-Kb pathway[29].

Analytical method:

Some of the common analytical methods used to determine the strength, purity and for the standardization of phytoconstituents are UV spectrophotometric methods [30], HPTLC, HPLC and RP-HPLC[31]. The first and most commonly used analytical technique of embelin is HPLC. Using various solvents LOD and LOQ values are determined. Solvents like methanol, tri-fluro acetic acid and water shows a very high sensitive LOD and LOQ values for EMB. The TLC method using chloroform as solvent system has been reported. HPTLC with chloroform, ethyl acetate and acetic acid as solvent system and LC-MS as detector is also been reported. UV double beam spectrophotometric method is also developed with methanol as solvent and max absorption at 289 nm with a linear range of 10-90µg/ml.

Recent literature on analytical methods for EMB are summarised in table 1.

Table 1: quantitative analytical method of EMB

Analytical method	Research objective/title	Matrix and sample preparation method	Results	Reference
TLC	Estimation of EMB by UV spectrophotometer in *Embelia ribes*	Air dried grinded sample(powder) extracted using chloroform	Rf value observed at above as 0.65, the calibration curve was found to be linear b/w 5-20 µg	[32]
HPLC	Development and validation of a liq chromatographic method for determination of EMB from crude extract of E. *ribes*	Methanol and chloroform as solvent crude EMB extract using soxhlet and maceration	Of integrated peak area and concentration of EMB was found to be linear over a range of 6.25-200 µg/ml. The LOD is 1.5and LOQ (µg/ml) levels is 4.5. The recovery of EMB was 98.6%.	[33]
RP- HPLC	Development and validation of RP-HPLC method	Embelin powder in vidangi churna in reverse phase C18 eluted with acetonitrile and water as solvent	Calibration curve showed good linear correlation coefficient(r^2>0.995)	[34]
HPLC	To obtain the extraction parameters of EMB and to quantify the EMB content in different parts	Extracts of E.*ribes* using solvents like acetone, methanol, chloroform, ethyl acetate, diethyl ether, hexane through sonication	The extraction of EMB through RSM extraction time 27.50 min temp 45°C and drug: solvent ratio 8:1 and maximum EMB content was found ethyl acetate 23.74%	[35]
HPLC	Estimation of EMB by photo diode array dectector method	The EMB was separated by using isocratic mode consisting of 0.1 % trifluoroacetic acid in water and methanol (in proportion of 88:12) at a flow rate of 1.0 mL/min	EMB content in various solvent extract a mean content of 0.44-33% w/w, EMB was found to be linear over the range of 5.0-75.0 µg/mL and the relative standard deviation is 0.61-0.96 %. The limit of detection was 20 ng on column and the limit of quantitation was 50 ng on column	[36]
HPTLC	Determination of EMB from methanolic extract of leaves, fruits and stem	Dried powder, using the solvent system of chloroform: ethyl acetate: acetic acid (5:4:1 v/v/v).	Rf=0.58, confirmation was done by LC-MS analysis in which EMB was observed at 295.1904 m/z ratio and retention time (RT) was 11.546 minute	[37]
RP-HPLC DAD	Quantitative estimation of EMB by DAD detection	Dried powder, homogenized at 200rpm separately with hexane, ethyl acetate, chloroform and methanol (2 ¥ 250mL) for 20min at room temperature	hexane with high extractability, linearity (15–250 mg/mL), LOD (3.97 mg/mL), LOQ (13.2 mg/mL), recovery (99.4–103.8%) and precision (1.43– 2.87%)	[38]
UV spectrophotometry	Development of double wavelength UV spectrophotometry	Dried powder dissolved in methanol using UV double beam spectroscopic method	maximum absorption at 289 nm, linear over the range of 10-90µg/ml with correlation coefficient 0.9991, The % relative standard deviation (RSD) value < 2 indicate that the method was precise. The Limit of detection (LOD) and Limit of quantification (LOQ) of EMB were found 3.96 µg/mL and 12 µg/mL respectively	[39]

Table 2: embelin formulations and their *in vitro* or outcomes

Embelin activity	Formulation	*In-vivo/in-vitro*	Results	Reference
Ulcerative colitis	Enteric coated microspheres with polymer eudragit S 100	Both, 30 wistar rats were used	Time dependent and pH-dependent sustained release with drug delivery specified to colon	[47]
Microfibre containing EMB	Solubilizing embelin in a biodegradable and biocompatible polymer matrix of poly(e-caprolactone) (PCL	*in vitro*	Crystallinity of EMB is decreased, *in situ* bioavailability of EMB proved topical drug delivery	[48]
Diabetes	Self solid nano emulsified DDS using polymers Capmul® MCM, NEUSILIN® UFL2, PEG 400	Both, wistar rats	Percentage cumilative release was increased from 34.29±1.20% to 90.63±3.67%	[49]
Ulcerative colitis	Lipid nano spheres with soya bean oil and virgin coconut oil	Both, 42 male wistar rats	The mechanism of drug release were non-fickian, in vivo studies revealed that it is suitable for ulcerative colitis	[50]
Enhancement of dissolution	liquisolid system using solutol hs -15 and synperonic PE/L6	*in vitro*	Significant enhancement of drug release from the formulation	[51]
ulcerative colitis	guar gum micro particles	Both, wistar rats	The drug release is site-specific and sustained, with less side effect	[52]
Skin cancer	Transferosome with span 80 and tween 80, carbopol 934 and propylene glycol	*in vitro*	Higher entrapment efficiency and can be used for transdermal preparations	[53]
Enhancement of solubility	Phospholipids	*in vitro*	EMB content increased up to 92% and solubility increased from 2.3-39µg/ml	[54]
diabetes	Oral niosomes	Both, wistar rats	EMB show higher antioxidant activity and also useful for diabetes in wistar rats	[55]
Ulcerative colitis	Eudragit coated microspheres	*In vitro*	pH dependent and delayed release for targeting drugs specifically to inflamed local site of colon	[56]
Topical delivery	Emulgel with tween and propylene glycol	*in vitro*	Emulgel formulation having potent antioxidant activity and EMB is well incorporated in the formulation	[57]
Improvement of solubility and dissolution property	Nano suspension with pluronic F68 as stabilizer and zirconium beads as milling agent	*in vitro*	Significant improvement of dissolution property after several in vitro studies like X ray and DSC	[58]
Dissolution enhancement	EMB compressed tablets	*in vitro*	Solubility tested in different dissolution medium and find out pH 7.4 as good dissolution medium	[59]
Brain targeting	Nano lipid carriers with cetyl-palmitate and octayl- dodecanol as excipients	Both, wistar rats	Sustained release pattern from drug formulation, high drug concentration reaches brain compared to plain drug	[60]
Improve solubility and micrometric property	spherical microcrystals with hydrophilic polymer PVP K30	*in vitro*	Improves the dissolution rate and flow property without changes in the crystalline structure of EMB	[61]
To improve oral bio availability	Self emulsiyfing with ethyl oleate, tween 80 and span 20	*in vitro*	EMB SEDDS shows highest cumulative drug release as compared to conventional dosage form with uniform drug release	[62]
Solubility enhancement	Solid dispersion with carriers like PVP,PVPK30, PEG 4000 , PEG 6000	*in vitro*	Using phosphate buffer of pH 7.4 shows improved solubility and dissolution rate within 2 hours	[63]
Dissolution enhancement for porous based S-SNEDDS	Self nano emulsifying system with caproyl 90 as oil phase	*in vitro*	Enhancement of dissolution without any changes in the physiochemical characteristics after 6 months of accelerated stability studies	[64]

Dosage forms:

Improvement of bioavailability and aqueous solubility can be achieved by formulating into phospholipid complexes[40], SEDDS[41], SNEDDS[42], nanoparticles[43], solid dispersion techniques[44], liquisolid technique[45] and microemulsions. Site specific drug delivery of phytoconstituents through nanoparticles and microparticles. Based on the reported bioactivity of EMB, immediate release, topical release, controlled and targeted drug delivery approaches has been developed. Most of the reported literature aims at improving aqueous solubility or poor solubility issues[46] of EMB. EMB emulgel was prepared using carbomer as the gelling agent and evaluated for its antioxidant potential. Release of EMB from microparticulate system of eudragit showed pH dependent and sustained drug release. Most of them were for targeted drug delivery for ulcerative colitis. Nanolipid carriers of EMB for

targeting to colon and brain has been reported. Solubility enhancement was also observed with SEDD, microcrystals, SNEDDS and with phospholipids. Formulation approaches for EMB are summarised in table 2

CONCLUSION:

EMB is a promising molecule and major physiochemical property limiting its use is low aqueous solubility. Mechanisms of anticancer and neurological activities of EMB are well exploited and provide potential targets for the treatment of cancer and neurological disorders. Mechanism of anti-inflammatory, wound healing and antimicrobial activities are yet to be unveiled. Formulation approaches are limited and their efficacy has to be confirmed by in vivo studies or clinical trials. Reports on successful brain and colon targeting and drug delivery of EMB need further scale up. Although various analytical techniques are reported, a model solvent system for chromatographic techniques is yet to develop.

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REVIEW ARTICLE

A Review on the Need of Advanced Clinical Pharmacy Education Services for Diabetes Prevention and Management in India in comparison with International Standards

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ABSTRACT:
Clinical pharmacy is a health care discipline which focuses on clinical pharmacist's involved patient care programmes to provide rationality in drugs use, to improve health and prevention and management of diseases. Clinical pharmacist directly can communicate with health care professionals and can resolve various health care issues related to patients. Clinical pharmacy services in the hospitals includes detecting of drug interactions, adverse drug events monitoring and filing, effective patient counselling, patient education, providing drug information services, monitoring of drug therapy, creating disease prevention and management awareness in the clinical setup. Diabetes mellitus is a chronic metabolic disease associated with elevated blood sugar levels which ultimate causes damage of vital organs of the human body. In western countries the practice of clinical pharmacy and education services for non-communicable diseases are well established effectively. In India, clinical pharmacy services are still to be flourished with advancement in delivering of high standard pharmaceutical care and effective clinical services in all the tertiary care hospital settings. This review mainly aimed to highlight the western practices of advanced clinical pharmacy services and to bring into limelight the same in Indian, the clinical pharmacy practice for a better health outcome for the future.

KEYWORDS: Clinical Pharmacist, Diabetes mellitus, advanced clinical pharmacy services, diabetes education.

INTRODUCTION:
Diabetes is a chronic metabolic disorders, it develops due to imbalance in insulin production or resistance for utilization in the body. The higher levels of glucose in the blood can damage vital organs in the body. The WHO also estimates around 80 percent of deaths due to diabetes occur in low and middle income countries. It was estimated that the global burden of diabetes mellitus rise to 438 million by 2030[1]. In India, diabetes is one of the major chronic diseases and the rural populations are the most affected. Diabetes screening, identification and management of co-morbid conditions at early stage are a must to reduce the prevalence of diabetes complications.[2,3]

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Diabetes burden in India:
Currently, India has 62 million populated diagnosed with diabetes. In 2000 India had topped the world's highest diabetes mellitus patients. People with diabetes require at least 2-3 times the health care resources than who do not have diabetes. Diabetes care accounts for up to 15% of national healthcare budgets[4-6]. The etiology of diabetes in India is multifactorial and includes genetic factors coupled with life style influences such as obesity associated with rising living standards, urban migration, and food style modifications. Illiteracy, poor sanitation and dominance of communicable diseases may also contribute directly or indirectly to diabetes. It could be render to both the policy makers and local governments should initiate warning alert to prioritise the looming threat on diabetes.

Diabetes and its co-morbid complications:
A study on International status of diabetes reported that the diabetes control worsened with longer diabetes duration neuropathy as the most common complication

(24.6 per cent) followed by cardiovascular complications (23.6 per cent), renal issues (21.1 per cent), retinopathy (16.6 per cent) and diabetic foot ulcers (5.5 per cent). These results were similar with other results from the studies of South Indian population, however further more data from different states of India are required to confirm whether patterns of complications vary across the country[7,8]. A study compared waist-to-weight ratio (WHtR) with traditional anthropometric indices in healthy women aged 21 to 45 years from urban slums of Mumbai city, India found mean value of WHtR 0.50± 0.1, but a little more than half (51.9%) of the women had WHtR ≥0.50. Poor glycaemia control is a main factor that is responsible for micro and macro vascular changes in the Indian diabetic population and can predispose diabetic patients to other complications such as diabetic necrosis and muscle infarction.

Indians are genetically predisposed to the development of coronary artery disease due to dyslipidaemia and low levels of high-density lipoproteins. These determinants makes Indians more prone to development of diabetes at an early age of 20-40 years as compared to Caucasians which indicate that diabetes must be carefully screened and monitored regardless of patient age in India[9,10].

Challenges in the management of Diabetes in India:

There are number of challenges that plague diabetes care in India. While HbA1c is the gold standard test around the world for insulin initiation and intensification, it is not easily available to a large section of Indian population. There is a clinical apathy for the commencement of insulin therapy in both the clinical and patient communities. The most common apprehensions are related to the complexities of the insulin regimen and concerns about weight gain, hypoglycaemic events and fear of insulin prick. Lack of adequacy in Indian guidelines is also responsible for wide variation in treatment preferences across the country. The creation of simple and practical insulin guidelines can be incorporated into routine clinical practice to facilitate treatment and the initiation of insulin therapy throughout the country[11].

International scenario of Diabetes mellitus:

The United States of America developed a number of public and private funded programmes to prevent and manage diabetes that had proved to be successful. Similarly, the Australian government runs programmes called "National Health Priority Areas initiative" to provide focussed and continuum of care and attention on chronic disease like diabetes. The United Kingdom government places special emphasis on diabetes care in patients, with the National Health Service conducting various patient education programs and trials to improve quality of life of patients. The United Arab Emirates has set up an expert panel to form guidelines for diabetes management and public awareness programmes. This has resulted in positive health effects which may arrest rising trend in diabetes cases in that country. In India, similar efforts and services are required at 'grass roots' level to contain the new-age diabetes pandemic. Many health systems are now adding clinical pharmacists to meet these standards and these numbers supposed to increase rapidly in the next several years.

Diabetes care should be a person-centered and it should aim to empower individuals to manage their own diabetes conditions. A study form Philippines described the situations of a diabetes patient and identified possible barriers to diabetes care and medications. They concluded with Insurance out-patient coverage and application of standard treatment/management guidelines that would help to encourage for providing and receiving regular care. Professionals providing diabetes care should support individuals to manage their own diabetes and help them to adopt and maintain a healthy lifestyle[12]. They should actively encourage partnership in decision-making and enabling people with diabetes to have choice, voice and control over what happens to them at each step of their care. A care plan, negotiated and agreed with each individual in an appropriate format and language preferred by the individual and reviewed as part of the care planning process.

The responsibilities of the diabetes patient include:

- Take as much control of their diabetes on a day-to-day basis as possible
- The diabetic patient must know about self-care, which includes dietary management, exercise, the monitoring of blood glucose levels
- To examine the feet regularly
- To know how to manage their diabetes
- To build into their daily life a regular discussion with the health care team
- To clear questions on health issues during consultations
- Attend the scheduled appointments and inform the healthcare team if they are unable to attend within the schedule
- A special care to be provided for the patients like,
  - Children and young people with diabetes
  - Women with diabetes who are considering pregnancy or who are already pregnant.
  - Any person with diabetes the specialist advice is required regarding the management of metabolic control, cardiovascular risk factors or diabetic complications.
  - People with complex psychological problems.

Need for Implementation of Nationalized Awareness Programme (NAP):

Indian guidelines should be improved adequately with due responsible for wide variation in treatment preferences across the country. Creation of simple and practical insulin guidelines that can be incorporated into routine clinical practice by primary health care physicians are desperately required to facilitate treatment and the initiation of insulin therapy throughout the country[13]. To reduce the diabetes disease burden in India, appropriate support from public, health care practitioners and more awareness programmes by the government are required. Clinicians may be targeted to facilitate the awareness programmes and effective implementation of screening and early detection programmes relates to meet the diabetes preventions and self-management counselling and therapeutic management of diabetes[14]. Approaching the diabetes guidelines and its application in the clinical practice helps to controls the diabetes in epidemic. Early screening and detection of pre-diabetes especially in pregnant women, children and adults may impacts the positive health outcomes in society. Continuing education programmes for pharmacists can unveil the "clinical inertia" required to initiate programme adherence, and may be a major step in achieving diabetic control and help for prevention of disease complications. Education on aggressive clinical measures in terms of early insulin initiation combined with optimal doses of oral hypoglycaemic agents and appropriate lifestyle modification to the patients by pharmacists could also have a long-term positive effect in disease management[15-16]. Currently existing diabetes mellitus management programmes are listed in figure1.

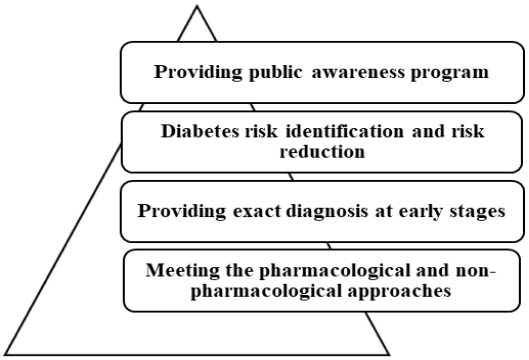

Fig 1: Diabetes mellitus management program

Role of health care personnel to support and encourage diabetes self-care and self-management[14]

- Treating individuals with respect and dignity.
- Ensure that patients with diabetes know how to contact members of the team providing their diabetes care.
- Provide high quality care and regularly review their clinical and psychological needs.
- Answer any questions about the quality of services received.
- Provide interpreting services, if English is not the person's first language and seek appropriate services for those with sensory impairment or learning disability[60-62].
- Provide information and structured education about diabetes management and local health related services.
- Remain up to date about diabetes and its care and treatment in order to keep patient with diabetes up to date about their condition.
- Facilitate access to a second opinion where required (subject to the agreement of the person's GP or consultant).
- Give information about local government services if any and details of local support groups.

Clinical Pharmacy Services:

The pharmacist role in health care system is a continuous patient care services ensuring the rational use of medications. The development of clinical pharmacy services helps to establish new treatment, screening programmes, patient education and follow-up counselling particularly for life style diseases. They can provide recommendation of evidence-based medication selection and offer drug information services to the health care providers and to the patients[17,18]. However, the expected out comes depends on the proper use of treatment guidelines and reach-out of health care suggestions beneficial to the patient community. The clinical pharmacist should meet with the relevant legal, ethical, social, cultural, economic and professional principles during their clinical practice. Clinical pharmacist requires the continuous training programmes in the clinical pharmacy practice areas and should have the widest knowledge on Pharmacokinetics, Pharmacodynamics, Pharmaco-therapeutics, clinical toxicology and pharmacology arena.

Clinical Pharmacy Services in diabetes management:

The clinical pharmacist may meet more challenges during his/her practice and most of the time they work in collaboration with multiple health care team in hospitals. Hence, every pharmacy practitioner requires proper training and good academic background on clinical pharmacy education and services. The evidence based clinical pharmacy practice requires continuous learning and training in various aspects of patient care. The clinical pharmacist should possess a basic qualification of Pharm.D, or post graduate in clinical pharmacy or in hospital pharmacy with adequate training in clinical pharmacy servicing to act as a preceptor in clinical pharmacy[19].

Table 1: Type of services and its purposes[22-26]

Name of the Department	Type of Clinical care	Clinical Pharmacy services
Teaching Hospitals attached research institutes	Hospital pharmacy practice, teaching activities, drug selection, distribution, management.	Making effective changes in education, training, teaching programmes in medical and health sciences.
Emergency Medicine	Drug therapy monitoring and drug information services	Determine types of critical conditions of the patient, solving the drug related problems, providing evidence based information to the physicians[27].
Community pharmacies[73]	Conducting health screening, awareness programmes, training programmes.	Educational and training opportunities for community pharmacists, thereby improve patient-centred knowledge providing advanced pharmacy services[28].
In-patient department	providing Pharmaceutical care services	Provision of pharmaceutical care, identification of drug therapy problems, prevention of adverse drug reactions and monitoring of drug therapy management[80].
Out-patient department	Ambulatory care[29-30], patient education	Advancing ambulatory care practice, to achieve the national priorities of improving patient care, adherence, patient health, and affordability of care.
General medicine	Identification of various diagnosis cases	Providing counselling to the chronic, non-communicable cases.
General surgery	Identification antibiotics prescribing pattern in operative cases	Providing drug related information to the physician, antibiotic alternatives.
Pulmonology	Identification of chronic inflammatory lung diseases associated co morbidities.	Providing counselling to the pulmonary disease associated co morbidities patients towards prevention and management.
Obstetrics& Gynaecology	Identification of gynaecological cases	Assessing drug use pattern in a post-operative patients, Providing treatment alternatives to the health care professionals[31-35].
Psychiatry	Identification of psychiatry related disorders	Providing patient counselling, drug related services to the patients.
Orthopaedics	Identification of bone related disorders	Providing antibiotic information, lifestyle interventions to the patients.
Paediatric department	Identification and evaluation of multiple diseases[81]	Provision of advanced paediatric care services.
Intensive care units and oncology department	Palliative care services[64,82-83]	Introducing the concept of advanced practice roles in pharmacy within the new integrated regionalized palliative care service[36-40].

The advanced clinical pharmacy services can be implemented in facilities like teaching hospitals attached in research institutes, community pharmacies and in various departments. Some of those facilities and their services are listed in Table1.The challenges on managing the chronic conditions of the patients to provide health care management which was previously termed as disease-state management.

Western University health systems include education to support basic medication management services on a consistent basis. Internationally several educational institutions focussing on better clinical services to reach the community and offers a specialized care for the patients on the various clinical situations. In India, apart from University curriculum there are no any special medication management trainings under use. In some Indian educational institutions there are some important software installed which are being utilized by the students free of cost.

A rapid development of technology in health care like drug information provider software Micromedex, Drug interaction checker Software, Medline, Medscape, E-medicine, Webmed etc. are required to update the various diseases and drug information which can be provided to the patients at appropriate level that can meet the health care demands. Advanced clinical pharmacy interventions are focused on medication reconciliation during the admission and discharge of the patients.

The diabetic patients are provided with continuous patient tailored education supported by periodic counselling[20]. The intervention was started to the patients by selecting them on the basis of diabetes severity and treatments patterns are designed accordingly during their hospital stay. The medications prescribed to the patient include antibiotics, pain killers, antithrombotic or anti-arrhythmic drugs and chemotherapeutic agents[21]. Clinical pharmacist deals on medication therapy management, patient counselling, disease prevention and management and follow up of patients etc.

According to National service frame work for Diabetes, UK structured patient education means, it is a planned course that:

- Covers all aspects of diabetes
- Flexible in content
- Relevant to a person's clinical and psychological needs
- Adaptable to a person's educational and cultural background

Advanced Clinical Pharmacy Services in the Community Practice

An array of advanced clinical pharmacy services in the community practice is depicted in figure 2.

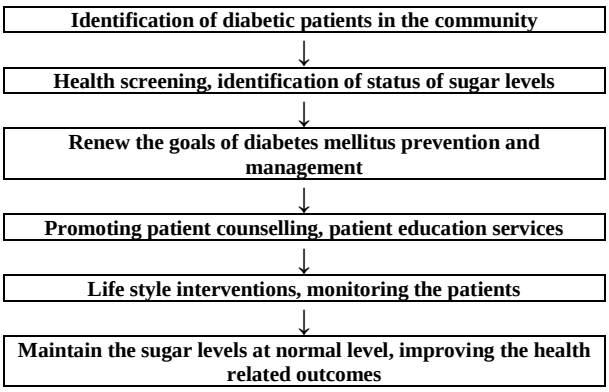

Fig 2: Advanced Clinical pharmacy service in diabetes

Structured Education Programmes (SEP) for diabetes patient[41-46]

There are two national patient education programmes in U.K that meet all the key criteria for structured education are

- DAFNE for Type 1 diabetes mellitus
- DESMOND for Type 2 diabetes mellitus

DAFNE:

Dose Adjustment for Normal Eating (DAFNE) is skills based course in which people with type 1 diabetes learn how to adjust their insulin dose to suit what they eat, rather than having to eat to match their insulin dose.

DESMOND:

Diabetes Education and Self-Management for On-going and Newly Diagnosed (DESMOND) is a new course for people with type 2 diabetes which helps to identify their own health risks and to set their own specific treatment goals.

Principles of good clinical practices in Structured Education Programme[47-50]:

The following figure 3 lists and explains the principles of good clinical practices in Structured Education Programme

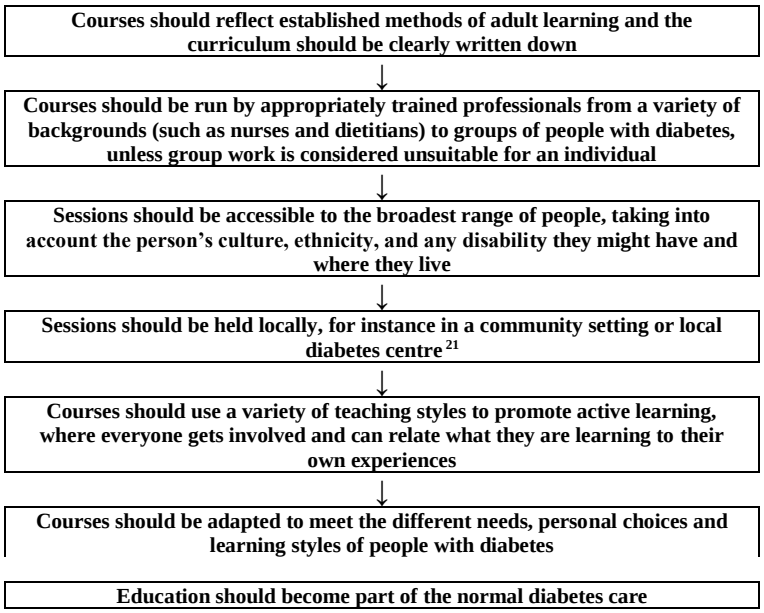

Fig 3: Principles of Good clinical practices in Structured Education Programme

Benefits and scope of Structured Education Programme:

Diabetes affected children, young people and adults shall be given necessary services which can encourages partnership, decision-making supports them in managing their diabetes and helps them to adopt and maintain a healthy lifestyle[51-55].

Structured education is one of the key interventions needed to achieve the standard care. According to the National Institute for Clinical Excellence (NICE) recommends that structured patient education should be made available to all people with diabetes at the time of initial diagnosis and should then be available as required on an ongoing basis which includes:

1. Prevention of Type 2 diabetes, practices should have systems in-place for identifying people at increased risk for developing diabetes so that they can be provided support by offering them appropriate advice on how to reduce the disease risks[56-58].
2. Identification and diagnosis of people with diabetes, people with diabetes remain undiagnosed and a high

index of suspicion amongst all members of the primary healthcare team is therefore essential. In addition, practices should have systems in place to actively identify people with undiagnosed diabetes their priority should be to focus on those known to be at high risk of developing diabetes[65-67].

3. Initial assessment and care at the time of the diagnosis, once the diagnosis of diabetes had been confirmed. Patients should be assisted to get referral to diabetes specialized team and if needed treatment and care should be initiated. This should include the provisions of education about diabetes and its management, including the provision of dietary advice[59-63].
4. Initial and on-going education mechanisms for ensuring that all people with newly diagnosed diabetes receive initial and on-going education about diabetes and its management should be agreed. The provision of education should be based on adult learning principles that promote active learning which is ideally provided within a group format, unless considered inappropriate[64]
5. Dietary advice by a registered dietician, general practitioners and community nurses on diabetes must be provided to all newly diagnosed patients[68-71].
6. Continuing care of once their diabetes has been stabilised, people with newly diagnosed diabetes should be invited to attend for regular reviews of their day-to-day metabolic control and on-going education, as frequently as required to meet the needs of the individual. In addition, they should be recalled at least once a year for a formal review of their metabolic control and the quality of their daily life, and should be offered annual surveillance for cardiovascular risk factors and long-term complications. Further follow-up appointments should be offered as appropriate to focus on any issues raised during annual reviews[72-79].

CONCLUSION:

Diabetes mellitus is reaching potentially epidemic proportions in India. The level of morbidity and mortality due to diabetes and its potential complications are enormous, and pose significant healthcare burdens on both families and society in the world[84-86]. Therefore, there is a demanded need to implement population-based interventions that prevent diabetes, enhance its early detection, lifestyle and pharmacological interventions to prevent the complications. In India, regular migration of people from rural to urban areas, the economic boom and corresponding change in life style are all affecting the status of diabetes. It is now highly developing across all sections of society within India, there is now the demand for immediate initiation of research and intervention programmes at regional and national levels to reduce the potentially catastrophic increase in diabetes that is predicted for the upcoming years.

Internationally clinical pharmacy services are well established but in India still these activities are at infant stage. The rapid change in pharmacy services is the need of hour and should upgrade the pharmacist activities to clinical pharmacist. It includes not only compounding and supplying drugs roles and also directly involving in the patient care. Advanced clinical pharmacy education and services should be done by clinical pharmacists at various facilities where standard pharmaceutical care is made available and preceptors and educators should be actively engaged with their clinical services[87-89]. Efforts to reduce the global health and economic burden of diabetes among the high-risk individuals should emphasise to delay the onset of the disease through enhancing healthy behaviours and diets. Identifying people at early risk stage of diabetes especially those with impaired glucose tolerance through the primary care system can be provided with proper medical advice and support to reduce the disease worsening in the community[90-92]. Early detection and management, lifestyle changes will be the short-term approach for diabetes prevention. Effective implementation of advanced clinical pharmacy services can helps to prevent the development of diabetes and its associated complications much in future[93-95].

CONFLICT OF INTEREST:

Author declares no conflicts of interest.

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REVIEW ARTICLE

A Review on Nanosponges – A Promising Novel Drug Delivery System

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ABSTRACT:
The major problem facing the investigators is targeted drug delivery to specific sites. The emergence of a new nanoparticles carriers known as nanosponges should address these problems. Nanosponge is a novel and arising innovation that provides for topical use controlled substance delivery. The implementation of nanosponges, its prep techniques, and assessment were addressed in this review paper. Nano-sponges play a crucial role in the controlled delivery of drugs. To guide drug delivery a wide range of drugs could be packed into nanosponge. All lipophilic and hydrophilic medicinal products could be loaded into nanosponges. The drug delivery system Nanosponge has appeared to be one of life modern science many successful sectors.

KEYWORDS: Nanosponges, topical application, targeted drug delivery, controlled drug delivery, colloidal carrier, Emulsion Solvent Diffusion Method.

INTRODUCTION:
Targeted drug delivery systems have been an undertaking for a long period of time to achieve the expected outcome. In the start, the drug delivery method of Nanosponge emerged only as a topical delivery system, but in the 21st century, both oral and intravenous (IV) route can be used to administer nanosponges[1]. Nanosponge is a traditional component group consisting of small particles with several nanometers narrow cavity. There are different types of materials that can fill these small cavities[2].Nanosponges are a 3D scaffold or polyester network that can naturally degrade. In a mixture to form nanosponges, these polyesters are blended with a crosslinker. The polyester is typically environmentally friendly here, so it starts to break down gradually in the body. When the nanosponges scaffold dissolves, it exposes the loaded drug molecules in a pejorative way.

Many of the developments made using nanomedicine are ·

1. Production of one dose per day of ciprofloxacin
2. Tumor guided taxol delivery[3]
3. Enhanced ophthalmic delivery utilizing wise hydrogel nanoparticles[4]
4. Oral insulin formulation using nanoparticles[5]
5. Liposomal-based amphotericin B formulation

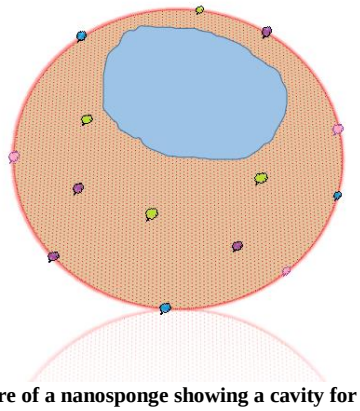

Fig 1: Structure of a nanosponge showing a cavity for drug loading

Some of the major benefits of this method is reliable release relative to other nanoparticle delivery systems under growth. Many other delivery systems of nanoparticles unload many of their drug in quick and unpredictable fashion when they hit their target. It is termed the burst effect, making it hard difficult to

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establish successful dosage levels, while nanosponges are difficult to determine when they hit their target. Controlled release of nanoparticles drug delivery system, that can be an enhanced method of delivery for anti-cancer treatments, including direct injection into the sitting tumor[6]. The nanosponges encompass the form of nanoparticles that encapsulate the molecules of drugs within their center. The nanoparticles can be categorized by the process of interaction with drugs into:

1) **Encapsulating nanoparticles:**

Nanosponges and nanocapsules reflect these. Nanosponges involve alginate nanosponges that have several gaps that keep the molecules of the drug. Even encapsulating nanoparticles are nanocapsules such as poly (isobutyl-cyanoacrylate) (IBCA). Within their aqueous base, they can capture drug molecules.

2) **Complexing nanoparticles**:

Such nanoparticles bind electrostatic charges to the molecule.

3) **Conjugating nanoparticles:**

Such nanoparticles are connected by a tight covalent bond with drug molecules[7]

Nanosponges are an unique class of nanoparticles that are generally derived from natural derivatives. Tunable polarity and high exclusively emit a diverse range of substances attributable to its 3D structure comprising cavities of nanomeric size. In addition, in combination with can nanoparticles, nanosponges present a remarkable benefit.

Nanosponges are still being used for all these properties in various fields of use, including the cosmetic and pharmaceutical sectors[8]. Such nanosponges' biggest drawback is their tendency to produce only small molecules. The nanosponges can be either crystalline or paracristalline. Nanosponges 'loading capability depends primarily on the extent of crystallization. There are various loading capabilities for paracrystalline nanosponges. Once processed in the absence of materials with electrical properties[9], these nanosponges can be permanently affixed. The small size enables nanosponges to be distributed pulmonarily and venously[10,11].

ADVANTAGES OF NANOSPONGES[12-15]:

1. Location-specific delivery of drugs.
2. This system offers a wide variety of items to be processed and common side effects.
3. Increased stability, increased beauty, and increased flexibility in the formulation.
4. Nanosponge systems are anti-irritating, anti-mutagenic and anti-allergenic.
5. A Nanosponge offers up to 12 hours of actual operation, i.e. extended release.
6. This reduces discomfort and provides good tolerance, leading to better compliance with the patient.
7. Allows the introduction of immiscible liquids that enhance the handling of materials. It is possible to transfer liquid to powder.
8. Such preparations are robust in a diverse range of PH (1-11) and temperatures (up to 130^0c).
9. They are auto-sterilizing as a total pore size is 0.25μm because bacteria are unable to penetrate.
10. These are free flowing, fully efficient and price-effective with a broad range of products.
11. We have increased stability in terms of environmental, physical and chemical.
12. Particles of nanosponge are water soluble, so that they can be encapsulated in the nanosponge by adding a chemical called an enzyme inhibitor.

PREPARATION METHODS OF NANOSPONGES:

A. Emulsion Solvent Diffusion Method[16]:

Nanosponges can be processed using different strengths of ethyl cellulose (EC) and polyvinyl alcohol (PVA). The dispersed product comprising ethyl cellulose and drug was diluted in 20ml dichloromethane and gradually applied in 150ml of aqueous persistent solution to a certain volume of polyvinyl alcohol The solution of the process was stirring for 2 hours at 1000 rpm. The developed Nanosponges were obtained through filtering and drying for 24 hours in the oven at 400c. To insure the elimination of excess solvents, the drained nanosponges were placed in vaccum desiccator

B. Solvent method[17]:

In a polar aprotic solvent such as dimethylformamide (DMF), dimethylsulfoxide (DMSO), combine the polymer with an appropriate solvent. Transfer this solution to the cross linker in excess quantity and suggest a ratio of 1:4 for cross linker / molar ratio. The reaction was conducted at temperatures ranging from 100°C to the solvent reflux temperature, ranging from 1 to 48 hours. Dimethyl carbonate and carbonyl diimidazole are the cross linkers that may be favored. The process is performed and mixture is cooled at ambient temperature, then product is applied to the large amount of bi-distilled water and product is removed by vaccum filtration and then extracted with ethanol by sustained soxhlet extraction. Eventually, the stock is vaccum-dried and grinded to produce homogeneous powder in a mechanical press[17].

FACTORS INFLUENCE NANOSPONGE FORMATION:

Type of polymer[18]:

The variety of polymer itself can affect the formation of

nanosponges and also the efficiency. The space size of nanosponge should be sufficient for complexation to fit a specific size drug molecule.

Type of drugs[19]:
Drug molecules ought to have those attributes described below in order to be complexed with nanosponges.

1) The drug's molecular weight must be between 100 and 400 Daltons.
2) There are less than five compact rings in the drug molecule.
3) Water solubility should be below 10mg/ml.
4) The material's melting point should be below 250°C.

Temperature[20]:
Changes in temperature will affect the complexation of the drug/nanosponge. Ultimately, rising temperature reduces the magnitude of the Drug/Nanosponge complex's evident stability variable may be related to possible decrease of drug/nanosponge interacting forces, such as van-der Waal forces and temperature-rising hydrophobic forces.

Degree of substitution:
The nanosponge's complexation capacity may be severely affected by the substituent's form, number, and location on the parent molecule.

CHARACTERIZATION OF NANOSPONGES:
Complex interaction between the drug and nanosponges may be defined by the following options:

1. **Loading efficiency[20]:**

The loading efficiency of compounds of nanosponge is to be immersed in an acceptable solvent, sonicized to split the structure, diluted appropriately and then examined using UV spectrophotometer & HPLC methods.

2. **Microscopy studies[21]:**

Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM) could be used to study the drug, nanosponges and product microscopic features (drug / nanosponge complex).

3. **Particle size and polydispersity[22]:**

Using 90 Plus particle sizer equipped with MAS OPTION particle sizing software, dynamic light scattering will evaluate particle size. It is possible to identify that mean diameter and the index of polydispersity.

It is also possible to observe the polydispersity index (PDI) from dynamic light dispersion instruments. PDI is a measure of the particle size distribution range or range or variance. Monodisperse samples get a smaller PDI value; while PDI's higher value suggests a wider range of particle size and the sample's polydisperse nature.

4. **Zeta potential:**

Zeta potential is a surface charge indicator. It could be estimated in the particle size apparatus when using additional electrode.

5. **Fourier Transform Infrared (FTIR) Analysis:**

In order to ascertain the potential for interference of chemical bonds between drug and polymer, Fourier transform infrared analysis was performed. Samples from 400-4000 cm-1 and carbon black reference were tested. Clean dry helium gas has deliberately purged the detector to raise the signal level and decrease humidity.

6. **Thin Layer Chromatography[23,24]:**

The Rf value of a drug molecule decreases dramatically in Thin Layer Chromatography, which helps to recognize the specific structure between drug and nanosponge. Complexing the incorporation of molecules among guest and host is a reversible process.

7. **Thermo-analytical methods[25,26]:**

Thermo-analytical methods assess if there is any improvement in the product material before the nanosponge thermal deterioration. Melting, evaporation, decomposition, oxidation, or polymorphic transformation may be the drug substance's shift. The product substance's transition represents the dynamic creation.

It is possible to observe the thermogram generated by DTA and DSC for the growth, shift and emergence of new peaks or the absence of certain peaks. Progress in weight loss may also provide proof to support the production of inclusion systems.

8. **Single crystal X-ray structure analysis[27]:**

This approach was used to establish the precise design of the inclusion and the mode of contact. It is possible to identify the interaction between both the host and guest molecules and to determine the precise geometric relationship. Such information gathered during most of the study contributes to knowledge about either the creation of systems of inclusion.

9. **I*n-Vitro* drug release study[28]:**

Utilizing Franz Diffusion cell with something like a diffusional region of 2.26 cm^2, the escape of the drug from the engineered nanosponge formulation can be analyzed using multi-compartment rotating cell with dialysis membrane. The donor process comprises of a nanosponge system filled with drugs in distilled water. The step of the receptor also have the same environment

Applications of nanosponges:

1) Cancer Therapy[29]:

Nanosponges that could be used as a tumor drug delivery device for anticancer drugs. Researchers say the approach is 3-5 times more efficient then direct injection of the drugs in reducing tumor growth. The small nanosponges are packed with a drug loading and display a binding peptide which binds to the cell surface targets due to radiation on the tumor. Such stick to the surface once the sponges contact tumor cells and are stimulated to expel their load. Targeted drug delivery advantages provide more accurate diagnosis at the very same dosage and less health issues. Studies have been conducted as a sponge load in animals with paclitaxel.

2) Antiviral application[30] :

Throughout the pathways of head, nasal, pulmonary treatment, nanosponges can be beneficial. In order to recruit viruses that infect RTI including respiratory sincytial virus, influenza virus & rhinovirus, nanocarriers can selectively deliver antiviral drugs or small interfering RNA (siRNA) to the nasal epithelia & lungs. These could be used for HIV, HBV, and HSV as well. Zidovudine, saquinavir, interferon-α, acyclovir (Eudragit-based) are the medications commonly used only as a nano delivery system.

3) Encapsulation of gases[31,32]:

Nanosponge dependent on cyclodextrin was used to form inclusion complexes with 3 separate gasses, i.e.1-methylcyclopropene, oxygen and carbon dioxide. For several biomedical applications, complexing of oxygen or carbon dioxide may be useful. The oxygen-filled nanosponge, in particular, could supply the hypoxic tissues contained in different diseases with oxygen. Due to its high extremely porous nature; as an efficient gas carrier, the Nanosponge was also explored. The composition of nanosponge shows the ability to monitor the accumulation and release of oxygen. They might be a valuable tool for supplying some critical gasses in the future.

4) Other applications of Nanosponges[33-36]:

Cyclodextrin-based nanosponges can bind organic molecules strongly and extract them even at these low levels from water. The same concept can be beneficial when selectively combining polymer and crosslinker to eliminate bitter ingredients from grape fruit juice. The 3Dimensional nanosponges for proteomic applications can play an important role in fractionalizing peptides. For gases such as oxygen and carbon dioxide, nanosponges may be used as transport.

For several biomedical applications, such nanosponges may have been beneficial. The oxygen-filled nanosponges in general will provide oxygen to the hypoxic tissues contained in different illnesses. For the examination, nanosponges may exclusively soak biomarkers. One study found that Nanosponges was able to harvest rare blood cancer markers.

CONCLUSION:

The nanosponges are able to distribute the medication to the target site in a controlled manner. These are indeed able to carry molecules that are both lipophilic and hydrophilic. These can be formulated as different dosage forms such as oral, parenteral, and topical preparations due to their small particle size and spherical shape. Nanosponge can be efficiently integrated into a topical drug delivery process for the persistence of dosage type on the skin and for the oral delivery of drugs using bioerodible polymers, particularly for the delivery of colon-specific and controlled-release drug delivery system. Nanosponge technology provides drug trapping and therefore minimized side effects, enhanced stability, increased beauty and increased flexibility in the preparation.

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REVIEW ARTICLE

An Overview on Post Approval Changes to an Approved ANDA in US-FDA

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ABSTRACT:
Post approval changes are the non-avoidable changes due to the many reasons for improving products quality and safety. According to US-FDA the changes which are made after the approval of the product are supposed to be reported as major, moderate and minor changes based on the impact on the process and filings. The post approval changes to approved generic products according to US-FDA regulations are the changes which includes the contents and composition, production sites changes, manufacturing or production process changes, container and closure system changes, specification changes, labeling changes and other miscellaneous changes. Hence United States regulatory authority requires that all changes to be thoroughly reviewed by the regulatory team before any changes are implemented for the particular commercialized product. The current study endeavours on the role post approval changes to the approved generic product or ANDA application.

KEYWORDS: Post Approval change, Generic Drugs, US-FDA, ANDA.

INTRODUCTION:
A generic drug is a drug defined as "a comparable drug product in the form of dosage, quality, strength and performance features and anticipated use with a drug product listed as a brand / reference. It can even well-defined as the generic drug product which is similar to the brand product in the same strength, dosage, chemical composition and route of administration. Though generic medication might not be related to a specific company, they are subject to the laws of the governments of the countries wherever they're distributed. Non-Brand medicines are tagged with the manufacturer's name and also the drug's adopted name (non-proprietary name).[1]

A generic drug is comparable or similar to a branded drug in dosage, protection, strength, route of administration, reliability, performance characteristics and intended use. While generic medicine is chemically adequate to their branded counterpart and they're generally well sold-out from the branded value.

Change is outlined as "A modification to any aspect of a pharmaceutical product, as well as however not restricted to a modification to formulation, manufacturing process, site of production of product, conditions for the finished pharmaceutical goods and ingredients, container closure system, labeling and products information".

Modifications or alterations to licensed product must be checked to determine their effects on product quality, effectiveness and safety of the product. The modifications or changes which will be implemented to the particular product must be recorded in detail without altering the original data. All changes may require the organization authorization, depending on the degree of effect on the product before implementing the changes to the product and those changes are evaluated for the safety and effectiveness of the product. There are different mechanisms for documenting these modification, and they can range from an annual report to a request for modification/variation to a new license application. Manufacturers should review the jurisdiction-specific guidance documents to follow proper enforcement procedures.[2]

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The post-approval changes are the modifications made to the approved pharmaceutical products and to provide the data to support a change that would be considered sufficient to evaluate the effect of the alteration on the value of the approved drugs as it relates to the health, efficacy and/or successful use of the products. The organization can make post-approval changes to the approved product after the approval of NDA or ANDA and which are conveyed to the FDA in the related classes.

After obtaining the original regulatory approval, there are several factors to make changes to drug products. Company / organization change management processes will explain how changes are assessed and enforced and how the change affects continuity and what information is desired to maintain the change. The regulatory group shall determine the submission strategy based on reviewing the change's technical evaluation and the appropriate regulatory guidance.

Abbreviated-New Drug Application-(ANDA):

The Hatch-Waxman Act created a new form of the regulatory authorization process for a generic drug. This application is named as "ANDA" which is submitted by the applicant, helps to provides the information of the particular product to the FDA for evaluation or review and approve a generic drug product. An applicant can start the production of the product for commercialization after the approval from the authority and once approval is granted then product is allowed to release in the market.[3]

If the drug substance of the generic drug is the bioequivalent of the approved drug, an ANDA may be filed and regulatory authority permits a generic drug producer to rely on the safety and effectiveness information provided by the unique producer. Also, the Hatch-Waxman Act continued the earlier practice of the document NDA" of the FDA by establishing what became known as a request under section 505(b)(2). Such an application depends, at least in part, on information on safety and efficiency that have not been established by the aspirant itself but are available in the available writings. ANDAs and section 505(b)(2) requests enable a generic supplier to reduce costs and delays in filing a complete NDA. Those two advanced marketing approval processes will allow a generic manufacturer to sell its bioequivalent drug approved by the FDA in many cases as soon as any related patents expire.[4]

Post-Approval changes in USFDA:

The reporting categories given under section 506A of the Act and CFR 314.70 are outlined in the following sections.

A. Major change
B. Moderate change
C. Minor change

A. Major changes:

A major change is the modification or alteration that has significant potential to adversely affect the identification, intensity, quality or excellence, purity, or potency of a drug product since these factors may be linked to the integrity or efficacy of the drug product. A major change includes an update and approval of the FDA to be requested before the alteration is used to sell the drug product. These supplements is named as a Prior approval supplement (PAS) and should be clearly labeled. An applicant may request the FDA to speed up its review of a PAS for public health reasons or if a delay in making the specified change causes the applicant extraordinary hardship. More likely, the FDA would approve applications for accelerated approval on the basis of exceptional difficulty in making changes made inevitable by terrible events or incidents that could not be foreseen and could not be prepared by the applicant.[5]

B. Moderate changes:

Moderate changes may be the modification which encompasses a modest potential to adversely have an effect on the drug product identification, strength, performance, purity, or efficiency as these factors might contribute to the drug product safety or effectuality.

These changes can be classified as 2 sub categories which are

- **CBE-30**
- **CBE-0**

Other form of moderate change which needs a supplement to submit the FDA within 30 days prior to the pharmaceutical product being sold using the modification. These form of supplement is named and must be clearly marked as a Supplement that is Changes being effected in 30 days.

If, FDA notifies the applicant that a PAS is essential within 30 days of receiving the supplement and the drug product which is commercialized by using particular change cannot be distributed to the market. This supplement must include the information defined by the FDA as necessary for each change and shall contain the information produced by the applicant in determining the effects of the change. If, FDA notifies the applicant of missing information within 30 days of receiving the supplement, distribution shall be postponed until the addition has been updated to provide the data that is lacking. FDA may recognize some moderate modification for which delivery can occur upon receipt by FDA of the supplement. This form of supplement should be clearly labelled and is termed as a Supplement

- CBE. The FDA may order the manufacturer to stop selling the pharmaceutical drug products made using the disapproved modifications if, after inspection, the FDA criticizes a change being made in 30 days' supplement or change being made supplement.

C. Minor change:

A minor change is a modification which may have limited ability to affect the identification, product quality, strength potency and purity, as these problems may be linked to the safety and efficiency of the drug product. In its next coming Annual report, the applicant must notify the minor changes to the authority.

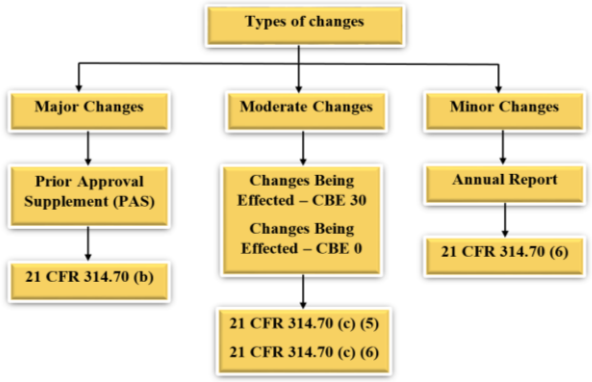


Fig 1: Classification of changes according to FDA

Reporting categories of changes according to 21 CFR 314.70:

Under the 21 CFR 314.70 individual post-approval change in the production process, quality control, facilities, product, equipment or labeling established in the approved NDA/ANDA should be reported using the submission type associated with one of the three tier-based reporting categories depending upon the potential (i.e., risk) of the change to adversely affect the product's performance, strength, identification, potency or purity as it may relate to the product's safety or efficacy (i.e., minimal potential, moderate potential, or substantial potential). Each reporting categories in this regulatory provision also specify when the product made using the change can be distributed. The submission types associated with each reporting category are the following [6]

Prior-Approval Supplement-PAS:

Changes that have a significant ability to undesirably affect the quality of the pharmaceutical product (i.e., major changes) need an applicant to report the change to the FDA in a supplement to the approved NDA or ANDA. Before releasing the product to the market using the amendment 21 CFR 314.70(b), a PAS must be approved by the FDA.

CBE -30 / CBE - 0:

CBE-0:

- No FDA approval is required before distributing the product using the change
- Used for proposal of certain types of modest changes

CBE-30:

- It is a modification that will be submitted 30 days before the pharmaceutical drug is put on the market using the adjustment
- Used for the application of a moderate change
- The pharmaceutical product cannot be sold or marketed if the FDA notifies the applicant needs to file a PAS, which is necessary for the change within 30 days of receiving the supplement

Annual Report-AR:

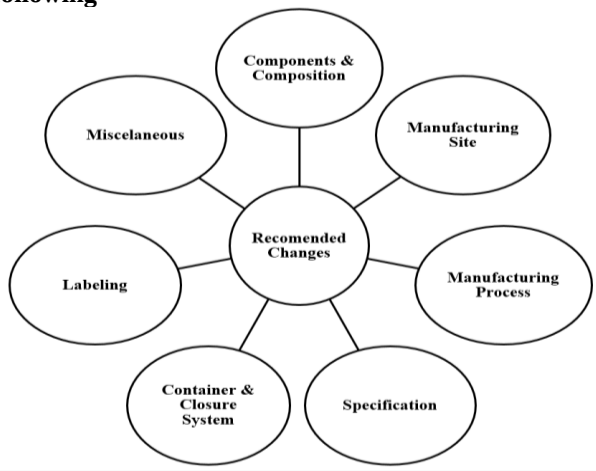
- Changes with negligible potential to adversely affect the quality of the product (i.e. minor changes) shall be reported by the applicant in the annual report using 21 CFR 314.70(d).
- No FDA approval is required prior to the distribution of the drug product made using the modification and used to make a minor change
- A minor change is defined as having some negligible possibilities to have a confrontational effect on the quality, identification, purity and / or potency, strength, of a product

Table 1: Reporting categories of changes and estimated timeline for approval according to USFDA regulations

Change	Major Change	Moderate Change		Minor Change
Supplement to be filed	Prior approval supplement (Tell, wait and do after getting approval procedure)	CBE-30 (Tell, wait and do procedure)	CBE-0 (Tell and do procedure)	Annual report (Do and tell procedure)
When to Notify to Agency	Before implementing the change	Before implementing the change	Can be implemented simultaneously	List of changes should be annually reported right from the drug approval date till withdrawn from the market.
Estimated timeline for Approval (6)	• 6 months (no pre-approval inspection) • 10 months (with pre-approval inspection)	30 days (Timeline may vary based on queries addressing to agency in due course)	Change can be implemented the very next day after notifying to the agency, but best wait for 15 days)	NA

CBE: Changes being effected NA: Not applicable

Different categories of Post-Approval changes in the following



A. Components and composition changes:

Components and composition changes are the changes in the pharmaceutical product ingredients or excipients. The regulation for changes to approved NDA or ANDA does not discuss changes in the amount of product content. Improvements in components or composition that allow a new excipient to be added or an excipient to be removed are specified at a different level. [7]

Category of changes in components and composition with the examples:

1. **Major changes-PAS**
- A qualitative or quantitative change in the formulation, including inactive ingredients [8]

2. **Moderate changes-CBE**
- Any modifications in the excipients grade or excipient specifications

3. **Minor changes-AR**
- Reduction or removal of an ingredients intended to affect the pharmaceutical product colour only

B. Manufacturing Sites changes:

Manufacturing site means a facility or establishment used to manufacture, process or package a finished dosage form or pharmaceutical product including an accessory of any finished product. Any changes in the manufacturing site the organization should notify the FDA before initiating the commercialization of the product. If the manufacturing site is not inspected by the FDA team or not cGMP certified manufacturing site Prior approval supplement to be filed by preparing the regulatory strategy.[9]

Category of Changes in Manufacturing Sites with examples:

1. **Major changes–PAS:**
- Change or move to another manufacturing site which is not inspected by the FDA
- A switch to another well-developed production site for the production, storage or primary packaging of medicinal products

2. **Moderate changes:**

a. CBE-30
- A switch to another production site for any drug product's primary packaging

b. CBE-0
- A switch to another production site for the final intermediate processing or manufacturing

3. **Minor Changes-AR:**
- Change to another production or manufacturing location for labelling
- Change to another or different production site for secondary packaging

C. Manufacturing process Changes:

Manufacturing process changes are the changes in process of manufacturing of the pharmaceutical products or finished products. The manufacturing changes may or may not affect the identification, strength, quality, efficiency and safety of the pharmaceutical product. These modifications that may also impact on the release of the medication after the product has been given. Under certain circumstances, there may be significant potential for adverse effects regardless of the drug ingredient or drug material being tested directly in order to conform with the accepted specification. If there is a significant risk for adverse effects, a modification in a previous approval then Prior approval supplement must be filed or submitted.

Category of Changes in Manufacturing process with examples:

1. **Major Change-PAS:**
- Changes that may impact the dose of controlled release to the patient
- Method of sterilization is changed

2. **Moderate changes:**

a. CBE-30
- Any modifications in the manufacturing process, equipment and/or process parameters
- Modifications in process or terminal of sterilization from one suitable sterilization chamber to another

b. CBE-0
- Change in methods or controls that give the drug material or drug product greater assurance

3. **Minor Changes – AR:**
- Minor modification of the existing code for the dosage form
- Change in order of ingredients to be added

D. Specification Changes:

A specification also refers to a collection of defined specifications that a content, design, product or service must meet. A specification is often a technical standard

type. Specification Changes are the type of changes in the approved requirements, which are approval conditions, tests and analytical procedures, quality standards given in an accepted request to ensure the quality of drug products, drug ingredients, raw materials, reagents, components, intermediates, in-process materials, container closure systems and other materials used in production. [10]

Category of Changes in Specification with examples:

1. Major Change-PAS:

- Relaxing in an acceptance criterion.
- Deletion in part of a specification

2. Moderate changes:

a. CBE-30

- Relaxation of acceptance criteria and elimination of standards for raw materials used in the manufacture of pharmaceutical products

b. CBE-0

- Constricting of specification which provides greater certainty of the drug material or drug product

3. Minor Changes-AR:

- Tightening or narrowing of the acceptance criteria

E. Container and Closure System Changes:

A pharmaceutical container is a device in which the pharmaceutical product is inserted that can be in direct contact with the product and it is considered an immediate container. A closure prevents the container to exclude moisture, dirt, bacteria, microorganism, etc. and prevents the material from losing liquid and other hazardous substances. The main purpose of containers and closures is to carry a preparation to protect the product and to identify it. A modification in a packing element will frequently lead to a new or updated packaging component plan.

Category of Changes in Specification with examples:

1. Major Change-PAS:

- Any alterations in the primary packaging component of the product
- A single-unit dose container change to a multi-dose container system

2. Moderate changes:

a. CBE-30

- Any changes made to the dimensions and/or shape of a sterile product material container
- Any changes or modifications in container size and/or shape

b. CBE – 0

- An adjustment in addition or omission of a desiccant

3. Minor Changes-AR:

- A change in the cap liner or addition of a lid liner
- Increasing in the thickness of the container.

F. Labeling Changes:

Labeling changes are the type of changes in an approved NDA or ANDA application. Modifications in the package labeling package insert or container label include an alteration in the branding of a pharmaceutical product. Labeling alterations may include the change in the layout of the label, any changes in the label write up and changes in the dimension of the label.

Category of Changes in Labeling with examples:

1. Major Change-PAS

- Modifications based on postmarketing performance, including, not restricted to however, changes in the labeling of new indications and use

2. Moderate changes

a. CBE-30

- Introduces or reinforces a dosage form and administration instruction to increase the drug product's safe use.

b. CBE-0

- Updating of prescribing information with the RLD label

3. Minor Changes-AR

- Alterations in the box or bottle tag format without any alteration in the content

G. Miscellaneous changes

Miscellaneous changes are the category of changes for approve NDA or ANDA, where the changes which will not fall under the main category of changes which are considered as a miscellaneous change**.**

Category of Changes in miscellaneous changes with examples:

1. Major Change-PAS

- Adding of a comparability protocol or stability protocol

2. Moderate changes

a. CBE-30

- Reduction of an expiry date duration to provide more assertion of the drug products identification, purity, strength, quality and effectiveness.

b. CBE – 0

- No modifications have been recognized

3. Minor Changes-AR

- Extension of the expiry date period on the basis of total shelf-life information on lots of output

Process of post approval changes in US-FDA:

The regulatory authority shall approve the ANDA and, if any of the explanations given in 314.127 for refusing to just accept the ANDA submitted by the applicant, send a letter of approval to the applicant holder. Therefore, if associate ANDA satisfies all the approval necessities of the Federal FD and C Act, office can issue the conditional approval letter. The ANDA cannot, however,

be approved as there's a seven-year orphan exclusivity period for the medication observed in section 527 of the Federal FD and C Act. 21 CFR 316.31 or might not be approved till the requirements of 21 CFR 314.107(b)(3) are met, as there is an amount of exclusivity for the drug observed in 21 CFR 314.108, as there is an amount of exclusivity for medical specialty use.

If a pharmaceutical product is given a provisional approval means, it's not associate approved drug and cannot be approved till when the requisite further ANDA examination has been issued by the regulatory agency. The regulatory agency's tentative approval of a pharmaceutical product relies on the knowledge obtainable to the FDA submitted by the mortal at the time of the letter of tentative approval (i.e. details found within the ANDA and also the standing of current good manufacturing practices (GMP) for facilities utilized in the manufacture and testing of the drug product it's insufferable to sell a brand new drug product till the date of approval. [11]

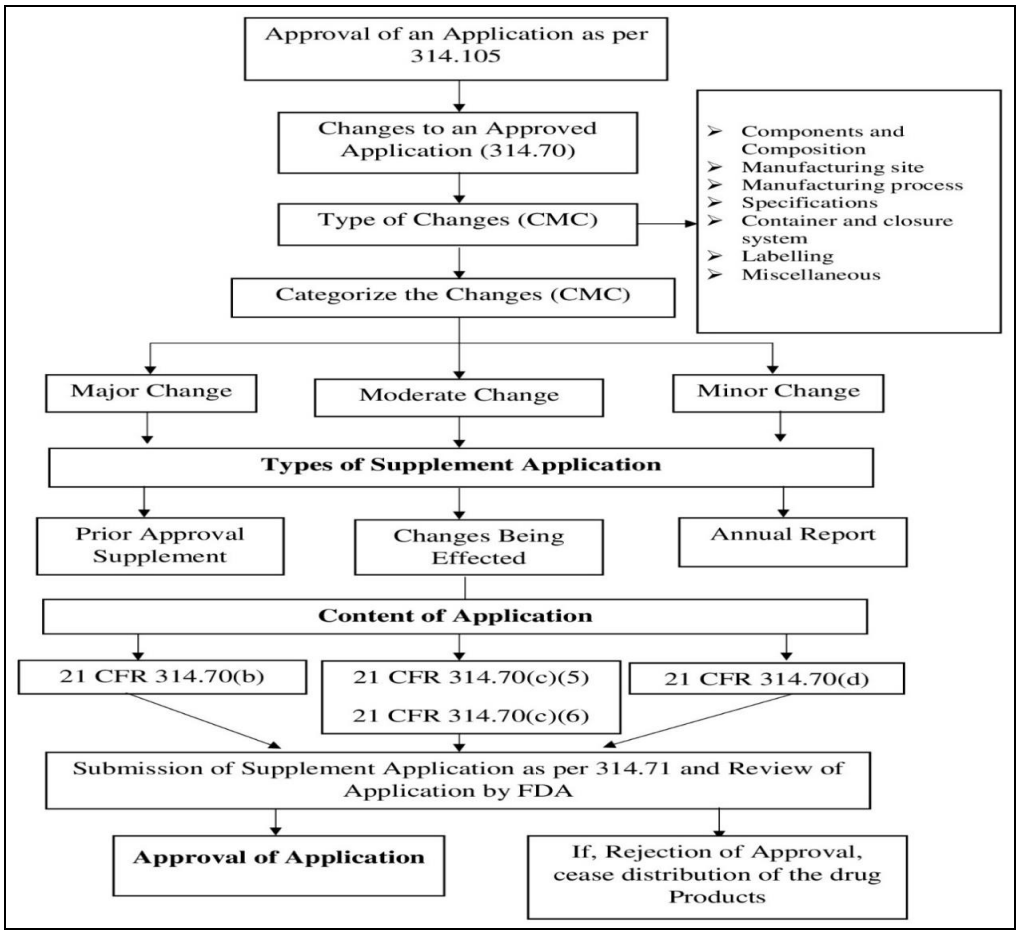


Fig 2: Process of post approval changes in US-FDA

CONCLUSION:

From the regulatory point of view, the FDA's primary concern is to protect the American public on drug products that are not interchangeable due to bioequivalence, clinical equivalence or other concerns that would not require a drug product to be therapeutically equivalent to another drug product. Upon the implementation of the changes which are approved by the regulatory authority which helps to increases the product safety for the patients as well as meets the organization's requirements.

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REVIEW ARTICLE

Phytoestrogens are emerging medicine in Prevention and Management of Cognitive deficits in Postmenopausal Women

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ABSTRACT:

Estrogen is reported to have multi-dimensional neuroprotective activity against cognitive dysfunctions in animals and human's despite of its classical hormonal effects. It has been well documented that postmenopausal women exhibit a higher risk of cognitive impairment than premenopausal females. In this context, estrogen replacement therapy (ERT) has been established as one of the better alternative therapy to alleviate cognitive deficits in such females. As per Women's Health Initiative, though ERT is safe and effective strategy to mitigate cognitive dysfunction in postmenopausal ladies still it is reported to have a risk of developing breast and endometrial cancer in these women. Thus, there is a demand for developing suitable alternative medicine to conventional estrogen replacement therapy in the management of cognitive deficits. Recent phytopharmacological research suggests that phytoestrogens, naturally occurring non-steroidal compounds, from plants especially in soy and soy-based products exhibits estrogen-like physiological and pharmacological actions. Phytoestrogens primarily act through binding to estrogenic receptors (ERs) α and β, with a higher affinity for ERβ. Isoflavone phytoestrogens such as Daidzein and Genistein are widely studied herbal drugs in the management of cognitive dysfunction in postmenopausal women among other classes including lignans and coumestans. Experimental studies report that phytoestrogens exert neuroprotective activity with anti-tumor property to uterine growth. Further, clinical and in-vitro studies also corroborate the fact that phytoestrogens exhibit minimal anti-cancer activity. Epidemiological survey also documents that Asian population who consume phytoestrogen-rich diet have a lower risk of breast and prostate cancer compared to western individuals. Hence, it can be presumed that phytoestrogens could be used as a safe and alternative medicine to estrogen replacement therapy in the management of cognitive deficits in postmenopausal ladies.

KEYWORDS: Estrogen; phytoestrogens, cognitive deficits, postmenopausal women.

INTRODUCTION:

Estrogen is reported to have multifunctional neuroprotective activity in terms of facilitating axonal sprouting and synaptic transmission despite the classical hormonal effect[1]. The incidence to risk of dementia is more in women than men[2]. However, after menopause, the risk of dementia is reported to be twice in women as compared to premenopausal females[3]. Estrogen deficiency leads to several pathological events including neuronal cell death, synaptic damage and neuroinflammation in different memory-sensitive brain regionssuch as hippocampus, frontal cortex and amygdala[4-7]. Therefore, it can be assumed that estrogen deficiency may modulate memory formation.

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To recompense the cognitive dysfunction attribute able to dearth of estrogen in postmenopausal women, ERT has been considered as one of the major therapeutic approach till date[8-10]. Nevertheless, due to its lack of access to the poor and rural communities as well as to serious adverse effects the ERT was regarded as an undesirable technique. Thus, the production of synthetic drugs and hormonal preparations is increasing to achieve the oestrogen deficiency and to manage the related cognitive dysfunction[11]. Yet, despite their deficient effects on the target and documented adverse effects including malignancy, these products are not very common among the majority of women. In traditional medicine system, the use of herbal medicines is gaining popularity in the management of estrogen-deficiency related cognitive dysfunction and alzheimer's disease with no adverse effects[12-15]. Moreover, in general peoples use herbal medicines as a memory enhancer[16-18].Thus, several researches have been performed to provide a better therapeutic alternative in the management of cognitive dysfunction in such individuals.

Cognitive dysfunction is considered as one of the major clinical neurological manifestation in addition to various diseases in estrogen-deficient females[19-22]. Therefore; an attempt was made to perform a review on pharmacotherapy of postmenopausal cognitive dysfunction by phytoestrogens.

Methods:

Literature Search:

A systematic and detailed phytoestrogens literature search was conducted. A variety of open repositories, such as the Pub Med US National Medicine Library databases and journals such as Elsevier, Springer, Scopus etc., have assessed the existing scientific evidence.

Role of Phytoestrogens in cognitive dysfunction

Table no. 1: Effect of Phytoestrogens on memory formation in different animal models

S. No	Name of Phytoestrogen	Animals/Animal Models	Mechanism of Action
1	Daidzein	Effects of daidzein on adult hippocampal neurogenesis using female mice.	Daidzein administration increases the numerical density, but not apoptotic, of proliferating cells[23].
		Scopolamine-induced impairments of learning and memory in male mice	Daidzein may be useful for cholinergic dysfunction-induced cognitive impairment and this beneficial effect is mediated, in part, through an estrogen receptor[24].
		Scopolamine-induced impairments of learning and memory	Daidzein administration may play a role in the biosynthesis of acetylcholine as a ChAT active ingredient and it improves amnesia due to scopolamine[25].
2.	Genistein	Isoflurane-induced neurotoxicity and impaired learning and memory	Genistein administration suppresses neuronal apoptosis induced with isoflurane, and enhances signaling of cAMP/CREB-BDNF-TrkB-PI3/Act[26].
		Alzheimer's disease rat model was established using an ip. Injection of D-galactose combined with an intracerebral injection of amyloid-β peptide (25-35)	Pre-use of genistein reduced escape delays by the controlling CAMK4 to modulate tau hyperphosphorylation and exhibits an neuroprotective role in Alzheimer's disease[27].
		Lipopolysaccharide-induced memory impairment in mice	Genistein attenuates the cognitive function affected by LPS in Y-maze, and improves the expression of BDNF and phosphorylation by CREB[28].
		Diabetes associated cognitive decline streptozotocin-induced diabetic mice	30 days of genistein therapy in diabetic mice significantly lowered hyperglycaemia, recovered cognitive performance by affecting acetylcholinesterase activity and oxidative stress and improved neuro-inflammatory conditions by varying levels of TNF-α, IL-1β and nitrite as impaired in diabetic mice[29].

Table no. 2: Effect of Phytoestrogens on postmenopausal cognitive dysfunction

S. No	Name of Phytoestrogen	Animals/Animal Models	Mechanism of Action
1	Daidzein	Ovariectomy induced memory loss	22-day old rats were fed a customized diet containing Teklad 2016 complemented with the main flavonoid soy, daidzein, for 40 days. These results show that flavonoids have significant effects in the CA1 region of the hippocampus in a standard rat laboratory diet on the density of spinal synapse [30].
		Middle-aged ovariectomized rat model	Isoflavones- daidzein treatment enhance expression of calbindin D-28k immunoreactivity in the hippocampus and as well as memory[31].
2.	Genistein	Ovariectomy induced memory loss	Genistein improves the expression of BDNF and IGF-1 in the hippocampus, as well as the ovariectomized rat spatial memory[32].
		Ovariectomized animal model of Parkinsonism	Genistein displayed a neuroprotective effect on dopaminergic neurons[33].
		Ovariectomy induced neurodegeneration	Genistein provides OVX-induced neuro-degeneration protection through oxidant stress reduction, lipid peroxidation and an apoptotic mitochondrial pathway[34].

CONCLUSION:

Estrogen deficiency is known to cause significant cognitive dysfunctional changes. ERT is considered to be the major option to achieve estrogen deficiencies. In addition to an undesirable and expensive technique, however, some studies equate it with serious adverse effects. Many new medicines and hormone formulations are therefore being formulated and sold to replace ERT therapy. However, despite its low effect on the target and documented risks, including malignancy, most people are now positive about highly advertised natural products, including phytoestrogens. For menopausal problems linked to cognitive dysfunction, these drugs are shown to be health protection.

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REVIEW ARTICLE

A Review on Enzyme Activated Drug Delivery System

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ABSTRACT:

Enzymes are the bio-molecules that are present in the various sites of the body and are specific in the organs. The recent trend of drug delivery as nanoparticles has a handful of uses. The designing of the systems in small-sized particles like nanoparticles have merits as they can easily diffuse into the membrane through the intracellular pores of very small size. Such a kind of delivery system has its applications in various treatments, one of which is in cancer therapy. Even though the anti-neoplastic drugs are much potent, they may also act on the normal cells and thus inhibiting the normal physiological process leading to serious adverse effects. Such a kind of problem can be overcome by the enzyme activated drug delivery system (EADDS). An enzyme activated system, the discharge of drugs from the system is activated by the enzymatic process. Various approaches for enzymatic drug delivery include liposomes, nanoparticles, prodrug, microparticles, etc. The main components in this system are drug, nanocarrier, promoiety, coating polymer, ligand, etc. The present review is about to provide some ideology on how the drug can be delivered to a particular type of organ or site based on the presence of respective enzyme.

KEYWORDS: Enzyme, Bio-catalysts, Nanocarriers, Targeted delivery, Ligand.

INTRODUCTION:

Enzymes are the bio-molecules present in the body performing a variety of functions. Enzymes consist of the non-protein part (co-factor), which is responsible for binding to a substrate and catalyze the reaction. An enzyme without co-factor (apo-enzyme) is a protein, whereas an enzyme with all necessary components (holoenzyme). Enzymes hasten the rate of a biochemical reaction and consists of active sites on their surface to which substrate binds. The main functions of enzymes viz., bio-catalysts, synthesizing the molecules, etc.

Some of the enzymes are specific in particular organs (E.g., Renin in kidneys, rennin, pepsin and chymotrypsin from Gut, Ptyalin in mouth oral cavity and others having significant functions are carbonic anhydrase, hexokinase, glycogen phosphorylase and protease in a certain type of specialised cells.

Proteases regulate most of the biological and pathological processes by proteolysis[2]. Proteolysis, a type of irreversible regulatory mechanism, is accountable for discerning cleavage of certain substrates[3]. The levels of proteases can act as biomarkers in the finding and prediction of tumors[4]. Proteases are involved in controlling various pathways viz., DNA replication and transcription, cell proliferation and differentiation, cell mobilization, inflammation, immunity, necrosis, apoptosis, hemostasis and blood coagulation[5]. Therefore, abnormalities in proteolytic actions result in cancer, cardiovascular and neurodegenerative disorders.

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Another class of enzymes called matrix metalloproteinases (MMPs) are mainly involved in the cancer initiation and act as biomarkers and therapeutic targets. Recent emerging trends deal with the ideal expansion of MMP receptive drug delivery thereby targeting the tumour cells[6]. It was established that the diseases befalling by the dysfunctioning of enzymes laid the foundation for drug development to act in such sites or conditions of the physiological status.

Now a day, nanoscience is an emerging technology including EADDS finds its significance in drug delivery to various sites to the enzymes present in that region. The designing of the systems as small-sized entities like nanoparticles (NP) have advantages that they can easily diffuse into the membrane through the intracellular pores of very small in size.

It was a known fact that certain type of drug molecules shows rather an enhanced action when their size gets reduced to the nanometer level. Hence the NP are described as the good carriers of drug delivery owing to their small size, shape and surface characteristics. It also offers the prolonged release of entrapped drug by the aid of an external or internal impact[7].

Another type of well-known strategy is the prodrug mechanism, which is described as the inactive form of drug molecules. Upon the action of certain enzymes, chemical or environment stimuli, prodrugs transform to release the active drug *in-vivo*. With this approach, the features of the drug-like solubility, stability, permeability, and distribution can also be enhanced[8]. It includes the chemical conjugation of the drug with a suitable moiety, can be described as the prodrug[9]. Because of these significant aspects, the prodrug approach has been in practice in developing various novel formulations. Approximately 5-7% of currently approved drugs as a prodrug. The designing of prodrug should be such that the promoiety should get removed after the respective enzymatic action. It provides an idea that the prodrug gets transformed into its active form only when the enzyme of interest, having the promoiety as substrate, cleaves it. So, the drug will be released at a specific site where the enzyme is in overexpressed condition. There are so many difficulties arising that even though the anti-neoplastic drugs are much potent, they may also act on the normal cells and thus inhibiting the normal physiological process like cell division leading to serious adverse effects. Due to the drug resistance developed by the patients, it leads to the let-down of treatment. Such a kind of problem can be overcome by the aid of several approaches of which one of them is EADDS[10].

Now the current article gives a clear idea of how the drug can be delivered to a particular type of organ or site based on the enzyme present at that respective site. The drug is supplied to the particular site, showing action on that site thus avoiding the unsolicited side effects on other types of tissues or organs. Before studying the EADDS, it is necessary to have a brief idea on rate-controlled drug delivery systems (RCDDS), it refers to the delivery of the drug to a particular site of which the rate can be determined by the various factors. The RCDDS can be classified as illustrated in chart 1.

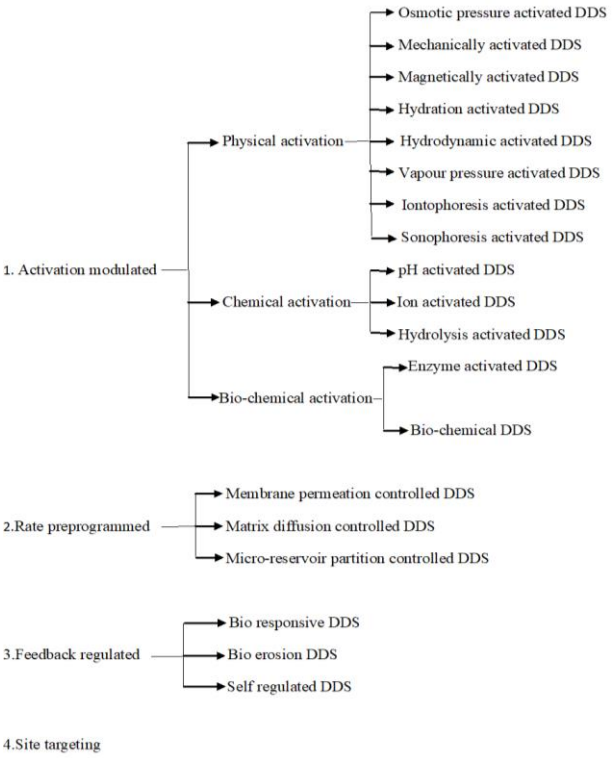

Chart 1: Classification of rate-regulated drug delivery systems

MATERIAL AND METHODS:

Enzyme activated drug delivery system (EADDS):

In this article, the authors highlighted EADDS, which is a bio-chemical activated type of rate-controlled system. This type of drug delivery is utilized for the treatment of various ailments including cancer. However, the traditional system of cancer treatment called chemotherapy was in practice, it shows rapid toxicity to other types of cells because of its non-specificity. In ESDDS, the discharge of drugs from the system is activated by the enzymatic process[11]. Here the drug is initially dissolved in a suitable medium to form the drug reservoir and can be enclosed physically as microspheres or by chemical means which includes the using of biopolymers like albumins or polypeptides to which the drug is bounded to their polymer chains. The chief procedure involved in the hydrolysis of biopolymers by the distinguishing type of enzyme present in the target

site. E.g., Albumin microspheres of 5-Fluorouracil can be made targeted to the cancer cells based on the enzyme called protease present in the cancer cells, that act specifically on the proteins like albumin. In this system, the degradation of albumin microspheres occurs by the action of the protease enzyme.

Past successful attempts on EADDS:

The scientific report by Dzamukova et.al. 2015,[12] revealed the successful delivery of brilliant green (an anti-neoplastic and anti-septic agent) delivered into the human cells by using the halloysite nanotube (HNT) carriers of 50nm size. They have used dextrin end stoppers, which are physically adsorbed for regulating the discharge of brilliant green. The interpenetration of the carrier particles can be achieved and their uptake by the cells is reliant on upon the cellular growth rate and creation. Glycosyl hydrolase is the enzyme present in the cells that act on the system and causes the dextrin tube end stoppers to undergo decay to release the loaded-brilliant green to act upon the human lung carcinoma cells rather than on the hepatoma cells thus the hepatic damage can be evaded. In this study, they have gone through the assortment of two kinds of cells i.e., Adenocarcinomic human alveolar basal epithelial cells and human hepatoma cells (Hep3b) that are explored for the cellular uptake as a purpose as they display diverse rates of proliferation. These cells were cultured in a manner of increasing concentrations of halloysite formulation of DX-HNTs (25-100μg/1, 00,000 cells, BG-free) for incubation up to 24 h. The cells were analysed microscopically by using the enhanced dark-field (EDF) microscopy and was found characteristic uptake nature of A549 cells and Hep3b, such that A549 cells appeared as obviously evident aggregates, while Hep3b cells have random delivery. The results obtained from TEM images revealed that A549 is arbitrarily dispersed in lysosomes, defining the enzymatic breakdown of dextrin tube-end stoppers with an improved discharge. The results were drawn from various other investigations and prophesied that the dextrin stoppers substantially reduced the toxicity of the formulation towards Hep3b and an improved release at the A549 cells. Their study has proven that the discharge of brilliant green by using the dextrin-coated halloysite nanotubes (HNT) is one of the most appropriate methods for handling cancer.

Bernardos et al., 2010,[13] designed a system with Doxorubicin, entailing of hydrocarbon molecules which are covalently attached to the silences. Doxorubicin release from mesoporous silica NP that are capped with saccharides, occurs by the enzyme called glycoside hydrolase. This system upon coming into the cancer cells comprising the enzyme glycoside hydrolase undergoes putrefaction to discharge the drug into the cells thus producing the effect.

Yildiz et al., 2018,[14] came with another work using Doxorubicin prepared by polymeric NP for the treatment of cancer. Here, the system gets activated by the enzyme called protease that acts on proteins. They have employed poly (lactic-*co*-glycolic acid)-*b*-poly-l-lysine and poly (lactic acid)-*b*-poly (ethylene glycol), among which the first polymer with Poly-l-lysine covalently adapted with near-IR 750 molecules. Doxorubicin delivery primed by the nanoprecipitation of copolymer blends were called as Theraneustic nanomedicines with a mean size of 60-80nm and was shown a controlled release of drug for 30 days. The discharge of NP to breast cancer cells was studied by fluorescence microscopy and was concluded that they were seemly as controlled release systems and contrast agents in the areas of imaging of cancer cells.

Sun et al., 2019,[15] worked on the improvement of the anti-tumour competence of Gemcitabine as a prodrug by FAPα-mediated activation. Gemcitabine agonizes with low uptake by tumor cells and low competence. So, the 4-amino group of Gemcitabine was modified to form Z-GP- Gemcitabine that advances the specificity and cleavage via FAPα-enzyme activation in tumor environment. In contrast, the prodrug form was found to have an improved uptake of tumor cells and enhanced inhibition effect on both growths of the 4T1 tumor cells and pulmonary metastasis in mice bearing orthotopic type of 4T1 breast tumors along with a reduction of tumor-associated fibroblast (TAF) was observed during animal testing. Therefore, it was established as an anticipated tactic for the treatment of cancer treatment by the route of intravenous administration.

Phillips and Pombeiro 2016,[16] described the transition metal-based prodrugs for anticancer drug delivery by taking Cisplatin as a model. The adverse effects of Cisplatin can be overcome by converting them into prodrug that gets activated and released by the differences in oxygen concentration or pH, by the action of overexpressed enzymes, by differences in metabolic rates, etc., through which the cancer cells can be easily differentiated from normal ones. By this technique, the pharmacological activity of the Cisplatin will be enhanced and becomes more inert.

Qing et al., 2018,[17] provided the novel idea of MMP (Matrix Metallo Proteinases) responsive smart drug delivery and tumor targeting systems. These are the extracellular enzymes that become overexpressed in the neoplastic conditions. However, under normal physiologic conditions, MMPs get regulated by the tissue inhibitors of metalloproteinases and they will be in limited quantity. Three types of MMPs are described in which MMP-2, MMP-7, and MMP-9 are found in cancer conditions. This concept can be utilized in developing the MMP inhibitors for targeting as nanocarriers of size

<200nm. The nanocarriers can be covalently linked to drug and coated with PEG (PEGylation). The mechanism is such that this system will reduce the apprehending of the Mono Nuclear Phagocytic (MNP) system, thereby promotes circulation and improved permeability and retention effect.

Paul et al., 2007,[18] described the role of polymer hydrogel particles in the case of bio-responsive polymer hydro gel for prolonged discharge. As per the view, the major advantage is that the active ingredient was protected from premature degradation using polymeric carrier. In this study, they described the carriers as "smart" materials because of their nature of altering their physical possessions in rejoinder to the applied stimuli, involving enzyme activity. The main components he used in emerging the system are enzyme cleavable linkers and hydrogels which are chemically cross-linked and are sensitive to a disease state enzyme. Upon the enzymatic action, these gels undergo a macroscopic change and get disintegrate to release the drug molecules that are entangled in it. For this work, he used PEGA-800 (copolymers of polyethylene glycol and acrylamide, 800). These PEGA particles were then modified using diglycine or dialanine as ECL (Enzyme Cleavable Linkers). They were found to respond for three types of enzymes thermolysin, chymotrypsin, and elastase. Later, they were evaluated for size characteristics, swelling nature of the hydrogel in response to the enzymatic action, and release physiognomies upon hydrolysis of the hydrogel by the enzymes. The enzymatic response of the PEGA particles was determined by three complementary methods.

- Analysis of the hydrogel accessibility to a fluorescently labeled dextran marker by two-photon microscopy
- Analysis of cleaved peptide fragments by HPLC
- Optical microscopy for particle diameters

Rationale:

The basis in the development of EADDS is the activation of the delivery system by the physiological enzymes present at respective sites to which the drug is envisioned to be delivered. Usually, in certain disease conditions like cancer, intracellular enzymes become overexpressed to uphold the rapid cell proliferation and obtaining more nutrients for cellular growth. Such an altered cellular enzymatic expression can be made as the target for drug delivery by which the system gets activated at the respective site or organ. Abnormal functioning of enzymes leads to the severe diseases that laid the basis for drug development that act in such sites or conditions of the physiological status[19].

Delivery carriers:

These are the substances used to deliver the drug, in which the drug molecule gets entrapped/linked to its polymer chains. Nanotubes of carbon molecules are the effectual carriers of the drug because they get easily internalized by mammalian cells and acts as the room for drugs until they reach the target cell. They are less preferable owing to its toxic effects[20].

Strategies:

Polymer type nanoparticles:

One of the methods for preparing the NP is by using the polymers of concerned physicochemical properties. The extensively used polymers as nanocarriers are polyethylene glycol (PEG), dextran, hydroxypropyl methyl acrylamide (HPMA)[21-23]. Various types of polymers can be used for preparing the NP that offers a targeted drug delivery especially in the case of cancer conditions. They not only meant for site targeting but also provides an enhanced and retention type of effect. During the time of designing, there is a need to incorporate a molecule that can be identified and acted by the respective enzyme to satisfy enzyme-based drug release[24]. Moreover, the polymer should have its properties, non-toxic, no influence on drug action and a chemical modification can be done[25]. This technique of combining healing and diagnostic drugs within a single nanoscale "theranostic" offers significant potential for personalized nanomedicine to cancer patients[26]. Theranostic type of NP employed in various conditions including cancer treatment, diabetes[27], neural disorders[28], cardiovascular[29], inflammatory or autoimmune disease[30] and pulmonary[31] afflictions.

Over 10 years, several types of NP have been established for imaging and treating cancer[32], based on organic and inorganic materials including quantum dots, carbon nanotubes, gold, silica, iron oxide, and polymers. NP approach for enzymatic drug delivery was shown in figure 1.

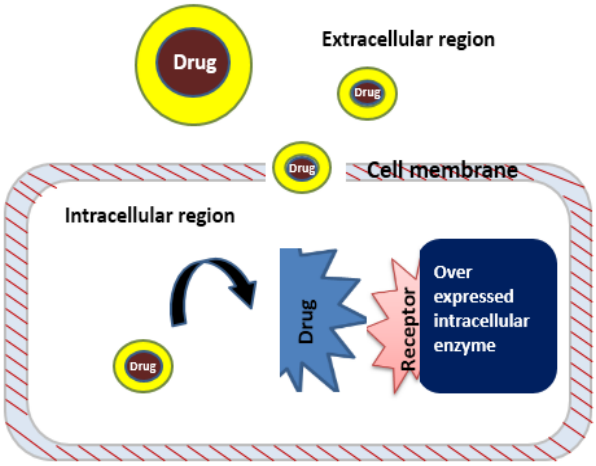

Fig 1: Nanoparticulate approach for enzymatic drug delivery

Liposomes:

Liposomes are the combination of phospholipids in water, by which many types of drugs can be delivered and offers the most efficient targeting of drugs to the particular site[33]. The drugs need to contain both lipophilicity and hydrophilicity for effective absorption as well as dissolution in the biological membrane. It was conversant that the cell membranes of most of the organs covering the lipid layers and followed by the hydrophilic layers due to which the permeability of drugs get limited and accounting a lot of difficulties to permeate into the membrane. This can be easily overcome by drug delivery through liposomes in which both hydrophilic and lipophilic drugs can be incorporated and delivered[34]. Due to their bio-compatibility and feasibility, liposomes are used in drug delivery[35].

Mesoporous Silica Nanoparticles:

They have a variety of applications in the delivery of drugs due to their large surface area, pore volume and high stability[36]. An organo-silane precursor was used for their preparation and the drug is incorporated through the pores. Along with these, the enzyme responsive materials should also be present which include lipase responsive, hydrolase responsive, and protease responsive NP etc.[37]

Another type of nanocarriers is clay nanotubes where the drug gets encumbered into the lumen of tubule and also provides surface modification[38]. An example of clay nanotubes is halloysite which is an aluminosilicate clay tubule having its external diameter of 50–60nm. At a neutral pH, the surface of silicon dioxide has a negative charge and the aluminium oxide inner lumen has positive charge[39]. It has good bio-compatibility. Another example of the polymer used for surface modification is PEG and thereby increase the aqueous solubility. Another type of polymer which is in the clinical study at present is HPMA (n- hydroxypropylmethacrylamide)[40]

Microspheres:

Here the drug is initially dissolved in a suitable medium to form the drug reservoir and can be enclosed physically as microspheres or by chemical means which includes the using of biopolymers like albumins or polypeptides to which the drug is bounded to their polymer chains. The main process involved in the hydrolysis of biopolymers by the characteristic type of enzyme present in the target site. Here, is an example that albumin microspheres of 5-Fluorouracil can be made targeted drug delivery to the cancer cells based on the enzyme called protease present in the cancer cells, that act specifically on the proteins like albumin. In this system, the degradation of albumin microspheres occurs by the action of the protease enzyme[41]. Representation of the mechanism of EADDS is represented in figure 2.

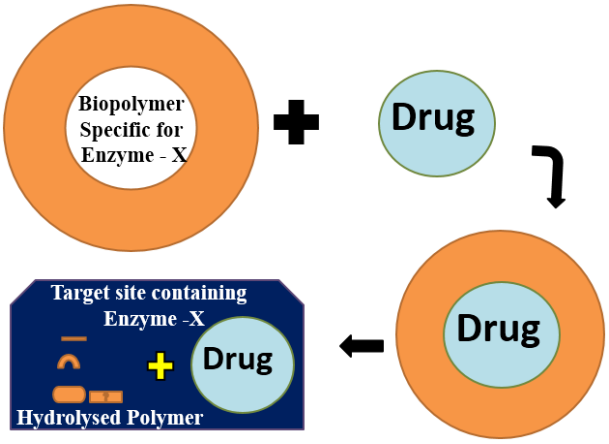

Fig. 2. Representation of mechanism of Enzyme activated drug delivery system

Prodrug:

Another type of well-known strategy is the prodrug mechanism, which is described as the inactive form of drug molecules. Upon the action of certain enzymes, chemical or environment stimuli, prodrugs transform to discharge of the active parent drug *in-vivo*[42]. With this approach, the pharmaceutical properties. It includes the chemical conjugation of the drug with a suitable moiety, can be described as the prodrug**.** Because of these significant aspects, the prodrug approach has been in practice in developing various novel formulations. Approximately 5-7% of currently approved drugs as a prodrug. The designing of prodrug should be such that the pro moiety should get removed after the respective enzymatic action. It provides an idea that the prodrug gets transformed into its active form only when the enzyme of interest, having the pro moiety as substrate, cleaves it. So, the drug will be released at a specific site where the enzyme is in overexpressed condition. The Prodrug approach for EADDS was illustrated in figure 3.

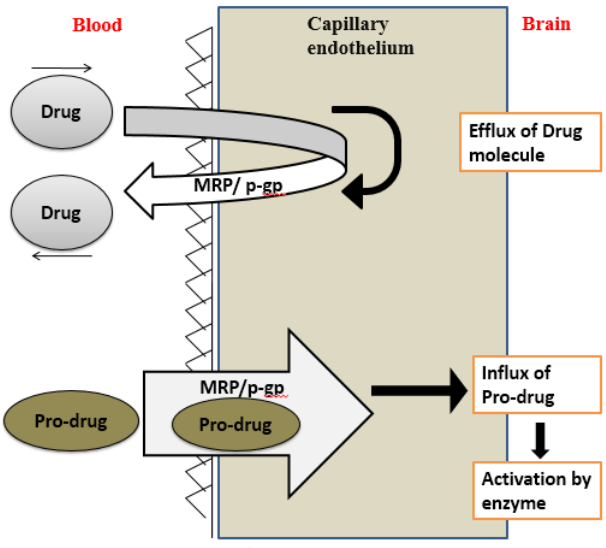

Fig. 3. Pro-drug approach for Enzyme activated DDS

Co-drug:

Conjugating the active moiety with those drugs that will inhibit the functioning of certain efflux transporters will improve the drug absorption into the target site or cell with overexpressed enzyme functioning. One of such examples is conjugating the anti-neoplastic drug-like Doxorubicin with the drugs quinidine, verapamil that inhibit the efflux transporters like p-gp or MRP will promote the cellular uptake of doxorubicin by temporary inactivation of efflux transporter.

Preparation:

Initially, the drug was loaded into the lumen of carrier nanotube with the help of vacuum suction technique[43]. These drug-loaded carrier nanotubes were then coated with the polymer-like dextrin for achieving the enzymatic response and surface functionalization as well as clogging at the tube end. The drug may be loaded by mixing with the solvents like ethanol and this liquid state drug was further conditioned to take a dry powder form. The drugs can be loaded into carrier nanotube as concentrated solutions or by melting. The loaded nanotubes are dried and were kept for a long period, the discharge of drug occurs in around 10 h when exposed to water. Examples of the drugs that achieved prominent results through the EADDS are dexamethasone, gentamycin, tetracycline, ciprofloxacin, and brilliant green. The coating with polymeric material ceases the tube ends and controls the rate of drug release for prolonged period[44].

CONCLUSION:

A study on these works revealed that enzyme activated drug delivery offers the targeted release in the controlled manner. When compared to the uncoated tubes, those coated with the stoppers will delay the release of drugs and thus allows for enhanced delivery of the drug. This technique is very easy when compared to the covalent linkage of nanotubes**.** Hence, this type of system can have its importance in the treatment of various diseases especially cancer to which targeted drug delivery is required to prevent the unwanted side effects associated with traditional chemotherapy. It provides a clear idea that the drug gets released only when the enzyme of interest, having the respective substrate, cleaves it. So, the drug will be released at a specific site where the enzyme is in overexpressed condition.

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CONFLICT OF INTEREST:

The authors declare no conflict of interest.

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REVIEW ARTICLE

Algae- Pollution Indicator and Control

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ABSTRACT:
Pollution is something that causes harmful effect on humans, animals, marine organisms and many factors are involved in the control of pollution. Interestingly, algae involves in the control of pollution. Algae are mostly aquatic and found in marine habitat. They are classified on basis of pigmentation such as green algae, brown algae, yellow-green algae, euglenophyta, golden brown, red algae and fire algae. It plays an important role in stabilizing carbon-di-oxide level and used in food supplements. Their mode of nutrition is an autotrophic and has the ability to perform photosynthesis. Algae contains an extensive bioactive compounds like dietary fiber, carotenoids, proteins, auxins, cytokinins. Colonies of algae which grow out of control causes an algal bloom. Some bloom produce toxin effects on fish, marine mammals. In certain cases some algae like aphanizomenon, microcystis acts as water pollutant.

KEYWORDS: Micro algae, Bioactivities, Pollution control, Pollution indicator, Water pollutant.

INTRODUCTION:
The idea of wastewater treatment utilizing microalgae was proposed by Oswald in the 1950 and the idea was later extended to propose utilization of this framework for vitality creation through reaping and usage of algal biomass[1,2,3]. The advancement in marine biotechnology has their own mark on producing natural marine products. The bioactivity includes pharmocological and biochemical studies[4]. Cyanobacteria consists of many features and metabolism produces nitrogenous compounds. They differ morphologically and they are conical, unicellular in shapes[5]. Mass culture of algae in squander water can fundamentally add to the administration of freshwater biological systems by giving an all the more ecologically solid way to deal with decreasing the eutrophication. Consolidating algal frameworks into regular wastewater treatment can possibly improve the water nature of the profluent by diminishing the supplement and metal burdens into freshwater biological systems[6].

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Metals are brought into the biological systems because of enduring of soil and shakes, from volcanic emissions and from an assortment of human exercises including mining, handling and utilization of metals and additionally substances containing metal contaminants[7]. Industrial discharge of waste has prompted high metal fixations on the earth, which are all in all 100—1000 overlap higher than those in the Earth's outside layer, and locally, living creatures can be presented to considerably more significant levels[8]. Mercury is a component and it was constantly present in the earth, worldwide human movement has prompted a critical increment of mercury discharged into the climate, oceanic condition and land[9]. Ongoing investigations indicated that suitable techniques for algal determination and development are utilized, it is conceivable to utilize microalgae to create the O_2 required by acclimatized microscopic organisms to biodegrade dangerous poisons, for example, polycyclic sweet-smelling hydrocarbons, phenolics, and natural solvents[10]. Microalgae have been demonstrated to be productive in expelling dangerous metals, phosphorus and nitrogen from a wide assortment of wastewaters[11,12,13]. In algal wastewater treatment, the subsequent slop with algal biomass is vitality rich which can be additionally prepared to make biofuel or other important items[14]. This incorporated wastewater treatment and biofuel generation would thus be able to profit the environment. There are studies of algal

development in modern wastewaters and agriculture[15,16,17]. Anabaena was the most lethal and non-harmful blossoms and it was available in every neurotoxic sample. Measurable affiliations were found among hepatotoxicity and incidence of *Microcystis aeruginosa*, *M. wesenbergii*, Anabaena spiroides, *M. viridis* and *Anabaena flos-aquae*[18]. Development in the human populace, contamination, overexploitation of land and absence of freshwater will support utilization of seaweeds[19]. Algae acts as sustainable power source. This review paper will increment natural consciousness of issues encompassing the creation of algal growth and will help the algal growth industry create to its maximum capacity[20].

TYPES AND CHARACTERISTICS:

Penostains F-1 isolated from strained species penicillium and originally isolated from enteromorpha intestinalis and the compounds present in it exhibits cytotoxicity towards p388 cells[21]. A brominated ether isolated from *Odonthalia corymbifera* inactivates alpha-glucosidase[22]. Methoxybifurcarenone shows an antibacterial and antifungal activity isolated from specimen *Cystoseira tamariscifolia*[23]. The green alga *Ulvella lactata* acts as a precursor of 2,4,6-tribromophenol and proposal to that of 4-hydroxybenzoic acid[24]. Sansalvamide showed cytotoxicity towards sk mel 2 cell lines, colo205 and inhibition of molluscum contagiosum virus topoisimoerase[25]. Amphidinium sp. contains colopsinols A-C exhibits cytotoxicity in which colopsinols-A inhibits DNA polymerase alpha, beta. Colopsinols consists of three ether rings, sulfate ester, glucoside moiety isolated from marine dinoflagellate[26,27]. Constantinea simplex and farlowia mollis shows antiviral activity which inhibits the herpes simplex virus. These are more active in prophylactic activity not therapeutically[28]. Mixes and concentrates with hostile to HIV movement are additionally dynamic against different retroviruses, for example, herpes simplex infection (HSV), yet the measure of antiviral action fluctuates with the compound and the infection[29]. Cell reinforcement and anticancer impact of fucoidan confined from dark colored ocean growth Sargassum polycystum was examined cancer prevention agent properties were controlled by DPPH rummaging the outcomes exhibited that the segregated fucoidan from S. polycystum had powerful cell reinforcement and anticancer properties[30].

POLLUTION INDICATOR AND CONTROL:

The utilization of marker creatures to think about follow metal contamination is inspected, with specific reference to the utilization of macroalgae, bivalve molluscs and teleosts. It is recommended that the macroalgae and bivalve molluscs are the most proficient and solid pointers created to the present time. However, the utilization of marker living beings presents organic factors which are absent in physico-concoction investigations of water or residue[31]. Grouping of zinc, copper, lead, cadmium and mercury were resolved in the darker alga Ascophyllum nodosum from two Norwegian fjords, Trondheimsfjord and Hardangerfjord. In the two zones checked nearby varieties in overwhelming metal fixations in the kelp. The outcomes demonstrate that follow component examination of kelp is a valuable and cheap strategy for assessing the condition of a marine beneficiary with respect to substantial metal sullying[32]. The broad dispersion of a few marine macroalgal animal groups makes them a potential bioindicator device for observing existing degrees of various sorts of marine toxins in the district. Results demonstrate a solid bioaccumulation potential in these species for both overwhelming metals and hydrocarbons[33]. The high take-up of metals in green growth (*Enteromorpha intestinalis* and *Ulva lactuca*) and algal growth (*Dictyota bartayresiana* and *Padina gymnospora*) recommended that these green growths might be utilized as potential biomonitors for overwhelming metal contamination. Three contamination markers Geochemical Index, Enrichment Factor and Contamination Factor were determined to decide the level of metal contamination in the marine coastline and the commitment of anthropogenic impact[34]. An endeavor has been made to utilize the development rate in culture of a portion of the algae for sign of contamination. *Ulva lactua* has a high potential starting here of view due to the simplicity with which it very well may be refined and furthermore as a result of its responses to contamination by sewage[35]. Green algae (Chlamydomonas sp. Chlorella sp.) acts as promising adsorbents for copper and zinc. Immobilization of the algae with sodium alginate made the partition and reuse of the algal globules extremely simple. Immobilization expanded the biosorption contrasted and free cells[36]. Spirulina platensis assume a significant job in the natural destiny of poisonous metals and metalloids with physico-compound and affecting changes among solvent and insoluble stages. Spirulina platensis fast cadmium adsorption rate and made them appropriate for the expulsion of cadmium in wastewater[37]. The take-up of uranium by *C. regularis* was quick, and was not all that influenced by light, temperature, and medications with metabolic inhibitors. It relies on the physical adsorption on the cell surface, however not upon the natural movement, and that uranium in the algal cells was combined with the ligands, which had the option to be effectively subbed with EDTA[38]. Different metals including the substantial metals can be expelled from effluents by bioaccumulation and biosorption by cyanobacteria. A portion of the effluents like molasses-based refinery effluents, may have an unsuitable shading segment

which additionally must be expelled and a portion of the cyanobacteria can help in this procedure[39]. Australian algae (*Ecklonia* and *Durvillaea potatorum*) based biosorbents (ER95Ca and DP95Ca) contains metal expulsion properties which removes heavy metals from water[40].

ADVANTAGES AND LIMITATIONS:

Absorption of heavy metals by microbial cells has been perceived as a potential option in contrast to existing advances for expelling substantial metals of waste waters from industry[41]. The biosorbent is set up by exposing biomass to different procedures like granulation, pre-treatment and immobilization. This innovation out-plays out its antecedents because of its cost viability as well as in being ecofriendly[42]. By utilizing the chlorophyll development of the algae, for instance, it was conceivable to evaluate spectrophotometrically all the nitrogen content in water gathered from water system giving us a thought on eutrophication levels[43]. The decision of algae to be utilized in wastewater treatment is controlled by their power against wastewater and by their productivity to develop in and to take up supplements from waste water[44]. Decrease in ooze Formation. Algae contain over half of oil in its biomass. They give a lot more significant returns of biomass and fuel[45]. The measures of uranium taken up by *C. regularis* additionally quickly diminished with concentration of sodium hydrogencarbonate in the uranium[46]. Both living and dead cells created by microorganisms can be powerful metal gatherers[47]. Mercury contamination in oceanic condition are barometrical statement, urban releases, agrarian material overflow, mining, petroleum derivative use and mechanical releases, consuming of coal, and pharmaceutical generation[48]. Eutrophication is one of a few systems by which poisonous algae growth seem to be expanding in degree and length in numerous areas[49]. Parameters like pH, light, lake profundity, strategies for blending, CO_2 conveyance influence the biomass profitability[50].

FUTURE PERSPECTIVES:

The particular highlights of algal water treatment can be acknowledged as a noteworthy minimal effort options in contrast to complex costly treatment especially for cleansing of metropolitan drinking waters. The numerous parts of metal-microorganism collaborations stay unexploited in biotechnology and further advancement and application is fundamental.

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REVIEW ARTICLE

Hydrogel based Nanosponges drug delivery for topical applications – A updated review

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ABSTRACT:
The invention of nanosponges (NSs) has become a significant step providing solutions for several formulation related problems. Main aim is to prevent overdosing and for control release of drug. Nanosponges are mesh like structures. The size range is below 1µm and highly porous with a three-dimensional network and nanometric cavity. Because of porous nature and small size they can bind poorly-soluble drugs and improve their bioavailability. Nanosponges are novel drug delivery system by avoiding circulation throughout the body. It provide therapeutic amount of drug to the site of action and maintain the desired drug concentration. Both hydrophilic and hydrophobic drugs can fit in to nanosponges. Nanosponges can also be an effective carrier for enzyme, proteins, antibodies and vaccine. These can be loaded to the formulations such as lotions, gel, ointments, cream, powder or liquid were orally and topically can be given.

KEYWORDS: Nanosponges, Prolonged release, Topical Application.

INTRODUCTION:
Nanotechnology is the study and use of structures among 1 nanometer and 100 nanometers in size. Nanotechnology carries different formulations like nanopsonges, nano-erythosomes, nano suspensions, nanoparticles, nanocapsules, nanospheres, nanocrystals, etc.[1]

Nanosponge technology was used for widely for the delivery of drugs for topical administration, parental administration, and oral administration. It is a targeted delivery of drug for extended period of time. The active ingredients such as emollients, fragrances, essential oils, sunscreens have high capacity to get entrapped in nanosponges. These are porous, non-toxic, insoluble in water and organic solvents and stable at high temperatures up to 300° C[2-3]

Hydrogels are hydrophilic polymers which are not soluble in water but in aqueous media it absorbs a large amount of water.

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Hydrogel were established in many fields such as regenerative medicine, as food additives, drug delivery and tissue engineering[4]. Nanosponge incorporated hydrogel were given topically at targeted site the gel which absorbs into the deeper skin and gives cooler sensation at inflammation part[5].

Advantages of nanosponges[6,7]
- It provide extended release condition
- Protected from degradation of drug
- It is non-mutagenic, non-irritating, non-toxic and no side effects
- At the pH from 1 to 11 the formulations are stable
- The formulations are cost effective
- Dosing frequency can be reduced
- Better patient compliance.
- Act as a absorbent and remove toxic and venom substance from the body

Disadvantages of nanosponges[2,8]
- Large molecules cannot encapsulated into nanosponges
- Dose dumping
- May retard the release
- Depend only upon loading capacity

Applications of nanosponges:

Nanosponges have many applications in the pharmaceutical field. Nanosponges can be loaded in to the different locations such as pellets, tablets, suspensions, granules, suspensions, solid dispersions or topical dosage forms.

1) Cancer[9]:

When doctors injected the drug in to the cancer patients are ineffective, these is mainly because of the either they can't get to the tumor site or they are attacked by the immune system. These can be overcome by the use of nanosponge to certain extent. Paclitaxel was one of the important drugs formulated as nanosponges. The researchers conducted study in the two different tumor types in animals like slow-growing human breast cancer and fast-acting mouse glioma. In the both cases they got the result that delivery of drug through the nanosponges increased the death of cancer cells and delayed tumor growth.

2) Delivery of protein[10]:

Bovine serum albumin (BSA) was used as a model protein to study the encapsulating capacity of β-cyclodextrin-based nanosponges. Bovine serum albumin (BSA) protein solution is stored in lyophilized form due to unstable form. The major drawback for the formulation and development of protein is maintains its native form and long-term storage. Stability of the proteins can be increased by nanosponges and also have the ability for controlled delivery, immobilization of enzyme, stabilization and enzyme, encapsulation of protein.

3) For treatment of drug delivery[10,11]:

Nanosponges are spherical shape and have nanomeric in size so they are used as carriers for water-insoluble drugs. BCS Class II drug having dissolution rate limited bioavailability and these is used to mask the unpleasant flavors, to increase the dissolution rate, solubility and stability. From the inclusion complex the highest solubility and in vitro drug release was observed were prepared from nanosponges and $NaHCO_3$.

4) For delivery of gases[12,13]:

For the treatment and diagnostic purposes gas plays an important role in medicine. Due to the inflammation of cancer there is a deficiency of oxygen supply called hypoxia. Cavalli et al who developed the nanosponge formulation for deliver oxygen at the topical application. Nanosponges have the ability to store and releases oxygen slowly over time.

5) Role of nanosponges for treatment of poison in blood[10,14]:

Instead of using antidotes, nanosponges which have the ability to absorb the toxins from the blood. Nanosponges are delivers to the blood by the injection and they are look like red blood cells. Each nanosponge absorb toxin molecules depends on the number of the toxin molecules.

6) Enzyme immobilization of nanosponges[14,15]:

For lipases enzyme immobilization is particularly relevant to progress their stability and modulates properties in the case of reaction rates and enantio selectivity. As a result, the demand for new solid chains, proper for this family of enzymes is continuously growing. For this Boscolo et al., statement is Pseudomonas fluorescents lipase have high catalytic performances on a new form of cyclodextrin-based nanosponges.

Factors considering for nanosponge development[13]:

a) Polymer: It affects the pre-formulation of nanosponges and influences its formulation.

b) Temperature: Increase the temperature decrease the stability of nanosponges by reduction of hydrophobic forces and Van der Waal forces of drug/nanosponges

c) Degree of substitution: The ability of complexation of the nanosponges was affect by the number, position, and type of the substituent of the parent molecule.

d) Method of preparations: Complexation of drug and the nanosponges may cause a change by method of drug loading into the nanosponges; here it can be succeed by the nature or the characteristics of the drug and polymer. Freeze drying was found to be most effective for drug complexation in many cases

Structure of nanosponges:

Nanosponges are nano-sized porous light weight polymeric delivery system with more or less spherical in nature. The cavities of the framework have a tunable polarity. The nanosponges can encapsulates the drug in its core. These are three-dimensional networks of polyester which are degrades naturally. Once the network of polyester breaks down it release the drug molecules. [16-17]

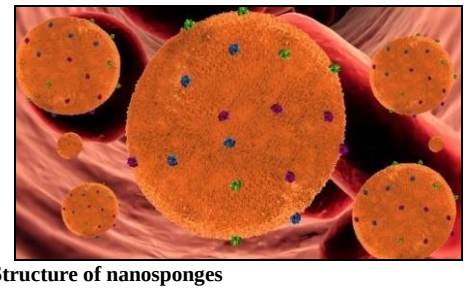

Fig 1: Structure of nanosponges

Formulation aspects of nanosponges,[18]:

A. The drug: Selection of suitable drug should be based on following characteristics. Conventional single dose of drug should be low, drugs have biological half life between 2 – 8 hours.

B. Polymer: The main step on the development of the nanosponges is selection of appropriate polymers. The wild used polymers are ethyl cellulose, poly venyl

alcohol, Eudragit, Chitosan, Poly (Valerolactoneallylvalerolactone), Alkyloxycarbonyl Cyclodextrins, 2-hydropropyl β-cyclodextrine, Hyper cross linked Polystyrene.

C. Cross linking agent: cross linking agent is used for rigidisation of polymer they are Diarylcarbonates, Carbonyldiimidazoles, Dichloromethane, Disocyanates, Carboxylic acid dianhydrides

D. Stabilizing agent: Poly Venyl Alcohol, PVP

Methods of nanosponge prepration:

Nanosponges can be prepared by following methods

- **Quassi emulsion solvent diffusion method**[8,10]**:**

In this method both the organic and aqueous phases are using for the preparation of the nanosponges. Different proportions of organic and aqueous phases are taking. Polyvinyl alcohol is used as the aqueous phase and organic phase include drug and polymer. The drug and polymer was dissolved in the particular organic solvent then this phase slowly added to aqueous phases containing polyvinyl alcohol. The above mixture stirred for two or more hours at 1000 rpm. Then by the filtration the nanosponges was collected, washed, dried and stored in the vacuum desiccators.

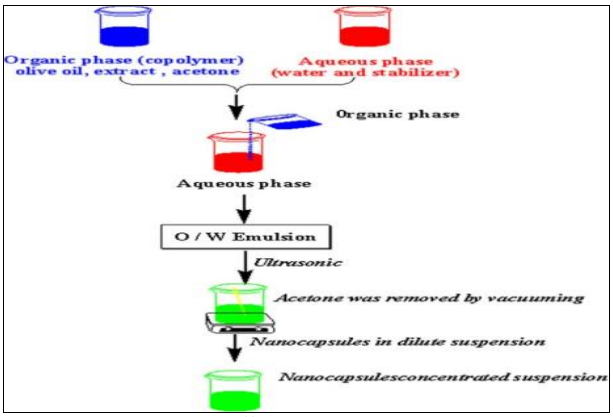

Fig 2: Emulsion solvent diffusion method

- **Ultrasound-assisted synthesis**[11]

In this method, by the reaction between the polymers with the cross-linkers in the absence of solvent and under the sonication the nanosponges were preparing. Spherical and uniform in the sized nanosponges are obtained by this method which is smaller than 5 microns. In this method cross-linkers are di-phenyl carbonate or pyromellitic anhydride are mixed with polymer at particular molar ratio in the flask. This flask containing mixture is heated to 90°C in ultrasound bath which was filled with water, after this mixture was cooled. To remove the excess of non-reacted polymer the mixture is washed with water. The mixture is purified by prolonged Soxhlet extraction using ethanol.

- **Solvent method**[9]**:**

In this method a polar solution of polymer was added to the excess quantity of crosslinker, here the temperature should maintain at the 10°C for 48 hours. Then the mixture cool down to room temperature and add the excess quantity of water. By the addition of water nanosponges were developed, then it collected by the filteration under vaccum. The mixture is purified by prolonged Soxhlet extraction using ethanol.

- **Hyper cross-linked method**[20]**:**

In a round bottom flask 100 ml of anhydrous DMF was taken and to this add 17.42 g of anhydrous β-cyclodextrin then stirred for complete dissolution. To this mixture add 9.96 g of carbonyldiimidazole then kept the solution for reaction at 100◦C for 4hours. After the condensation polymerization is over, a hyper-cross-linked cyclodextrin is developed in RBF. To remove excess of DMF an excess of deionized water should added to above mixture. Finally, unreacted reagents were completely eliminates by Soxhlet extraction with ethanol.

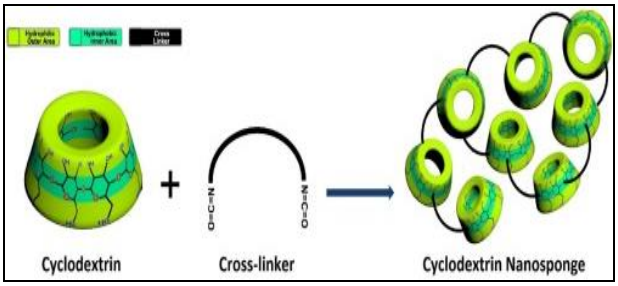

Fig 3: Cyclodextrin nanosponges

Evaluation of nanosponges[21, 22]**:**

- **Solubility studies:** Solubility and bioavailability of the drug can determine by the technique is inclusion complexes.
- **Microscopic study:** Scanning electron microscopy (SEM) and transmission electron microscopy (TEM)
- **Particle size and poly-dispersity:** Particles size (90Plus particle size determining software), Size distribution profile of nanoparticles (Dynamic light scattering (DLS))
- **To determine the formation of a complex between drug and nanosponges:** Thin layer chromatography (TLC)
- **The interaction between nanosponges and the drug in the solid state:** Infrared spectroscopy
- **Loading efficiency:** UV spectrophotometer
- **Stability of the colloidal dispersion and the difference of potential between two layers (dispersion medium and immobile layer):** Zeta potential determination
- **Extent of interaction between and drug molecule:** Single crystal X-ray structure analysis nanosponge
- **To measure surface charge:** Laser doppler

anemometry, Zeta potential meter

Mechanism of drug release[15]:

The nanosponges are open structure and drug had the ability to move out of the particles into the vehicle until equilibrium is reached. In topical delivery, once the product containing nanosponge with drug is applied to the skin, the drug which is already present in the vehicle will be absorbed into the skin. These will decrease the concentration of drug in the vehicle and reaches to the equilibrium. At the time flow of the drug from the sponge particle into the vehicle will starts into the skin and vehicle is either dried or absorbed. This will provides prolonged release of drug to the stratum corneum of the skin.

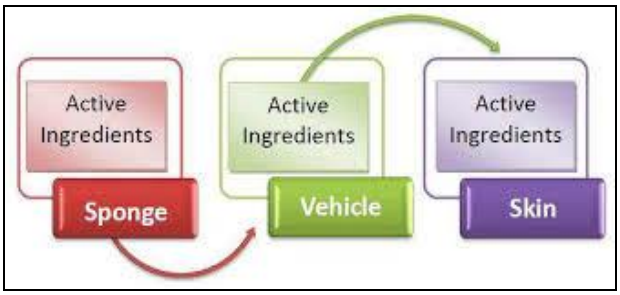

Fig 4: Mechanism of drug release

Table 1: Examples of nanosponges currently under research [8, 23]

Drug	Nanosponge vehicle	Indication
Camptothecin	β-cyclodextrin	Cancer
Paclitaxel	β -cyclodextrin	Cancer
Econazole nitrate	Ethyl cellulose Polyvinyl alcohol	Fungal infections
Dexamethasone	β -cyclodextrin	Brain tumors
Tamoxifen	β -cyclodextrin	Breast cancer
Temozolamide	β-cyclodextrin Poly (valerolactoneallyl-valerolactone)	Inflammation Breast cancer Cardiovascular disease
Antisense oligonucleotide	Sodium alginate Poly L-lysine	Cancer therapy

CONCLUSION:

Nanosponges can be formulated into different dosage forms they are parenterals, aerosols, topical, capsules and tablets due to its size and shapes. For effective drug delivery the drug has to reaches target site instead of circulating through the body. This can be possible when the drug is converted in to topical dosage forms. Hence, this stated that nanosponge drug delivery systems ideal platform form for topical use and prolonged delivery systems.

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REVIEW ARTICLE

A review on the total synthesis of (-)-Swainsonine natural product (2005-2020)

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ABSTRACT:
This review aims to cover the literature published methodologies of the total synthesis of the (-)-swainsonine alkaloid natural product that has been reported between the years 2005 and 2020. (-)-Swainsonine has been widely studied for many important biological activities and scientifically reported to possess such as anticancer, antimetastatic, antitumor, antiviral and anti-HIV.

KEYWORDS: (-)-Swainsonine, Indolizidine alkaloids, Anticancer activities.

INTRODUCTION:

Alkaloid compounds have been found to possess a broad range of biological and pharmaceutical activities.[1-10] In recent years, polyhydroxylated indolizidine alkaloids have been found to possess a broad range of biological activities such as immunoregulatory activity, anti-HIV activity, antiviral, antitumor and anticancer activity.[11-19] This is due to the structure of these compounds, which are stereochemically rich (Figure 1).

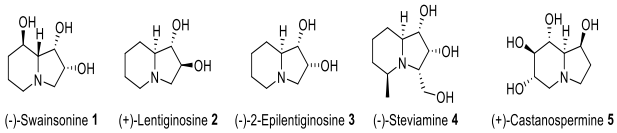

Figure 1: Some polyhydroxylated indolizidine alkaloids

Swainsonine is an example of a polyhydroxylated indolizidine alkaloid containing a sp^3 hybridised nitrogen atom and three hydroxyl groups. It has four stereogenic centres at C-1, C-2, C-8 and C-9, and therefore could exist as one of sixteen stereoisomers. (-)-Swainsonine **1** is a natural product, whereas (+)-swainsonine **6** is a non-natural product (Figure 2).

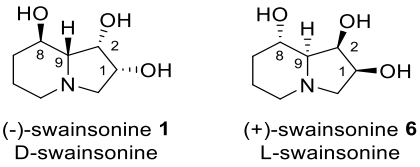

Figure 2: Structure of (-)-Swainsonine 1 and (+)-Swainsonine 6

(-)-Swainsonine **1** is a sugar analogue, the first isolation was in 1973 by Guengerich, DiMari and Broquist from the fungus plant pathogen *Rhizoctonia leguminicolain*,[20] then from the legume *Swainsona canescens* (Australian plant),[21,22] and from fungus *Metarhizium anisopliae* F-3622.[23,24] In addition, it was found in North American plants of genera *Astragalus* and *Oxytropis* (commonly called locoweed).[25,26]

(-)-Swainsonine **1** was found to be as a potent inhibitor of Golgi enzyme mannosidase II, which is an imported enzyme for the synthesis of glycoproteins *via N*-linked oligosaccharides.[27-29] Compound **1** is also a potent inhibitor of lysosomal α-D-mannosidase,[30,31] which lead to the accumulation of oligomannoside chains in cells exposed to the drug.[32-34] In addition, the natural product **1** has been found to possess a broad range of biological activities such as immunomodulatory,[23,35,36] antiviral activities,[37] antimetastatic,[38] immunoregulating,[39,40] antitumor-proliferative,[41,42] anticancer activities.[43,44]

Skelton and White in 1980,[45] have been deduced the relative stereochemistry of (-)-swainsonine **1** by X-ray crystallography. While, the absolute configuration of **1** was determined by Harris and co-workers in 1982 depend on the biosynthetic (also called anabolism),[46] two-dimensional nuclear magnetic resonance

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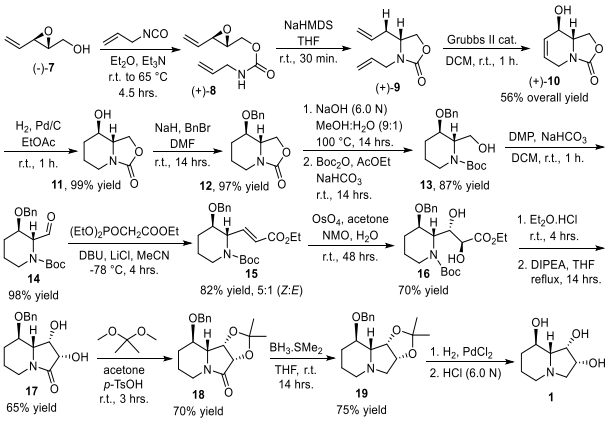
spectroscopy (2D NMR)[47] and asymmetric induction studies.[48] The analogues of (-)-swainsonine **1** have been used as biochemical tools and chemotherapeutic agents against cancer,[49] HIV[50] and diabetes.[51] Therefore, these interesting biological activities led to achieve many studies toward the total synthesis of (-)-swainsonine **1** and its analogues.

Total synthesis of (-)-swainsonine 1:

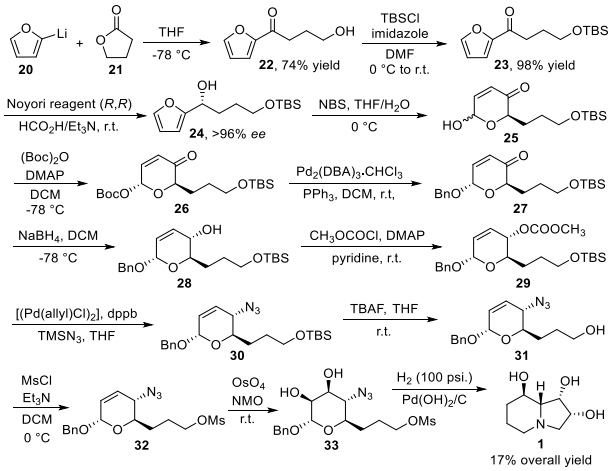
El Nemr[52] has reported a review including the synthetic methods of the (-)-swainsonine **1** and its analogues since its first total synthesis until the year 2000. Following this, Pyne reported another review, which covered the literature up to 2005.[53] Whereas, in this review, it will summarise all the total syntheses of the (-)-swainsonine **1** between 2005 and 2020 years.

Riera and co-workers,[54] reported an efficient total synthesis of (-)-swainsonine **1** enantioselectively. Their methodology was based on the use of enantiomerically pure epoxy alcohol (-)-**7**, which was prepared from (*E*)-2,4-pentadien-1-ol *via* Sharpless epoxidation.[55,56] The product (-)-**7** was used to prepare the key intermediate (+)-**10** in this synthetic route (Scheme 1). Allyl carbamate derivative (+)-**8** was then conveniently obtained *via* treatment of (-)-**7** with allyl isocyanate/Et_3N. Followed by intramolecular ring opening of (+)-**8** using NaHMDS afforded oxazolidinone derivative (+)-**9** as a single isomer.[57,58] Thereafter, the product (+)-**9** was subjected in ring-closing metathesis to provide the desired intermediate (+)-**10** in 56% overall yield from (-)-**7** over three steps. The alkene bond at (+)-**10** was hydrogenated to give **11** in 99% yield, followed by protection of the hydroxyl group with BnBr afforded **12** in 97% yield. Subsequently, hydrolysis of carbamate using basic conditions (NaOH 6.0 N) gave the corresponding amino alcohol, then the free amine was protected with a Boc group in a one-pot reaction to provide **13** in an 87% overall yield. Oxidation of primary alcohol **13** using a Dess-Martin periodinane oxidant gave the corresponding aldehyde **14** in a 98% yield. The desired unsaturated ester *Z*-**15** was then isolated in 82% yield as a major isomer (5:1) when **14** subjected to a still reaction.[59] Next, *syn*-diols derivative **16** was obtained in a good yield (70%) *via* *syn*-dihydroxylation of *Z*-**15** using OsO_4/NMO. Deprotection of the Boc protecting group at **16** using acidic conditions (Et_2O.HCl) gave amine derivative, followed by the addition of Hünig's base, which activated the cyclisation to afford indolizin-3-one **17** in 65% yield. Hydroxyl groups at **17** were then protected with 2,2-dimethoxypropane to provide **18** in 70% yield as a single diastereoisomer. Following this, reduction of cyclic amide **18** by $BH_3.SMe_2$ furnished the protected desired natural product **19** in 75% yield. Finally, deprotection of two groups (benzyl and acetonide) was then successfully accomplished in a one-pot reaction by hydrogenation of **19** in the presence of $PdCl_2$ then HCl hydrolysis gave (-)-swainsonine **1** (Scheme 1).



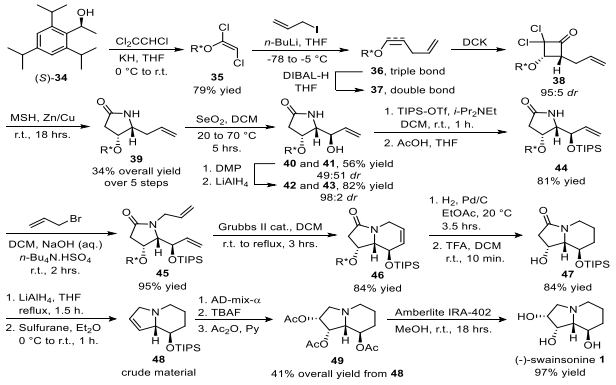
Scheme 1: Enantioselective total synthesis of (-)-swainsonine 1 by Riera and co-workers

In 2006, a new enantioselective synthesis of (-)-swainsonine **1** was achieved successfully by Guo and O'Doherty (Scheme 2).[60] Their approach was based on the reduction of acylfuran **23** enantioselectively to give the intermediate **24** using Noyori conditions.[61] The synthetic route involved 13 steps to obtain the desired (-)-swainsonine **1** starting from furan **20**. The reaction between a solution of 2-lithiofuran **20** and γ-butyrolactone **21** provided furyl ketone **22** in a 74% yield. Protection of hydroxyl group at **22** was then performed using TBSCl/imidazole to afford **23** in 98% yield. Following this, asymmetric reduction of ketone at **23** using Noyori's reagent yielded the corresponding furyl alcohol **24** with an excellent *ee* (>96%). Achmatowicz conditions (NBS in THF/H_2O) were then applied on **24** to give pyranone derivative **25**. Thereafter, compound **25** followed by nine consecutive steps to afford the desired natural product **1**in an overall yield of 17% (Scheme 2).[60]



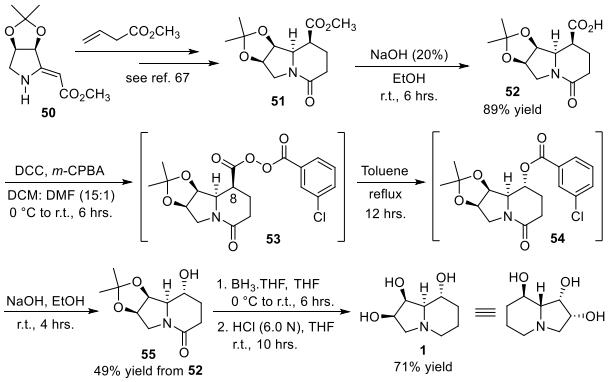
Scheme 2: Synthesis of natural product (-)-Swainsonine 1 by Guo and O'Doherty

In the same year, Ceccon, Greene and Poisson, reported a stereoselective total synthesis of (-)-swainsonine **1**.[62] Their synthetic route involved the preparation of **38** as a key intermediate *via* asymmetric [2+2] cycloaddition reaction (Scheme 3). The potassium alkoxide analogue of (*S*)-1-(2,4,6-triisopropylphenyl) ethanol **34** was treated with trichloroethylene to furnish the corresponding enol ether derivative **35** in a good yield (79%). Ynol ether acetylide **36** was then prepared by reaction between **35** and *n*-BuLi, which was then directly reacted with allyl iodide in a one-pot reaction, which gave ynol ether **36**. Thereafter, selective reduction of the alkyne bond at **36** using DIBAL-H yielded enol ether **37**, which was used in the asymmetric [2+2] cycloaddition with dichloroketene without further purification.[63] This afforded the key intermediate **38** in 95:5 *dr*, which was followed by a five sequence steps to give pyrrolidinone derivative **39** in an overall yield of 34%. The product **39** was then used to prepare **40** *via* allylic oxidation, but a mixture of two alcohols **40** and **41** were obtained in 49:51 *dr* with 56% yield. To improve the *dr* ratio, oxidation of alcohols **40** and **41** with a Dess-Martin reagent afforded enone forms, followed by reduction with $LiAlH_4$ in a one-pot reaction provided **42** and **43** in an excellent *dr* (92:8) with 82% yield. Disilylation of **42** then selective hydrolysis of the silyl imidate in a one-pot reactiongave **44** in 81% yield. Treatment of **44** with allyl bromide provided *N*-allyl derivative **45** in an excellent yield (95%). Subsequently, ring closure of **45** *via* cross metathesis reaction afforded the corresponding alkene **46** in 84% yield. Hydrogenation of the alkene at **46** gave the saturated analogue of **46**, which was then followed by removal of the chiral auxiliary using TFA to give **47** in a very good yield (84%). Reduction and dehydration on **47** were then applied, which furnished the crude material **48**. Following this, a one-pot sequence of dihydroxylation, desilylation then triacylation afforded triacetate derivative **49** in an overall yield of 41% from **48**. Finally, hydrolysis of the acetyl groups yielded the desired natural product **1** in an excellent yield (97%) (Scheme 3).



Scheme 3: Ceccon, Greene and Poisson's stereoselective total synthesis of (-)-swainsonine 1

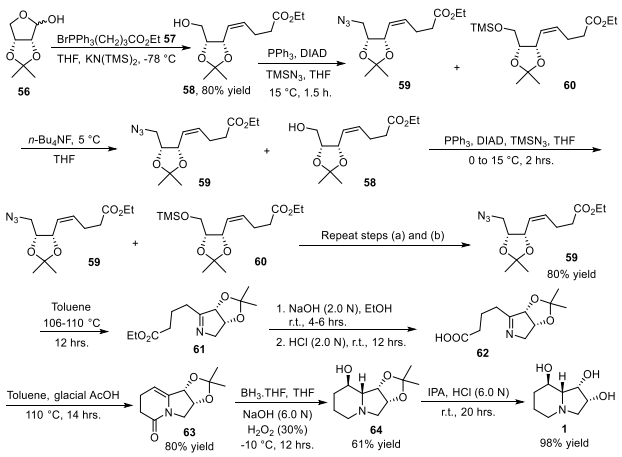
Two years later, a novel and facile stereoselective synthesis of (-)-swainsonine **1** has been reported by Shi *et al*.[64] This was done over five sequence steps from a chiral heterocyclic enaminoester intermediate **50** (Scheme 4). Compound **50** was synthesised according to literature procedure over six steps starting from D-erythronic acid γ-lactone.[65,66] The product **51** was prepared by reaction between **50** and methyl acrylate using a literature method.[67] The conversion of **51** to the corresponding carboxylic acid **52** was achieved successfully using aqueous NaOH (20%). Thereafter, treatment of **52** with *m*-chloroperbenzoic acid and dicyclohexylcarbodiimide afforded a diacyl peroxide intermediate **53**, which was then directly heated to reflux in toluene to give the intermediate **54**. This was followed by hydrolysis of the ester to the desired product **55** in 49% yield from **52**, with the desired stereochemistry at C-8. The carbonyl group at **55** was then reduced with BH_3.THF, followed by deprotection of acetonide group in a one-pot reaction using acidic conditions (HCl 6.0 N, THF), which gave the desired (-)-swainsonine **1** in a good yield (71% yield) (Scheme 4).



Scheme 4: Shi *et al*. Stereoselective total synthesis of (-)-swainsonine 1

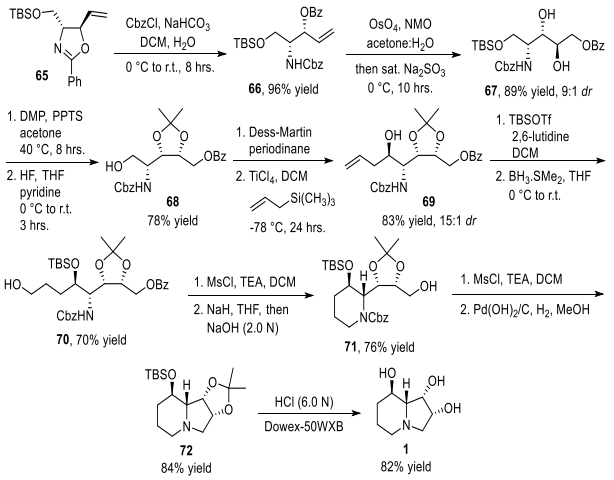
In the same year, Sharma, Shah and Carver,[68] reported a scale-up synthesis of (-) swainsonine **1** over 12 steps starting from lactol **56** (Scheme 5). Treatment of **56** with 3-(carbethoxypropyl) triphenylphosphonium bromide **57** *via* Wittig reaction afforded **58** in a very good yield (80%). Mitsunobu conditions were applied on **58** gave a mixture of two products; desired product **59** and undesired product **60**. A solution of *n*-Bu_4NF in THF was then added to remove the TMS protecting group at **60**. This gave a mixture of **58** and **59**, which was subjected to Mitsunobu conditions using 0.5 equivalents of the reagent to provide a mixture of **59** and **60**. The aim of using these conditions again is to improve the yield of **59**. Mitsunobu conditions were used for a third time on **59** and **60**, followed by adding an extra amount of *n*-Bu_4NF to access the desired product **59** in 80% yield and high purity (99%, determined by HPLC). The product **59** was then heated in toluene to yield **61**, which

was used in the next step without further purification. Hydrolysis of the ester group at **61** under basic conditions (NaOH 2.0 N, EtOH) yielded the corresponding acid **62**. Thereafter, treatment of **62** with glacial AcOH furnished **63** in a very good yield (80%). Compound **63** was treated with borane/THF, followed by the addition of solutions of NaOH (6.0 N) and H_2O_2 (30%), which provided **64** in 61% yield. The desired (-)-swainsonine **1** was then prepared by treatment of **64** with IPA, which afforded 98% yield of the desired product **1** (Scheme 5).



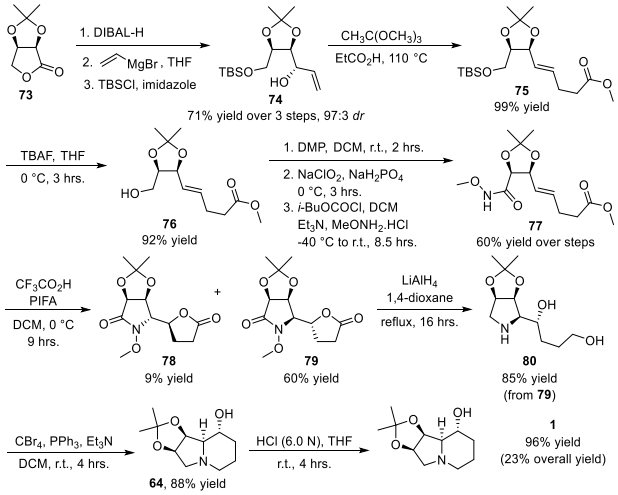
Scheme 5: A scale-up synthesis of (-)-swainsonine 1 by Sharma, Shah and Carver

Ham and co-workers,[69] reported an asymmetric synthesis of (-)-swainsonine **1** using a chiral *trans*-oxazoline **65**, which was prepared from D-serine according to the literature method.[70] The reaction between **65** and benzyl chloroformate provided a carbamate derivative **66** in an excellent yield (96%) (Scheme 6). Dihydroxylation of the terminal alkene of **66** was then performed stereo selectively using OsO_4 to give the desired *anti*-diol **67** in 89% yield with 9:1 *dr*. The product **67** was employed to synthesise **68** in 78% yield over two steps. The hydroxyl group at **68** was then oxidised with a Dess-Martin reagent to provide the corresponding aldehyde, followed by nucleophilic addition of allyltrimethylsilane in a one-pot reaction to provide *anti*-amino alcohol **69** in a very good yield (83%) with 15:1 *dr*. Thereafter, the product **70** was prepared from **69** in 70% yield over two steps. The hydroxyl group at **70** was then activated with methanesulfonyl chloride, followed by intramolecular cyclisation and benzoate hydrolysis in a one-pot reaction, which gave **71** in a good yield (76%). Following this, mesylation then hydrogenolysis were applied on **71** to provide protected (-)-swainsonine **72** in 84% yield. Finally, the TBS and acetonide protecting groups at **72** were then removed using acidic conditions (HCl 6.0 N) to afford the natural product **1** in a very good yield (82%) (Scheme 6).

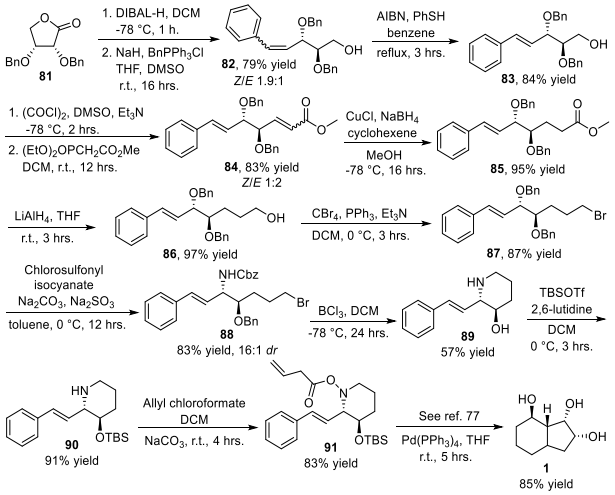


Scheme 6: Asymmetric synthesis of (-)-swainsonine 1 by Ham and co-workers

Two years later, Wardrop and Bowen,[71] conducted a new total synthesis of (-)-swainsonine **1** stereoselectively over 12 steps starting from 2,3-*O*-isopropylidene-D-erythrose **73**, which was prepared from sodium D-isoascorbate *via* oxidative cleavage with H_2O_2 (Scheme 7).[72,73] Reduction, stereoselective allylation and selective protection were then conducted on **73** over three sequence steps to afford allylic alcohol **74** in a high *dr* (97:3) and a good yield (71%). This was followed by a Johnson-Claisen rearrangement of **74**, which gave the ester derivative **75** in an excellent yield (99%) as a single *E*-isomer. Hence, deprotection of the TBS group using TBAF gave the desired alcohol **76** in 92% yield. Thereafter, double oxidation on **76** with a Dess-Martin periodinane and Pinnick conditions gave the corresponding acid, followed by treatment with isobutyl chloroformate in a one-pot reaction to afford isobutyl chloroformate **77** in a 60% yield. Cyclisation of **77** was then achieved successfully when **77** was treated with phenyliodine(III)bis(trifluoroacetate) in the presence of trifluoroacetic acid as a catalyst provided a mixture of **78** and **79** (about 6:1) in 69% yield. The desired product **79** was isolated in 60% yield from this mixture by flash column chromatography, followed by reduction of the three functional groups at **79** with $LiAlH_4$ to give the desired product **80** in a very good yield (85%). Appel conditions were then applied on **80**, which gave indolizidine derivative **64** in 88% yield. Finally, the desired (-)-swainsonine **1** was obtained in 96% yield when **64** was treated with HCl (6.0 N) (Scheme 7).



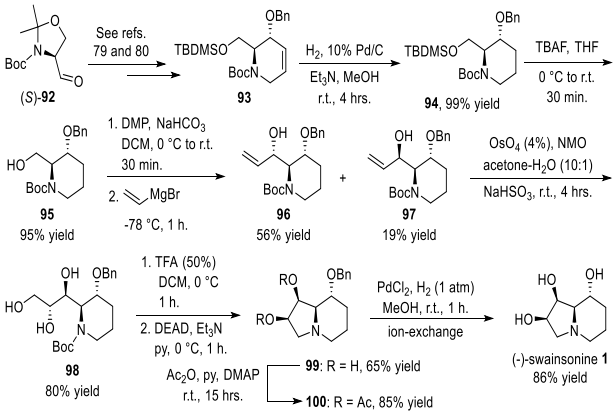
Scheme 7: Wardrop and Bowen's total synthesis of (-)-swainsonine 1 stereoselectively



Scheme 8: Formal synthesis of natural product (-)-swainsonine 1 by Li *et al*.

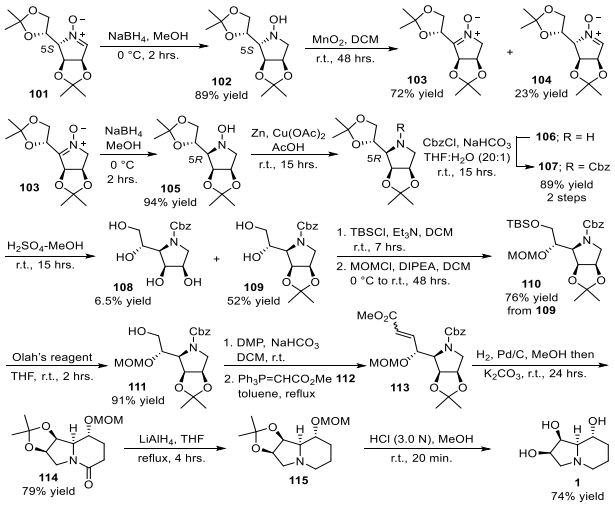
In 2013, Li *et al.*[74] reported a facile diastereoselective formal synthesis of (-)-swainsonine **1** using a lactone intermediate **81**, which was synthesised from D-erythronolactone according to the literature procedure.[75] A one-pot sequence of reduction with DIBAL-H then Wittig reaction on **81** gave an olefin derivative **82** in a low ratio of *Z/E*isomers (1.9:1), albeit in a good yield (79%) (Scheme 8). To improve this ratio, the isomerisation of the mixture **82** was then achieved successfully *via* treatment with AIBN and PhSH to provide a single *trans* olefin **83** in an 84% yield. Oxidation of the hydroxyl group at **83** using Swern conditions gave the corresponding aldehyde, followed by Wittig reaction in one-pot reaction, which afforded **84** in a very good yield (83%) and 1:2 of *Z/E*. The alkene bond at α,β-unsaturated ester **84** was then reduced chemoselectively with $NaBH_4$ to give **85** in 95% yield. Following this, reduction of ester group at **85** with $LiAlH_4$ afforded **86** in an excellent yield (97%). Appel conditions were employed on **86**, which provided bromide **87** in 87% yield. The product **88** was then prepared in 16:1 *dr* and 83% yield *via* treatment of **87** with chlorosulfonyl isocyanate using optimised conditions.[76] Thereafter, deprotection of Bn and Cbz groups were performed when **88** reacted with BCl_3, which activates the intramolecular cyclisation to afford **89** in a 57% yield. *O*-Protection on **89** gave **90** in 91% yield, followed by reaction with allyl chloroformate provided **91** in a very good yield (83%). Finally, the desired (-)-swainsonine **1** was obtained according to the literature procedure in an 85% yield (Scheme 8).[77]

After a year, Singh, Manna and Panda,[78] reported a new total synthesis of (-)-swainsonine **1** stereoselectively (Scheme 9). Their approach depended on using **93** as a key material in the synthetic route. The product **93** was prepared easily in some steps from (*S*)-Garner's aldehyde **92**.[79,80] The hydrogenation of the olefin bond at **93** gave saturated form **94** in a 99% yield. Thereafter, deprotection of the TBDMS group at **94** using TBAF afforded the corresponding alcohol **95** in an excellent yield (95%).The product **95** was then oxidised with a Dess-Martin reagent to give the corresponding aldehyde, and a subsequent nucleophilic addition of vinyl magnesium bromide in a one-pot reaction provided a mixture of two products **96** and **97** (*circa* 3:1) in a good yield (75%). The product **97** was then isolated from the mixture using flash column chromatography in about 19% yield. Following this, dihydroxylation of **97** using OsO_4 gave the triol derivative **98** in a very good yield (80%). Removal of the Boc group on **98**, under acidic conditions (TFA/DCM) furnished the desired amino triols, followed by cyclisation under Mitsunobu conditions, which afforded indolizidine derivative **99** in a 65% yield. Diacetylation on **99** was then performed to give **100** in a very good yield (85%). Finally, hydrolysis of acetyl and benzyl groups at **100** using $H_2/PdCl_2$ provided the desired natural product **1** in an 86% {7% overall yield from the intermediate **93** (Scheme 9)}.



Scheme 9: Stereoselective total synthesis of natural product (-)-swainsonine 1 by Singh, Manna and Panda

Another asymmetric synthesis of (-)-swainsonine **1** has been reported by Yu and co-workers,[81] which includes using nitrone **101** as a key intermediate in the synthetic route (Scheme 10). The product **101** was synthesised in a 43% overall yield from D-mannose using literature procedures.[82,83] The epimerisation of stereochemistry of C-5 at **101** was achieved successfully over a sequence of three steps. Reduction of **101** with $NaBH_4$ afforded hydroxyl amine **102** in 89% yield, followed by regioselective oxidation with MnO_2, which gave a mixture of **103** and **104** (around 3:1) in an excellent yield (95%). The desired regioisomer **103** was then reduced with $NaBH_4$ to provide *N*-hydroxypyrrolidine derivative **105** as a single diastereoisomer in 94% yield. Following this, reduction of **105** with $Zn/Cu(OAc)_2$ gave an amine derivative **106**, which was then directly protected with CbzCl in a one-pot reaction to afford **107** in a very good yield (89%). Thereafter, acetonide group at **107** was cleaved using H_2SO_4-MeOH, which provided the desired diol **109** in 52% yield with the undesired tetrahydroxylated pyrrolidine product **108** in only 6.5% yield. The primary hydroxyl group at **109** was protected with TBSCl, followed by protection of the secondary hydroxyl group with MOMCl in a one-pot reaction to furnish **110** in a good yield (76%). Deprotection of TBS group at **110** afforded a primary alcohol **111** in an excellent yield (91%), which was done using Olah's reagent {(pyridinium poly (hydrogen fluoride)}.[84,85] The alcohol **111** was then oxidised with a Dess-Martin reagent to give the corresponding aldehyde, and a subsequent Wittig reaction with methyl 2-(triphenylphosphoranylidene) acetate **112** in a one-pot reaction gave **113**. Hydrogenation and cyclisation of the crude material of **113** provided δ-lactam **114** in a 79% overall yield. Next, reduction of **114** with $LiAlH_4$ afforded the corresponding tertiary amine **115**, which was followed by removal of acetonide and MOM protecting groups using acidic conditions (HCl 3.0 N, MeOH) to give the desired (-)-swainsonine **1** in a good yield (74% from **115**) (Scheme 10).



Scheme 10: Asymmetric synthesis of (-)-swainsonine 1 by Yu and co-workers

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RESEARCH ARTICLE

A Review of Gastro-retentive Drug Delivery Systems for Antidiabetics and its present status

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ABSTRACT:
The present review provides concise information on status of gastro-retentive drug delivery systems for antidiabetic molecules. Present review emphasis on pharmacotherapy of diabetes via gastric retention of drug molecules. The gastro-retentive system is a proven useful tool for sustaining the drug release for the drugs having good absorption through the GIT, drugs with narrow therapeutic index and low dose drugs. The gastro-retentive dosage forms have also been developed for antidiabetic molecules and metformin hydrochloride, the first line drug in the treatment of diabetes is commercially available in the market in the form of sustained release formulations. As oral antidiabetic therapy is required for prolonged duration, the oral hypoglycemic may lead to side effects such as diabetic neuropathy, diabetic myopathy and many more. The primary reason for side effects is under-utilization of the drug molecule which can be improved using gastro-retentive drug delivery systems thereby minimizing the side effects. Despite being one of the most successfully systems, the commercial value gastro-retentive drug delivery systems is still below par. Gastro-retentive drug delivery systems for antidiabetic drug molecules are still awaited. The research is still in progress for gastro-retentive formulations which can attract industry utilizing these systems for humanely causes.

KEYWORDS: Antidiabetics; floating; mucoadhesive; raft forming; drug delivery systems (DDS).

INTRODUCTION:

Oral route of administration has benefits such as low treatment costs and ease of administration, high level of patient conformity and so far remain the preferred route of administration[1]. However drug absorption from oral route is not always uniform due to the physiological factors and gastrointestinal (GIT) system heterogeneity. Moreover, many involuntary variables influence drug uptake throughout the GIT such as variable pH, intestinal flora, gastrointestinal transit time, gastric secretions and absorption surface area[2]. The traditional immediate release drug delivery systems are not able to combat the problems associated with low drug absorption in the gastrointestinal transit as these systems do not possess any additional characteristics to counter stomach motility and thus are not suitable for the drugs which are to be absorbed in the upper part of GIT.

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Incomplete drug deliverance and subsequent reduction in bioavailability are the consequence that can be ascribed for the failure of traditional devices[3]. To overcome these issues, drug delivery systems that can control drug release and the residence time of the drug have been developed. Such systems are designed to reside in the upper GIT for a long period of time during which they regulated the release of the drug. The longer contact time with absorbing membrane of the gastro-retentive systems permits greater site absorption and greater bioavailability of drugs[4]. These systems are successfully developed and scaled up for commercial use. Additional benefits of gastro retentive drug delivery systems include: (i) Improved therapeutic effect for low-solubility drugs due increased drug solubility and absorption from stomach (ii) Reduction in drug dose and (iii) reduction in associated side effects[5].

Gasrtoretentive drug delivery systems:

Gastroretentive dosage forms are designed to remain in the stomach for up to several hours thereby significantly enhancing the drug's residence time in the stomach. The prolonged gastric residence results in improves

bioavailability of the drug due to higher solubility of drugs at stomach pH conditions thereby minimizing the drug loss. This system can also be utilized for local drug delivery to stomach and drug delivery to proximal small intestine[6]. The formulation technologies for gastroretention can be broadly classified based on different mechanism: high density (sinking) formulations, low density (floating) formulations, expandable systems, super porous hydrogel systems, mucoadhesive systems as well as magnetic systems.

Table 1: Techniques used for study gastroretention

Technique	Equipment used	Material used for gastroretention	Reference
Gamma Scintigraphy	E-Cam Single Head Gamma Camera (Company: Siemen's, Germany).	^{99m}Tc-labeled floating microspheres	Jain et al[12].
Gastroscopy	Gastroscope	Fibre optics or video system	Mandal et al[13]
X-ray	X-ray generators	12% BaS04 as opaqueing agent	Patel A. et al[14]
Ultrasonography	High resolution real time scanner (ALOKA SSD 250) with a 3.5-MHz linear array transducer.	-----	Bolondi l. et al[15]
Magnetic Resonance Imaging (MRI)	37-channel superconducting quantum interference device (SQUID) magnetometer.	Sucrose pellets were coated with powdered magnetite (Fe3O4) in poly (methyl methacrylate).	Weitschies W. et al[16]

These drug delivery systems localize the medication in the upper part of the GIT within a narrow absorption window. This enables the drug to act locally in the stomach and increases the formulation effectiveness through long intimate contact with the absorbing membrane. These are especially useful for drugs with poor absorption and stability in colon[7]. Various techniques such like Gamma, Gastroscopy, X-ray, Ultrasonography and Magnetic Resonance Imaging (MRI) have been adopted for testing gastro-retention time in the dosage form. These techniques are successful in tracing the movement of the dosage form throughout the GIT[8-11]. All these techniques along with the material required for their use is listed in the table 1.

Gastroretentive drug delivery system for antidiabetic drugs:

Diabetes is a physiological state associated with either non availability of insulin or its ineffectiveness within the human body. The complete absence of insulin characterizes type-1 diabetes while type-2 diabetes is characterized by resistance to the impacts of insulin within peripheral tissues of the body. Both types of diabetics have no insulin signaling impact on glucagon secretion which leads to hyperglycemia by enhancing hepatic glucose output from glucagon. Diabetes mellitus is caused by imbalanced carbohydrate metabolism and its impacts on other pathways[17]. According to the WHO, diabetes affects 285 million adult patients in 2010, 67% higher than in 2000 and an estimated 439 million in 2030 is 20% higher than the estimated 2030 study.[18]. Oral therapy is considered as the best path for administration of antidiabetic drugs owing to the highest patient compliance among other routes except insulin which is given as subcutaneous injection, owing to its degradation in GIT. Conventional drug delivery systems or modified drug release systems are the most commonly utilized drug delivery systems[19]. Modified release drug delivery systems are employed to obtain better therapeutic results, this concept is also applicable to the anti-diabetic drugs.

The modified release drug delivery systems that has gained interest in the last decade is gastroretentive drug delivery. The techniques employed for development of such systems in modern days are as under:

At present the drugs available in market for the treatment of diabetes can be classified in to the following categories (Table 2):

Gastroretentive drug delivery systems for anti-diabetic drugs can be broadly divided in to the following:

1. Floating drug delivery systems (FDDS):

FDDS is one of the many approaches employed for achieving extended gastric retention, improving drug bioavailability and drug targeting in the stomach and upper intestines (Figure 1). These systems were originally depicted by Davis in 1968. FDDS perfectly suited for the drugs having selective absorption in the gastric region. Bulk density of these systems is a less than that of gastric fluids (almost 1.004g/cm^3), so they show a good floating property. These devices float on gastric liquids for longer periods without influencing the gastric emptying rates and progressively discharge the drug at a required and regulated rate and the left over system is cleared from the stomach after releasing the drug, this results in minimal variation in plasma drug concentration and improved absorption of drugs via gastro-retention[20,21]. Drugs like metformin hydrochloride which has potential absorption from stomach when given as Controlled Release/Sustained Release dosage form pass through the stomach (absorption region) at faster rate than its release and most of the drug is release in colon where the drug is poorly absorbed[22,23]. Metformin hydrochloride should therefore be a given in gastro-retentive form while given as controlled release formulation for better efficacy[24], as gastro-retentive dosage form release the drug slowly for longer periods in the stomach for steady absorption in the intestines.

Table 2: Marketed anti diabetic product and their drug delivery systems

Category	Drugs	pKa (acidic)	pKa (basic)	Partition coefficient	Half life	Bioavail ability	Marketed products	Remarks
Sulfonyul ureas	Gliclazide	5.8	NA	1.09	10h	79-81%	Diamicron® (Servier Laboratories Limited)	Available as extended release tablets
	Glibenclamide/ Glyburide	6.3	NA	3.16	1.4-1.8 h	73%	Daonil® (Sanofi-Aventis)	Available as immediate release tablets
	Glimepiride	2.23	-0.36	3.16	2-3 h	100%	Amaryl® (Sanofi)	Available as immediate release tablets
Biguanides	Metformin	12.4		1.772	6.2 h	50-60%	Glucophage® (Merck Serono Limited)	Also Available as sustained release tablets
Thiazolidin ediones	Pioglitazone	6.66		2.94	3-7 h and 16-24 h	83%	Actos™ (Eli Lilly and Company)	Available as immediate release tablets
	Rosiglitazone	6.84	6.23	2.72–3.73	3-4 h	99%	Avandia® (GlaxoSmithKline)	Available as immediate release tablets
Meglitinides	Repaglinide,	4.16	6.01	3.81	1 h	56%	Prandin (Novo Nordisk)	Available as immediate release tablets
	Nateglinide	4.06	1.53	0.2 at pH 6.8	1.5 h	73%	Starlix® (Novartis Pharmaceuticals)	Available as immediate release tablets
Alpha-glucosidases inhibitor	Acarbose	5.1		NA	2 h	2%	Glucobay (Bayer AG)	Available as immediate release tablets
Dipeptidyl-peptidase-4 (DPP-4) inhibitors	Linagliptin	8.6	1.9	1.7	100 h	30%	Trajenta® (Boehringer Ingelheim)	Available as immediate release tablets
	Saxagliptin	7.3		0.607	3.1 h	67%	Onglyza® (Astrazeneca Pharma)	Available as immediate release tablets
	Sitagliptin	7.7		1.5	12.4 h	87%	Januvia® (Merck and Co)	Available as immediate release tablets
	Alogliptine	9.47		- 0.5	12–21 h	100%	Nesina (Takeda Pharmaceutical)	Available as immediate release tablets
Glucagon-like peptide-1 (GLP-1) agonist	Exenatide	NA	NA	NA	2.4 h	25%	Bydureon® Injection (AstraZeneca)	Available as extended release injectable suspension
	Liraglutide	NA	NA	NA	13 h	55%	Victoza® Injection (Novo Nordisk)	Available as injectable
	Dulaglutide	NA	NA	NA	Approx. 5 days	up to 65%	Trulicity® Injection (Eli Lilly and Co.)	Available as injectable
	Lixisenatide	NA	NA	NA	2- 4 hours	32%	Suliqua (Sanofi-Aventis)	Available as subcutaneous injection
	Semaglutide	NA	NA	NA	approx. 7 days	89%	Ozempic® (Novo Nordisk)	Available as injectable
Sodium glucose co transporter 2 (SGLT2) inhibitors	Canaglifozine	12.57,	- 3.0	NA	10-13 h	65%	Invokamet® (Johnson and Johnson)	Available as (immediate release) in combination with metformin (extended release)
	Dapagliflozine	12.6		NA	12.9 h	78%	Farxiga® (Astra Zeneca)	Available as immediate release tablets
	Empagliflozin	12.57	- 3.0	1.7	12.4 h	78%	Jardiance® (Boehringer Ingelheim)	Available as immediate release tablets
	Ertugliflozin	11.98	- 3.1	NA	11-17 h	Almost 100%	Steglatro® (Merck and Co)	Available as immediate release tablets

A slow but almost complete release of drug into the stomach is bound to increase drug bioavailability and its complete utilization, leading to minimizing doses and reducing gastrointestinal side effects. Multiunit floating dosage forms are supposed to release the drug at a predefined controlled rate and remain in the stomach for an extended period of time with very little dumping chances. In addition, they reduce associated GIT side effects and are unaffected by the concomitant intake of food, thereby reducing inter and intra-patient variation, improving patient compliance and increasing the uniformity of dosage form[25-27].

Patel *et al* developed floating metformin hydrochloride microspheres by nonaqueous emulsifying technique utilizing ethylcellulose as polymer to control drug release. Developed formulations were assessed for pharmacopoeial as well as non-pharmacopoeial tests including drug-polymer compatibility by FTIR, percentage yield, drug entrapment efficiency, particle size determination, surface topography, *in-vitro* floatation and drug release studies. The results showed that the yields of the developed floating microsphere metformin hydrochloride were 58 - 87% and drug release from microsphere was 47 - 87% after 8 hours and the floating time of > 8 hrs for the prolonged drug

release in the stomach, thereby enhancing bioavailability and patient compliance[28]. Encapsulated floating repaglinide microspheres were designed and developed by Sharma *et al* to improve the residence time of the drug in GIT and thereby improve its systemic availability. The emulsion solvent diffusion techniques were used to formulate floating microspheres of repaglinide with ethylcellulose (EC) and methylcellulose hydroxypropyl (HPMC) (5 and 100 cps). The floating capacity and *in-vivo* antidiabetic activity of microspheres was carried out in alloxane-induced diabetic rats. The optimized formulation remained buoyant for six hours and showed marked reduction in blood glucose in comparison to the group of pure drugs[29]. Kamila *et al* prepared floating rosiglitazone microspheres using non-aqueous emulsification/solvent evaporation techniques by encapsulation of medicine into Edragit RS-100. To attain predetermined target release, simplex lattice mixture layout was used. *In-vivo* formulation efficiency in streptozotocin-induced diabetic rats has been assessed, optimized formulations (microspheres) of rosiglitazone started to decrease blood glucose in the third hour until the ninth hour until blood glucose reached a standard level while in the case of pure medication the blood glucose declined from second hour to 5 hours[30].

In combination with the gums (sodium alginate, sodium CMC, xanthan gum and guar Gum) Thulluru *et al.* developed effervescent floating pioglitazone tablets with synthetic polymer HPMC K100 M that can extend the release of drug up to 12 hours. Studies of *in-vitro* buoyancy have shown that the optimized formulation remains buoyant for 12 hours and *in-vitro* drug release was found to be almost complete in the same time. *In-vivo* studies of rabbit x-ray imaging showed that the formulation is capable to withstand repeated gastric contractions and remain intact in the gastric region for 12 hours[31]. Ahmed *et al* developed gastro-retentive tablets of sitagliptin tablets based on floating, the tablets were prepared using the polymers HPMC K100, Poly vinyl pyrrolidine and polyacrylic acid in different concentrations by direct compression method. The bulk mixture was characterized for flow properties and finished formulations were evaluated for pharmacopoeial tests. *In-vitro* dissolution in the stimulated gastric pH 1.2 and intestinal fluid pH 6.8 were carried and drug releases for optimized formulation were found to be between 92.9-99.28%. The study concluded that good release was observed when a combination of polymers was used instead of single polymer and necessity of combining different class of polymers to get the desirable pharmacokinetic profile[32]. Jeganath *et al* developed a non-effervescent gastro-retentive tablets of linagliptin (dipeptidyl peptidase-4 enzyme inhibitor) based on floating. The tablets were prepared using a combination of Hydroxy Propyl Methyl Cellulose-K15, Hydroxy Propyl Methyl Cellulose-E15 and chitosan as polymers and Accurel, Gelucire as low-density drug carriers. The tablets were successfully prepared by direct compression method. The developed formulations remain floating for more than 20 hours in 900ml of simulated gastric fluid pH 1.2 and *in-vitro* drug release was found to be 98% in 20 hours for the optimized formulation[33]. Duggi *et al* developed poiglitazone hydrochloride floating matrix tablets for gastro-retention using a combination of polymers viz. hydroxy propyl methyl cellulose, xanthan gum and guar gum. The authors studied the effect of polymer concentration and viscosity on gastro-retention and drug release. Based on various combinations they developed the optimized formulation which started floating in 28 seconds and remain floated for more than 12 hours. The optimized formulation has swelling index of 91.8% and drug release up to 95.86% after 12 hours was attained. The authors concluded that floating, swelling and subsequent drug release is highly dependent on polymer concentration and its viscosity[34].

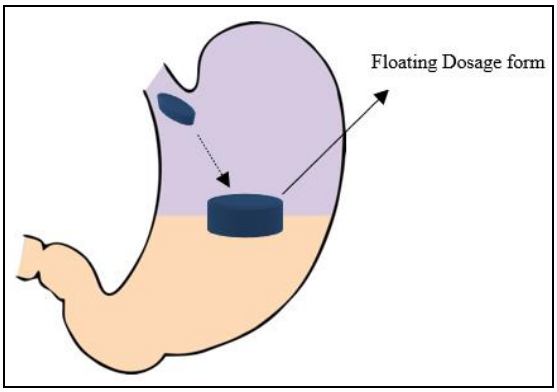

Figure 1: Depiction of floating drug delivery system in stomach

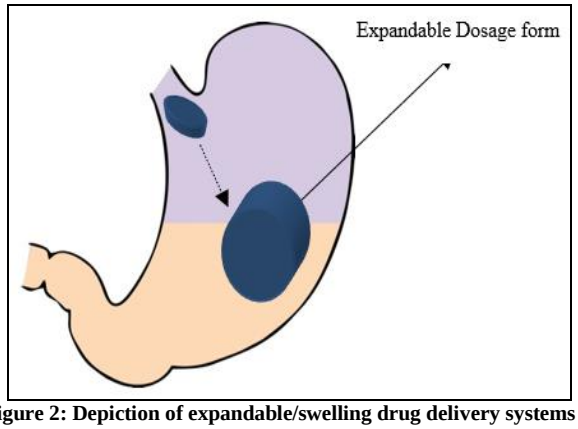

Figure 2: Depiction of expandable/swelling drug delivery systems

2. Expandable Systems:

Expanding drug delivery systems (figure 2) absorb water to increase their size and retain within the stomach due to their size which is usually larger than the diameter of the pyloric sphincter, thereby restricting its passage into the intestine. While formulating an expandable drug delivery system, following points should be taken care

of:

a) The dosage form should be small enough and convenient to swallow
b) It should expand quickly to an effective size in order to prevent its premature passage from the stomach to intestine.
c) The dosage form should erode so as to prevent a luminal blockage.

Expansion of delivery systems is usually accomplished by two mechanisms namely swelling and unfolding. Both these mechanisms result in an increase in size of the dosage form which restricts the passage of the delivery system through the pyloric sphincter into the intestine. Swelling occurs due to the absorption of water, usually by osmosis, whereas unfolding occurs due to the mechanical shape of the pharmaceutical carrier. Swelling systems offer an additional advantage over other gastro-retentive delivery systems in that they maintain a fed state in the stomach which suppresses housekeep waves offering a prolonged gastro-retention as the bulk of the system is located within the stomach[35,36]. Boldhane *et al* manufactured effervescent based floating and swelling gastro-retentive tablets of metformin hydrochloride using sodium alginate as a gelling agent, sodium CMC as release modifier and polymer Eudragit NE 30D as release retardant respectively. The authors used 3^2 full factorial design to study the effect of concentration of sodium alginate and sodium CMC on release of drug, they selected time required for 50%, time required for 90% of cumulative drug release, Flag and f_2 as 4 dependent variable. The statistical model was employed to make the optimization process very effective and quick. The authors also concluded that Low floating time and higher % swelling of the formulation are essential to increase its residence time in the stomach, thereby improving the bioavailability of the drug[37].

3. Mucoahesive drug delivery systems:

When a pressure sensitive adhesive material comes in contact with a surface, a bond is a formed by the contact between then two and phenomenon is called adhesion. The same phenomenon is termed as mucoadhesion when a polymer carrying a drug forms a bond with the mucosa membrane of stomach and the system is called mucoadhesive drug delivery system (figure 3). At the absorption or application site, these systems extend the residence time of the dosage form as the dosage form remain in intimate contact with the underlying surface of absorption that improves the drug's therapeutic performance. Till date these systems are developed for local as well as systemic effects and can be administered via oral, buccal, nasal, rectal and vaginal routes depending on the site of action and absorption at the site[38]. Polymers like carbopol, chitosan, polycarbophil, lectins etc. are usually incorporated in these systems for mucoadhesion. These naturally occurring bioadhesive polymers allow a device to stick to the mucous membrane and develop an interaction which is hydration mediated, receptors mediated or bonding mediated adhesion with the biological membrane[39].

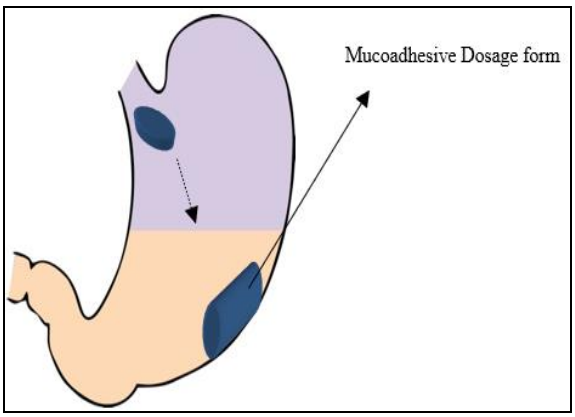

Figure 3: Depiction of mucoadhesive drug delivery system

While designing mucoadhesive drug delivery systems, dosage form must be small and flexible enough to be acceptable for patients and should not cause irritation. Other valuable features of a mucoadhesive dosage form include a high drug loading capacity, controlled drug release (preferably unidirectional release), better mucoadhesive properties, smooth surface, tastelessness and convenient application. Erodible formulations offer more benefits as they do not require system retrieval at the end of desired dosing interval. Numerous valuable mucoadhesive dosage forms have been developed for a variety of drugs. Several typical molecules such as peptides, including thyrotropin-releasing hormone (TRH), insulin, octreotide, leuprolide, and oxytocin have been successfully given via the mucosal route, though with relatively low bioavailability (0.1–5%) due to their hydrophilic characteristics and large molecular weight as well as the inherent permeation and enzymatic barriers of the mucosa[40]. Awasthi *et al* developed hollow floating gliclazide beads using a simple ionotropic gelation technique using a combination of low methoxyl pectin and hydroxypropylmethyl cellulose. Particle size analysis of beads revealed the size of dry beads in the range of 730-890µm based on the composition of polymer and concentration of calcium carbonate. The authors also concluded that the mean diameter of beads increases with increase in the concentration of gas forming agent which may be attributed to the increased viscosity of solution. The authors used fourier transform infrared (FTIR) spectroscopy, differential scanning calorimetry (DSC) and X-ray diffraction technique to confirm the drug's stable character. Formulations were assessed for mucoadhesion using goat stomach mucosal membrane. The optimized formulations showed excellent bioadhesive properties for a duration of 2h in the mucoadhesion experiment. The *in-vitro* drug release

represents Fickian diffusion with swelling[41]. Glipizide microspheres were also developed by Patel *et al* through simple emulsification phase separation method using chitosan as polymer for mucoadhesion and glutaraldehyde as a cross-linking agent. A full factorial 3^2 design was used using two independent variables (polymer-to-drug ratio and stirring speed) and four dependent variables (percentage mucoadhesion, t_{80}, drug entrapment efficiency). The best exhibited formulation showed entrapment effectiveness of 75%, a swelling index of 1.42, a mucoadhesion of 78% after one hour and the drug release also maintained up to 12 hours. The in vivo research in Wistar mice also reveals an important hypoglycemic impact up to 12 hours[42]. Prasanthi *et al* developed mucoadhesive microspheres of linagliptin by ionotropic gelation and single emulsion methods using synthetic polymers like carbopol 934P, guar gum, HPMC K100M and sodium carboxy methyl cellulose. Spherical free flowing microspheres of linagliptin were successfully prepared by emulsification method. The developed mucoadhesive microspheres has swelling index of 1.03, entrapment efficiency of 85±0.57% and the mean particle size of 135±6µm. Mucoadhesion strength was found to be 87% in 7 hrs and drug release was shown to be 98.2±0.63% in 8 hrs and release kinetics followed anomalous transport mechanism. Radiographic studies were performed in rabbits and microspheres retained for 7 h in the rabbit stomach as confirmed by the images[43]. Sarkar *et al* developed poly (acrylic acid)-grafted-gellan-based gastroretentive continuous release tablets of metformin hydrochloride based on swelling and mucoadhesion. Firstly authors synthesized poly (acrylic acid)-grafted-gellan by microwave-promoted cerric (IV) ion initiated graft copolymerization technique. The yield of polymer was found to be dependent on concentration of cerric (IV) ammonium nitrate and acrylic acid. Further they developed mucoadhesive sustained release tablet using metfomin hydrochloride as drug by wet granulation. The formulations developed showed sustained release potential in simulated gastric fluid (pH 1.2) over a period of 10 hours[44].

4. Dual (floating and bioadhesive) systems:

These systems are usually available in the form of mucoadhesive beads, ion resin complexes, microspheres, films, and tablets which based on the combination of floatation and mucoadhesion properties together in a single dosage form. Sonar *et al* developed a bilayer, floating-bioadhesive dosage form based on a unique combination of floating and bioadhesive properties to extend the residence of rosiglitazone maleate in the stomach. The formulation comprises of two layers one is floating layer and other one is sustained release layer. Floating layer was prepared using 5% w/v PVP ethanolic solution and hydroxyl propyl methyl cellulose (HPMC) and sodium bicarbonate were added in the preparation to sustain the drug release. The optimized formulation showed a satisfactory profile of dissolution, detachment force, floating properties and followed first order drug release. The tablets stayed floating in the stomach for up to 8 hours, as determined in healthy human volunteers by gamma scintigraphy[45]. Sah *et al* developed floating sitagliptin microsphere applying 3^2 factorial design to improve the gastric residence time and subsequent absorption of the drug in the stomach. The authors prepared microspheres using HPMC K4M and psyllium husk as swelling agents by ionotropic gelation method. In X-ray imaging in rabbits initially and after 24 hours of administration of dosage form, microspheres were found retained in the stomach. During *in-vivo* study sitagliptin was detected in plasma from swellable gastroretentive microsphere till the end of 24 hours post-administration, while the plasma concentration of conventional microsphere of sitagliptin was detectable till 12 hours post administration. The swelling of the developed formulation was found to be 191% to 240% and for the optimized formulation, *in-vitro* release of drugs was found to be 99.2% after 24 hours[46].

5. In-Situ Gelling systems (Raft Forming system):

Another method of gastroretention with excellent patient compliance is the in-situ gelling systems (also known as the raft forming system figure 4). These structures comprise of sodium alginate as polymer-forming gel in-situ with carbonate or bicarbonates as effervescent substances. These systems are in the solution form initially but when they come in contact the gastric fluid, they get swell to form viscous cohesive gel and generate carbon dioxide bubbles these bubbles are caught in the gel which cause drug delivery systems to float. Raft forming systems are used mostly for gastroesophageal treatment because they are likely to produce a layer on top of the gastric fluid[47,48]. Metformin hydrochloride floating gastro-retentive tablets of based on in-situ gel technique were developed by Senjoti *et al*. Box-Behnken experimental design was used to develop tablets with effervescent and swelling properties using a combination of sodium bicarbonate and HPMC-PEO (Poly Ethylene Oxide) polymer. The developed tablets were able to float within 4 minutes, remain in floating condition for 24 h and sustained the release of drug for 12 hours. The authors also concluded that amount of polymer matrix (amount of HPMC and PEO), effervescent agent (sodium bicarbontate), and swelling enhancer (SSG) affected and floating and drug release from the formulation[49].

In-situ miglitide formulations, using gellan gum and sodium alginate as gelling agent, propylene glycol as co-solvent and calcium carbonate were developed by Mahmoud *et al*. Calcium carbonate dissociates in the acidic environment to release carbon dioxide which gets

entrapped in the gel and helps in the floating. Developed formulations showed reasonable viscosity and formed a firm gel that floated over the surface in seconds and remained floating for 24 hours when it came into contact with simulated gastric fluid. *In-vitro* dissolution was achieved at 900 mL 0.1N HCl (pH 1.2), and the release of all formulations exceeds 70% in 24 hours. The *in-vivo* pharmacokinetic experiments were performed in 10 New Zealand rabbits and the results reveal 1.79 fold increase in C_{max} and 18.4 fold increase in AUC with the newly developed in-situ formulation compared to the one on the market. The authors concluded that use of propylene glycol in the formulation helped in solubilization the water insoluble drug[50].

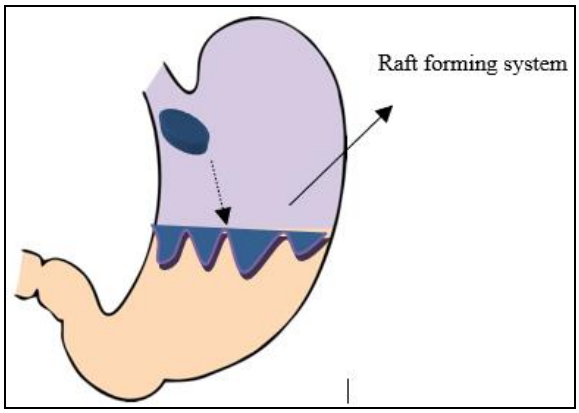

Figure 4: Depiction of raft forming drug delivery system

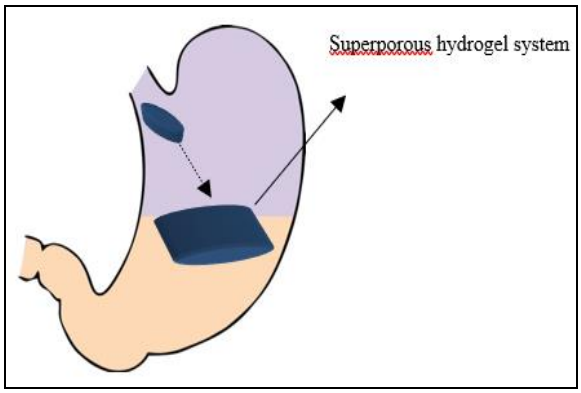

Figure 5: Depiction of superporous hydrogel swelling systems

6. Superporous Hydrogels:

Superporous hydrogel (figure 5) is characterized by a three-dimensional network of hydrophilic polymer, which absorbs a huge amount of water in a short period of time, due to the presence of interconnected microscopic pores. These highly swollen hydrogels remain in the stomach for a long period of time when used as drug carriers, releasing virtually all loaded drugs, large volumes and sheer bulk of these hydrogels does not allow them to be transported through the pylorus to the next organ. This distinct swelling characteristic enables them to be used as gastro-retentive carriers that provide a sustained release through extended gastric retention. Not only are hydrogels needed to swell rapidly, but they should also be biocompatible, biodegradable, better swelling properties, strong mechanical strength and remain static in acidic situation[51]. Park *et al* developed hydrogels of chitosan-glycol and chitosan by a gas blowing method and studied their swelling behaviours in acidic solution to explore their use as a stomach retention dosage form. Firstly the stock solution of chitosan and glycol-chitosan were prepared dissolving the former in 0.01M acetic acid and latter in water respectively. The pH of the solution was adjusted to 5 by adding acetic acid, then sodium bicarbonate (blowing agent) is added to the solution and mixed vigorously by stirring for 30seconds. Foaming started immediately and completed in 2 minutes. The formed hydrogels were stored at room temperature and freeze dried before use. Both the chitosan and glycol chitosan hydrogels revealed higher swelling ratio in acidic environment than in distilled water probably due to the cationization of amine groups in acidic environment. The results also indicated that swelling of hydrogels with increasing density of crosslinking agent[52].

Gupta *et al* formulated chitosan/poly(vinyl alcohol) interpenetrating polymer-type superporous hydrogels of Rosiglitazone maleate utilizing glyoxal as crosslinker. To induce the porous structure, sodium bicarbonate was used as a foaming agent. The swelling of the formulation depended on the amount of chitosan and crosslinker. The drug release from the superporous hydrogels was maintained for 6 hours successfully[53].

7. Magnetic Systems:

These devices contain a tiny inner magnet in the design and an external magnet is positioned on the abdomen above the stomach location. Gastro retention of the dosage form can be improved by applying an extracorporeal magnet for localization to a specific part. The site of magnet should be designated with very high precision to acquire the desired results in such systems. Thus, the factual significance of such systems is frequently doubtful[54]. Angelopoulou *et al* developed magnetically targetable nanocarriers of the sodium-glucose transporter protein (SGLT2) inhibitor dapagliflozin for the selective delivery of dapagliflozin in tumors. The dapagliflozin-loaded PMMA-g-PEGMA nanoparticles showed concentration-dependent toxicity against the A549 cancer cell line. The application of an external magnetic magnet increases the uptake of nanoparticles by cells, leading to increased cytotoxicity. The developed nanoparticles exhibited satisfactory drug loading efficiency and high colloidal stability. Dapagliflozin release from the nanoparticles responded to an AC magnetic field to induce dapaglifozin release in close proximity to the tumor area confirming accumulation of the dapaglifozin-loaded nanoparticles at the tumor cells due to magnetic targeting[55].

CONCLUSION:

Gastro-retentive drug delivery systems are successfully developed and reported for antidiabetic drugs by researchers and they can do wonders if the same are available commercially. These systems are very useful for the drugs which are absorbed from the stomach. Further investigation in human volunteers still needs to done before scaling up them in to commercials. However with the advent new molecules like DDP-4 and SGLT2 inhibitors there has been a significant progress in the field of drug discovery. The formulation work is still in progress to develop the gastro-retentive dosage form of these drugs which can be commercialized. With increasing population of diabetics there is further scope of development in this field for the economical products with better therapeutic results and reduced side effects especially for prolonged use.

CONFLICT OF INTEREST:

The authors confirm no conflict of interest.

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RESEARCH ARTICLE

A Review of Gastro-retentive Drug Delivery Systems for Antidiabetics and its present status

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ABSTRACT:
The present review provides concise information on status of gastro-retentive drug delivery systems for antidiabetic molecules. Present review emphasis on pharmacotherapy of diabetes via gastric retention of drug molecules. The gastro-retentive system is a proven useful tool for sustaining the drug release for the drugs having good absorption through the GIT, drugs with narrow therapeutic index and low dose drugs. The gastro-retentive dosage forms have also been developed for antidiabetic molecules and metformin hydrochloride, the first line drug in the treatment of diabetes is commercially available in the market in the form of sustained release formulations. As oral antidiabetic therapy is required for prolonged duration, the oral hypoglycemic may lead to side effects such as diabetic neuropathy, diabetic myopathy and many more. The primary reason for side effects is under-utilization of the drug molecule which can be improved using gastro-retentive drug delivery systems thereby minimizing the side effects. Despite being one of the most successfully systems, the commercial value gastro-retentive drug delivery systems is still below par. Gastro-retentive drug delivery systems for antidiabetic drug molecules are still awaited. The research is still in progress for gastro-retentive formulations which can attract industry utilizing these systems for humanely causes.

KEYWORDS: Antidiabetics; floating; mucoadhesive; raft forming; drug delivery systems (DDS).

INTRODUCTION:

Oral route of administration has benefits such as low treatment costs and ease of administration, high level of patient conformity and so far remain the preferred route of administration[1]. However drug absorption from oral route is not always uniform due to the physiological factors and gastrointestinal (GIT) system heterogeneity. Moreover, many involuntary variables influence drug uptake throughout the GIT such as variable pH, intestinal flora, gastrointestinal transit time, gastric secretions and absorption surface area[2]. The traditional immediate release drug delivery systems are not able to combat the problems associated with low drug absorption in the gastrointestinal transit as these systems do not possess any additional characteristics to counter stomach motility and thus are not suitable for the drugs which are to be absorbed in the upper part of GIT.

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Incomplete drug deliverance and subsequent reduction in bioavailability are the consequence that can be ascribed for the failure of traditional devices[3]. To overcome these issues, drug delivery systems that can control drug release and the residence time of the drug have been developed. Such systems are designed to reside in the upper GIT for a long period of time during which they regulated the release of the drug. The longer contact time with absorbing membrane of the gastro-retentive systems permits greater site absorption and greater bioavailability of drugs[4]. These systems are successfully developed and scaled up for commercial use. Additional benefits of gastro retentive drug delivery systems include: (i) Improved therapeutic effect for low-solubility drugs due increased drug solubility and absorption from stomach (ii) Reduction in drug dose and (iii) reduction in associated side effects[5].

Gasrtoretentive drug delivery systems:

Gastroretentive dosage forms are designed to remain in the stomach for up to several hours thereby significantly enhancing the drug's residence time in the stomach. The prolonged gastric residence results in improves

bioavailability of the drug due to higher solubility of drugs at stomach pH conditions thereby minimizing the drug loss. This system can also be utilized for local drug delivery to stomach and drug delivery to proximal small intestine[6]. The formulation technologies for gastroretention can be broadly classified based on different mechanism: high density (sinking) formulations, low density (floating) formulations, expandable systems, super porous hydrogel systems, mucoadhesive systems as well as magnetic systems.

Table 1: Techniques used for study gastroretention

Technique	Equipment used	Material used for gastroretention	Reference
Gamma Scintigraphy	E-Cam Single Head Gamma Camera (Company: Siemen's, Germany).	^{99m}Tc-labeled floating microspheres	Jain et al[12].
Gastroscopy	Gastroscope	Fibre optics or video system	Mandal et al[13]
X-ray	X-ray generators	12% BaS04 as opaqueing agent	Patel A. et al[14]
Ultrasonography	High resolution real time scanner (ALOKA SSD 250) with a 3.5-MHz linear array transducer.	-----	Bolondi l. et al[15]
Magnetic Resonance Imaging (MRI)	37-channel superconducting quantum interference device (SQUID) magnetometer.	Sucrose pellets were coated with powdered magnetite (Fe3O4) in poly (methyl methacrylate).	Weitschies W. et al[16]

These drug delivery systems localize the medication in the upper part of the GIT within a narrow absorption window. This enables the drug to act locally in the stomach and increases the formulation effectiveness through long intimate contact with the absorbing membrane. These are especially useful for drugs with poor absorption and stability in colon[7]. Various techniques such like Gamma, Gastroscopy, X-ray, Ultrasonography and Magnetic Resonance Imaging (MRI) have been adopted for testing gastro-retention time in the dosage form. These techniques are successful in tracing the movement of the dosage form throughout the GIT[8-11]. All these techniques along with the material required for their use is listed in the table 1.

Gastroretentive drug delivery system for antidiabetic drugs:

Diabetes is a physiological state associated with either non availability of insulin or its ineffectiveness within the human body. The complete absence of insulin characterizes type-1 diabetes while type-2 diabetes is characterized by resistance to the impacts of insulin within peripheral tissues of the body. Both types of diabetics have no insulin signaling impact on glucagon secretion which leads to hyperglycemia by enhancing hepatic glucose output from glucagon. Diabetes mellitus is caused by imbalanced carbohydrate metabolism and its impacts on other pathways[17]. According to the WHO, diabetes affects 285 million adult patients in 2010, 67% higher than in 2000 and an estimated 439 million in 2030 is 20% higher than the estimated 2030 study.[18]. Oral therapy is considered as the best path for administration of antidiabetic drugs owing to the highest patient compliance among other routes except insulin which is given as subcutaneous injection, owing to its degradation in GIT. Conventional drug delivery systems or modified drug release systems are the most commonly utilized drug delivery systems[19]. Modified release drug delivery systems are employed to obtain better therapeutic results, this concept is also applicable to the anti-diabetic drugs.

The modified release drug delivery systems that has gained interest in the last decade is gastroretentive drug delivery. The techniques employed for development of such systems in modern days are as under:

At present the drugs available in market for the treatment of diabetes can be classified in to the following categories (Table 2):

Gastroretentive drug delivery systems for anti-diabetic drugs can be broadly divided in to the following:

1. Floating drug delivery systems (FDDS):

FDDS is one of the many approaches employed for achieving extended gastric retention, improving drug bioavailability and drug targeting in the stomach and upper intestines (Figure 1). These systems were originally depicted by Davis in 1968. FDDS perfectly suited for the drugs having selective absorption in the gastric region. Bulk density of these systems is a less than that of gastric fluids (almost 1.004g/cm^3), so they show a good floating property. These devices float on gastric liquids for longer periods without influencing the gastric emptying rates and progressively discharge the drug at a required and regulated rate and the left over system is cleared from the stomach after releasing the drug, this results in minimal variation in plasma drug concentration and improved absorption of drugs via gastro-retention[20,21]. Drugs like metformin hydrochloride which has potential absorption from stomach when given as Controlled Release/Sustained Release dosage form pass through the stomach (absorption region) at faster rate than its release and most of the drug is release in colon where the drug is poorly absorbed[22,23]. Metformin hydrochloride should therefore be a given in gastro-retentive form while given as controlled release formulation for better efficacy[24], as gastro-retentive dosage form release the drug slowly for longer periods in the stomach for steady absorption in the intestines.

Table 2: Marketed anti diabetic product and their drug delivery systems

Category	Drugs	pKa (acidic)	pKa (basic)	Partition coefficient	Half life	Bioavail ability	Marketed products	Remarks
Sulfonyul ureas	Gliclazide	5.8	NA	1.09	10h	79-81%	Diamicron® (Servier Laboratories Limited)	Available as extended release tablets
	Glibenclamide/ Glyburide	6.3	NA	3.16	1.4-1.8 h	73%	Daonil® (Sanofi-Aventis)	Available as immediate release tablets
	Glimepiride	2.23	-0.36	3.16	2-3 h	100%	Amaryl® (Sanofi)	Available as immediate release tablets
Biguanides	Metformin	12.4		1.772	6.2 h	50-60%	Glucophage® (Merck Serono Limited)	Also Available as sustained release tablets
Thiazolidin ediones	Pioglitazone	6.66		2.94	3-7 h and 16-24 h	83%	Actos™ (Eli Lilly and Company)	Available as immediate release tablets
	Rosiglitazone	6.84	6.23	2.72–3.73	3-4 h	99%	Avandia® (GlaxoSmithKline)	Available as immediate release tablets
Meglitinides	Repaglinide,	4.16	6.01	3.81	1 h	56%	Prandin (Novo Nordisk)	Available as immediate release tablets
	Nateglinide	4.06	1.53	0.2 at pH 6.8	1.5 h	73%	Starlix® (Novartis Pharmaceuticals)	Available as immediate release tablets
Alpha-glucosidases inhibitor	Acarbose	5.1		NA	2 h	2%	Glucobay (Bayer AG)	Available as immediate release tablets
Dipeptidyl-peptidase-4 (DPP-4) inhibitors	Linagliptin	8.6	1.9	1.7	100 h	30%	Trajenta® (Boehringer Ingelheim)	Available as immediate release tablets
	Saxagliptin	7.3		0.607	3.1 h	67%	Onglyza® (Astrazeneca Pharma)	Available as immediate release tablets
	Sitagliptin	7.7		1.5	12.4 h	87%	Januvia® (Merck and Co)	Available as immediate release tablets
	Alogliptine	9.47		- 0.5	12–21 h	100%	Nesina (Takeda Pharmaceutical)	Available as immediate release tablets
Glucagon-like peptide-1 (GLP-1) agonist	Exenatide	NA	NA	NA	2.4 h	25%	Bydureon® Injection (AstraZeneca)	Available as extended release injectable suspension
	Liraglutide	NA	NA	NA	13 h	55%	Victoza® Injection (Novo Nordisk)	Available as injectable
	Dulaglutide	NA	NA	NA	Approx. 5 days	up to 65%	Trulicity® Injection (Eli Lilly and Co.)	Available as injectable
	Lixisenatide	NA	NA	NA	2- 4 hours	32%	Suliqua (Sanofi-Aventis)	Available as subcutaneous injection
	Semaglutide	NA	NA	NA	approx. 7 days	89%	Ozempic® (Novo Nordisk)	Available as injectable
Sodium glucose co transporter 2 (SGLT2) inhibitors	Canaglifozine	12.57,	- 3.0	NA	10-13 h	65%	Invokamet® (Johnson and Johnson)	Available as (immediate release) in combination with metformin (extended release)
	Dapagliflozine	12.6		NA	12.9 h	78%	Farxiga® (Astra Zeneca)	Available as immediate release tablets
	Empagliflozin	12.57	- 3.0	1.7	12.4 h	78%	Jardiance® (Boehringer Ingelheim)	Available as immediate release tablets
	Ertugliflozin	11.98	- 3.1	NA	11-17 h	Almost 100%	Steglatro® (Merck and Co)	Available as immediate release tablets

A slow but almost complete release of drug into the stomach is bound to increase drug bioavailability and its complete utilization, leading to minimizing doses and reducing gastrointestinal side effects. Multiunit floating dosage forms are supposed to release the drug at a predefined controlled rate and remain in the stomach for an extended period of time with very little dumping chances. In addition, they reduce associated GIT side effects and are unaffected by the concomitant intake of food, thereby reducing inter and intra-patient variation, improving patient compliance and increasing the uniformity of dosage form[25-27].

Patel *et al* developed floating metformin hydrochloride microspheres by nonaqueous emulsifying technique utilizing ethylcellulose as polymer to control drug release. Developed formulations were assessed for pharmacopoeial as well as non-pharmacopoeial tests including drug-polymer compatibility by FTIR, percentage yield, drug entrapment efficiency, particle size determination, surface topography, *in-vitro* floatation and drug release studies. The results showed that the yields of the developed floating microsphere metformin hydrochloride were 58 - 87% and drug release from microsphere was 47 - 87% after 8 hours and the floating time of > 8 hrs for the prolonged drug

release in the stomach, thereby enhancing bioavailability and patient compliance[28]. Encapsulated floating repaglinide microspheres were designed and developed by Sharma *et al* to improve the residence time of the drug in GIT and thereby improve its systemic availability. The emulsion solvent diffusion techniques were used to formulate floating microspheres of repaglinide with ethylcellulose (EC) and methylcellulose hydroxypropyl (HPMC) (5 and 100 cps). The floating capacity and *in-vivo* antidiabetic activity of microspheres was carried out in alloxane-induced diabetic rats. The optimized formulation remained buoyant for six hours and showed marked reduction in blood glucose in comparison to the group of pure drugs[29]. Kamila *et al* prepared floating rosiglitazone microspheres using non-aqueous emulsification/solvent evaporation techniques by encapsulation of medicine into Edragit RS-100. To attain predetermined target release, simplex lattice mixture layout was used. *In-vivo* formulation efficiency in streptozotocin-induced diabetic rats has been assessed, optimized formulations (microspheres) of rosiglitazone started to decrease blood glucose in the third hour until the ninth hour until blood glucose reached a standard level while in the case of pure medication the blood glucose declined from second hour to 5 hours[30].

In combination with the gums (sodium alginate, sodium CMC, xanthan gum and guar Gum) Thulluru *et al.* developed effervescent floating pioglitazone tablets with synthetic polymer HPMC K100 M that can extend the release of drug up to 12 hours. Studies of *in-vitro* buoyancy have shown that the optimized formulation remains buoyant for 12 hours and *in-vitro* drug release was found to be almost complete in the same time. *In-vivo* studies of rabbit x-ray imaging showed that the formulation is capable to withstand repeated gastric contractions and remain intact in the gastric region for 12 hours[31]. Ahmed *et al* developed gastro-retentive tablets of sitagliptin tablets based on floating, the tablets were prepared using the polymers HPMC K100, Poly vinyl pyrrolidine and polyacrylic acid in different concentrations by direct compression method. The bulk mixture was characterized for flow properties and finished formulations were evaluated for pharmacopoeial tests. *In-vitro* dissolution in the stimulated gastric pH 1.2 and intestinal fluid pH 6.8 were carried and drug releases for optimized formulation were found to be between 92.9-99.28%. The study concluded that good release was observed when a combination of polymers was used instead of single polymer and necessity of combining different class of polymers to get the desirable pharmacokinetic profile[32]. Jeganath *et al* developed a non-effervescent gastro-retentive tablets of linagliptin (dipeptidyl peptidase-4 enzyme inhibitor) based on floating. The tablets were prepared using a combination of Hydroxy Propyl Methyl Cellulose-K15, Hydroxy Propyl Methyl Cellulose-E15 and chitosan as polymers and Accurel, Gelucire as low-density drug carriers. The tablets were successfully prepared by direct compression method. The developed formulations remain floating for more than 20 hours in 900ml of simulated gastric fluid pH 1.2 and *in-vitro* drug release was found to be 98% in 20 hours for the optimized formulation[33]. Duggi *et al* developed poiglitazone hydrochloride floating matrix tablets for gastro-retention using a combination of polymers viz. hydroxy propyl methyl cellulose, xanthan gum and guar gum. The authors studied the effect of polymer concentration and viscosity on gastro-retention and drug release. Based on various combinations they developed the optimized formulation which started floating in 28 seconds and remain floated for more than 12 hours. The optimized formulation has swelling index of 91.8% and drug release up to 95.86% after 12 hours was attained. The authors concluded that floating, swelling and subsequent drug release is highly dependent on polymer concentration and its viscosity[34].

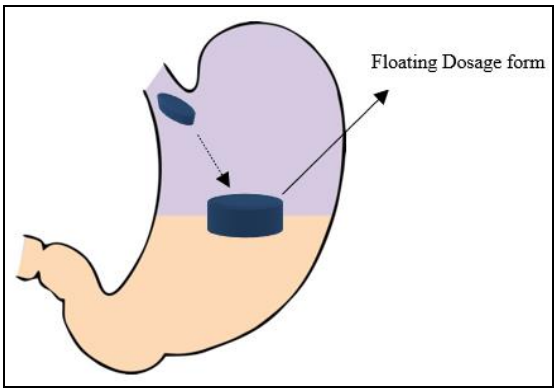

Figure 1: Depiction of floating drug delivery system in stomach

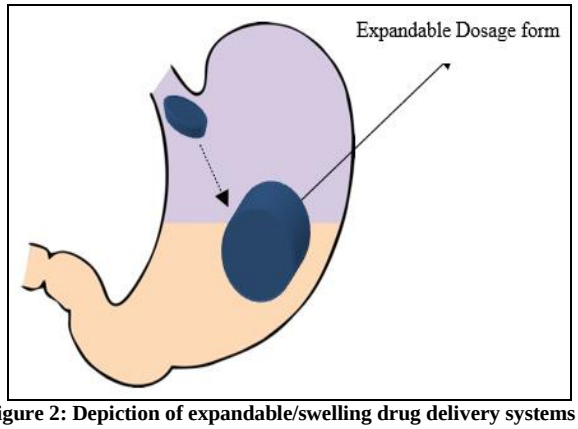

Figure 2: Depiction of expandable/swelling drug delivery systems

2. Expandable Systems:

Expanding drug delivery systems (figure 2) absorb water to increase their size and retain within the stomach due to their size which is usually larger than the diameter of the pyloric sphincter, thereby restricting its passage into the intestine. While formulating an expandable drug delivery system, following points should be taken care

of:

a) The dosage form should be small enough and convenient to swallow
b) It should expand quickly to an effective size in order to prevent its premature passage from the stomach to intestine.
c) The dosage form should erode so as to prevent a luminal blockage.

Expansion of delivery systems is usually accomplished by two mechanisms namely swelling and unfolding. Both these mechanisms result in an increase in size of the dosage form which restricts the passage of the delivery system through the pyloric sphincter into the intestine. Swelling occurs due to the absorption of water, usually by osmosis, whereas unfolding occurs due to the mechanical shape of the pharmaceutical carrier. Swelling systems offer an additional advantage over other gastro-retentive delivery systems in that they maintain a fed state in the stomach which suppresses housekeep waves offering a prolonged gastro-retention as the bulk of the system is located within the stomach[35,36]. Boldhane *et al* manufactured effervescent based floating and swelling gastro-retentive tablets of metformin hydrochloride using sodium alginate as a gelling agent, sodium CMC as release modifier and polymer Eudragit NE 30D as release retardant respectively. The authors used 3^2 full factorial design to study the effect of concentration of sodium alginate and sodium CMC on release of drug, they selected time required for 50%, time required for 90% of cumulative drug release, Flag and f_2 as 4 dependent variable. The statistical model was employed to make the optimization process very effective and quick. The authors also concluded that Low floating time and higher % swelling of the formulation are essential to increase its residence time in the stomach, thereby improving the bioavailability of the drug[37].

3. Mucoahesive drug delivery systems:

When a pressure sensitive adhesive material comes in contact with a surface, a bond is a formed by the contact between then two and phenomenon is called adhesion. The same phenomenon is termed as mucoadhesion when a polymer carrying a drug forms a bond with the mucosa membrane of stomach and the system is called mucoadhesive drug delivery system (figure 3). At the absorption or application site, these systems extend the residence time of the dosage form as the dosage form remain in intimate contact with the underlying surface of absorption that improves the drug's therapeutic performance. Till date these systems are developed for local as well as systemic effects and can be administered via oral, buccal, nasal, rectal and vaginal routes depending on the site of action and absorption at the site[38]. Polymers like carbopol, chitosan, polycarbophil, lectins etc. are usually incorporated in these systems for mucoadhesion. These naturally occurring bioadhesive polymers allow a device to stick to the mucous membrane and develop an interaction which is hydration mediated, receptors mediated or bonding mediated adhesion with the biological membrane[39].

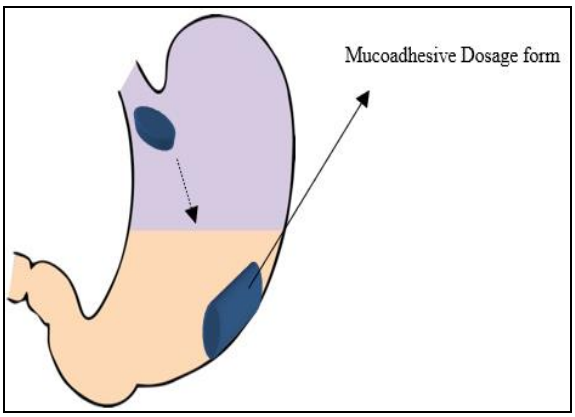

Figure 3: Depiction of mucoadhesive drug delivery system

While designing mucoadhesive drug delivery systems, dosage form must be small and flexible enough to be acceptable for patients and should not cause irritation. Other valuable features of a mucoadhesive dosage form include a high drug loading capacity, controlled drug release (preferably unidirectional release), better mucoadhesive properties, smooth surface, tastelessness and convenient application. Erodible formulations offer more benefits as they do not require system retrieval at the end of desired dosing interval. Numerous valuable mucoadhesive dosage forms have been developed for a variety of drugs. Several typical molecules such as peptides, including thyrotropin-releasing hormone (TRH), insulin, octreotide, leuprolide, and oxytocin have been successfully given via the mucosal route, though with relatively low bioavailability (0.1–5%) due to their hydrophilic characteristics and large molecular weight as well as the inherent permeation and enzymatic barriers of the mucosa[40]. Awasthi *et al* developed hollow floating gliclazide beads using a simple ionotropic gelation technique using a combination of low methoxyl pectin and hydroxypropylmethyl cellulose. Particle size analysis of beads revealed the size of dry beads in the range of 730-890µm based on the composition of polymer and concentration of calcium carbonate. The authors also concluded that the mean diameter of beads increases with increase in the concentration of gas forming agent which may be attributed to the increased viscosity of solution. The authors used fourier transform infrared (FTIR) spectroscopy, differential scanning calorimetry (DSC) and X-ray diffraction technique to confirm the drug's stable character. Formulations were assessed for mucoadhesion using goat stomach mucosal membrane. The optimized formulations showed excellent bioadhesive properties for a duration of 2h in the mucoadhesion experiment. The *in-vitro* drug release

represents Fickian diffusion with swelling[41]. Glipizide microspheres were also developed by Patel *et al* through simple emulsification phase separation method using chitosan as polymer for mucoadhesion and glutaraldehyde as a cross-linking agent. A full factorial 3^2 design was used using two independent variables (polymer-to-drug ratio and stirring speed) and four dependent variables (percentage mucoadhesion, t_{80}, drug entrapment efficiency). The best exhibited formulation showed entrapment effectiveness of 75%, a swelling index of 1.42, a mucoadhesion of 78% after one hour and the drug release also maintained up to 12 hours. The in vivo research in Wistar mice also reveals an important hypoglycemic impact up to 12 hours[42]. Prasanthi *et al* developed mucoadhesive microspheres of linagliptin by ionotropic gelation and single emulsion methods using synthetic polymers like carbopol 934P, guar gum, HPMC K100M and sodium carboxy methyl cellulose. Spherical free flowing microspheres of linagliptin were successfully prepared by emulsification method. The developed mucoadhesive microspheres has swelling index of 1.03, entrapment efficiency of 85±0.57% and the mean particle size of 135±6µm. Mucoadhesion strength was found to be 87% in 7 hrs and drug release was shown to be 98.2±0.63% in 8 hrs and release kinetics followed anomalous transport mechanism. Radiographic studies were performed in rabbits and microspheres retained for 7 h in the rabbit stomach as confirmed by the images[43]. Sarkar *et al* developed poly (acrylic acid)-grafted-gellan-based gastroretentive continuous release tablets of metformin hydrochloride based on swelling and mucoadhesion. Firstly authors synthesized poly (acrylic acid)-grafted-gellan by microwave-promoted cerric (IV) ion initiated graft copolymerization technique. The yield of polymer was found to be dependent on concentration of cerric (IV) ammonium nitrate and acrylic acid. Further they developed mucoadhesive sustained release tablet using metfomin hydrochloride as drug by wet granulation. The formulations developed showed sustained release potential in simulated gastric fluid (pH 1.2) over a period of 10 hours[44].

4. Dual (floating and bioadhesive) systems:

These systems are usually available in the form of mucoadhesive beads, ion resin complexes, microspheres, films, and tablets which based on the combination of floatation and mucoadhesion properties together in a single dosage form. Sonar *et al* developed a bilayer, floating-bioadhesive dosage form based on a unique combination of floating and bioadhesive properties to extend the residence of rosiglitazone maleate in the stomach. The formulation comprises of two layers one is floating layer and other one is sustained release layer. Floating layer was prepared using 5% w/v PVP ethanolic solution and hydroxyl propyl methyl cellulose (HPMC) and sodium bicarbonate were added in the preparation to sustain the drug release. The optimized formulation showed a satisfactory profile of dissolution, detachment force, floating properties and followed first order drug release. The tablets stayed floating in the stomach for up to 8 hours, as determined in healthy human volunteers by gamma scintigraphy[45]. Sah *et al* developed floating sitagliptin microsphere applying 3^2 factorial design to improve the gastric residence time and subsequent absorption of the drug in the stomach. The authors prepared microspheres using HPMC K4M and psyllium husk as swelling agents by ionotropic gelation method. In X-ray imaging in rabbits initially and after 24 hours of administration of dosage form, microspheres were found retained in the stomach. During *in-vivo* study sitagliptin was detected in plasma from swellable gastroretentive microsphere till the end of 24 hours post-administration, while the plasma concentration of conventional microsphere of sitagliptin was detectable till 12 hours post administration. The swelling of the developed formulation was found to be 191% to 240% and for the optimized formulation, *in-vitro* release of drugs was found to be 99.2% after 24 hours[46].

5. In-Situ Gelling systems (Raft Forming system):

Another method of gastroretention with excellent patient compliance is the in-situ gelling systems (also known as the raft forming system figure 4). These structures comprise of sodium alginate as polymer-forming gel in-situ with carbonate or bicarbonates as effervescent substances. These systems are in the solution form initially but when they come in contact the gastric fluid, they get swell to form viscous cohesive gel and generate carbon dioxide bubbles these bubbles are caught in the gel which cause drug delivery systems to float. Raft forming systems are used mostly for gastroesophageal treatment because they are likely to produce a layer on top of the gastric fluid[47,48]. Metformin hydrochloride floating gastro-retentive tablets of based on in-situ gel technique were developed by Senjoti *et al*. Box-Behnken experimental design was used to develop tablets with effervescent and swelling properties using a combination of sodium bicarbonate and HPMC-PEO (Poly Ethylene Oxide) polymer. The developed tablets were able to float within 4 minutes, remain in floating condition for 24 h and sustained the release of drug for 12 hours. The authors also concluded that amount of polymer matrix (amount of HPMC and PEO), effervescent agent (sodium bicarbontate), and swelling enhancer (SSG) affected and floating and drug release from the formulation[49].

In-situ miglitide formulations, using gellan gum and sodium alginate as gelling agent, propylene glycol as co-solvent and calcium carbonate were developed by Mahmoud *et al*. Calcium carbonate dissociates in the acidic environment to release carbon dioxide which gets

entrapped in the gel and helps in the floating. Developed formulations showed reasonable viscosity and formed a firm gel that floated over the surface in seconds and remained floating for 24 hours when it came into contact with simulated gastric fluid. *In-vitro* dissolution was achieved at 900 mL 0.1N HCl (pH 1.2), and the release of all formulations exceeds 70% in 24 hours. The *in-vivo* pharmacokinetic experiments were performed in 10 New Zealand rabbits and the results reveal 1.79 fold increase in C_{max} and 18.4 fold increase in AUC with the newly developed in-situ formulation compared to the one on the market. The authors concluded that use of propylene glycol in the formulation helped in solubilization the water insoluble drug[50].

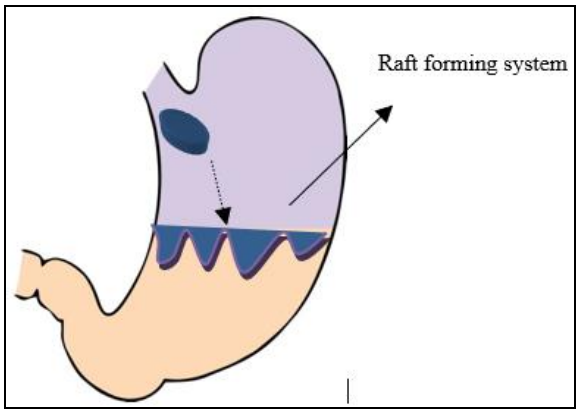

Figure 4: Depiction of raft forming drug delivery system

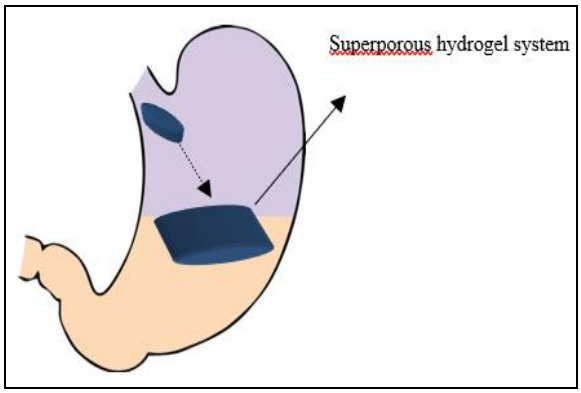

Figure 5: Depiction of superporous hydrogel swelling systems

6. Superporous Hydrogels:

Superporous hydrogel (figure 5) is characterized by a three-dimensional network of hydrophilic polymer, which absorbs a huge amount of water in a short period of time, due to the presence of interconnected microscopic pores. These highly swollen hydrogels remain in the stomach for a long period of time when used as drug carriers, releasing virtually all loaded drugs, large volumes and sheer bulk of these hydrogels does not allow them to be transported through the pylorus to the next organ. This distinct swelling characteristic enables them to be used as gastro-retentive carriers that provide a sustained release through extended gastric retention. Not only are hydrogels needed to swell rapidly, but they should also be biocompatible, biodegradable, better swelling properties, strong mechanical strength and remain static in acidic situation[51]. Park *et al* developed hydrogels of chitosan-glycol and chitosan by a gas blowing method and studied their swelling behaviours in acidic solution to explore their use as a stomach retention dosage form. Firstly the stock solution of chitosan and glycol-chitosan were prepared dissolving the former in 0.01M acetic acid and latter in water respectively. The pH of the solution was adjusted to 5 by adding acetic acid, then sodium bicarbonate (blowing agent) is added to the solution and mixed vigorously by stirring for 30seconds. Foaming started immediately and completed in 2 minutes. The formed hydrogels were stored at room temperature and freeze dried before use. Both the chitosan and glycol chitosan hydrogels revealed higher swelling ratio in acidic environment than in distilled water probably due to the cationization of amine groups in acidic environment. The results also indicated that swelling of hydrogels with increasing density of crosslinking agent[52].

Gupta *et al* formulated chitosan/poly(vinyl alcohol) interpenetrating polymer-type superporous hydrogels of Rosiglitazone maleate utilizing glyoxal as crosslinker. To induce the porous structure, sodium bicarbonate was used as a foaming agent. The swelling of the formulation depended on the amount of chitosan and crosslinker. The drug release from the superporous hydrogels was maintained for 6 hours successfully[53].

7. Magnetic Systems:

These devices contain a tiny inner magnet in the design and an external magnet is positioned on the abdomen above the stomach location. Gastro retention of the dosage form can be improved by applying an extracorporeal magnet for localization to a specific part. The site of magnet should be designated with very high precision to acquire the desired results in such systems. Thus, the factual significance of such systems is frequently doubtful[54]. Angelopoulou *et al* developed magnetically targetable nanocarriers of the sodium-glucose transporter protein (SGLT2) inhibitor dapagliflozin for the selective delivery of dapagliflozin in tumors. The dapagliflozin-loaded PMMA-g-PEGMA nanoparticles showed concentration-dependent toxicity against the A549 cancer cell line. The application of an external magnetic magnet increases the uptake of nanoparticles by cells, leading to increased cytotoxicity. The developed nanoparticles exhibited satisfactory drug loading efficiency and high colloidal stability. Dapagliflozin release from the nanoparticles responded to an AC magnetic field to induce dapaglifozin release in close proximity to the tumor area confirming accumulation of the dapaglifozin-loaded nanoparticles at the tumor cells due to magnetic targeting[55].

CONCLUSION:

Gastro-retentive drug delivery systems are successfully developed and reported for antidiabetic drugs by researchers and they can do wonders if the same are available commercially. These systems are very useful for the drugs which are absorbed from the stomach. Further investigation in human volunteers still needs to done before scaling up them in to commercials. However with the advent new molecules like DDP-4 and SGLT2 inhibitors there has been a significant progress in the field of drug discovery. The formulation work is still in progress to develop the gastro-retentive dosage form of these drugs which can be commercialized. With increasing population of diabetics there is further scope of development in this field for the economical products with better therapeutic results and reduced side effects especially for prolonged use.

CONFLICT OF INTEREST:

The authors confirm no conflict of interest.

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REVIEW ARTICLE

Bi-layer Tablets: A Flexible Technology for Oral Drug Delivery– An Updated Review

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ABSTRACT:
Bi-layer tablet is one of the new technologies for the successful development of controlled release formulations along with various features. Bi-layer tablet is suitable for sequential release of two different drugs in combination, separate the two incompatible substances and also suitable for the sustained release tablet in which one layer will be immediate release which serves as initial dose and second layer is maintenance dose. Nowadays the use of this bi-layered tablet technology has been enlarged. Bi-layer tablet is useful for gradual release of two active ingredients in combination. Two different incompatible drugs can also be formulated into a bi-layer tablet by adding an inert intermediate layer. Bi-layer tablets serves as one of the principle option to avoid chemical incompatibilities between the API's which is mainly achieved by physical separation, and also helps to enable the progress of different drug release profiles (immediate release with extended release). In spite of their advantages, the mechanical structures of this drug delivery system have become quite difficult, requiring complicated tablet architectures due to the use of different materials and also the complex geometric boundaries. Bi-layer tablets provide specific advantages when compared to the conventional release formulation of the same drug. A number of pharmaceutical companies are now currently developing bi-layer tablets.

KEYWORDS: Bi-layer tablets, Formulation, Controlled release, immediate release.

INTRODUCTION:
In most recent decade, more attention in developing a combination of two or more Active Pharmaceutical Ingredients (API) in a single dosage form has been improved in the pharmaceutical industries, thereby promoting the patient compliance. Bi-layer tablets can be the primary choice to avoid the chemical incompatibilities between the APIS and also to improve the release profiles (immediate release with extended release). This is achieved by physical separation of two different API.[1]

Bi-layer tablets are always suitable for sequential release of two drugs in combination and it also has the ability to separate two incompatible substances. Bi-layer tablets consists of two different layers in which one layer is immediate release layer for initial loading dose and sustained release layer for maintenance dose.

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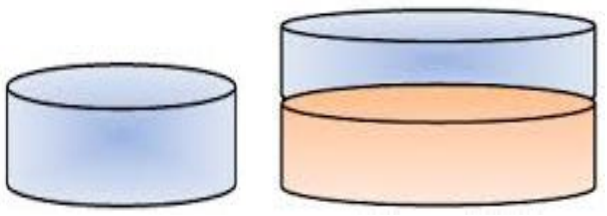
The immediate release layer are added with some super disintegrants like sodium starch glycolate, croscarmellose sodium etc., which promotes the drug release rate and also helps to attain the onset of action rapidly (loading dose) whereas, sustained release layer releases the drug in sustained manner for prolonged period of time (maintenance dose).[2]



Conventional tablet **Bilayer Tablet**

Need for Bi-layer tablets

- To control the rate of drug delivery either of single or two different API's.
- To spate the incompatible API from each other and also to control the release of API from one layer by using the functional property of the next layer such

as, osmotic property.

- To fabricate novel drug delivery systems such as chewing device, mucoadhesibve delivery systems, and floating tablets for gastro-retensive drug delivery.
- To administer fixed dose combinations of different APIs and also to prolong the life cycle.
- To modify the total surface area available for API layer either by sand witching with one or two inactive layers in order to achieve swellable/ erodible barriers for controlled release.[3]

Advantages of the Bilayer Tablet[1,4]

- Greatest chemical and microbial stability compared to other oral dosage forms.
- Cost effective when compared to other dosage forms.
- Bi-layer tablet is suitable for preventing direct contact of two drugs and thus to maximize the efficacy of combination of two drugs.
- Offer greatest precision and the least content uniformity.
- Easy to swallow.
- Bi-layer execution with optional single layer conversion kit.
- Bi-layer tablets can be designed in such a manner as to modify the release as either of the layers can be kept as extended and the other as immediate release.
- Flexible concept.
- The weight of each layer can be accurately
- controlled, in the contrast to putting one drug
- of a combination product in a sugar coating.
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- of a combination product in a sugar coating.
- The weight of each layer can be accurately controlled.

Disadvantages of the Bilayer Tablet:

- Some drugs resist compression into dense compacts, owing to amorphous nature, low density character.
- Drugs with poor wetting, slow dissolution properties, optimum absorption high in GIT may be difficult to formulate or manufacture as a tablet that will still provide enough or full drug bioavailability.
- Adds complexity and bi-layer rotary presses are expensive.
- Insufficient hardness, layer separation, reduced yield.
- Imprecise individual layer weight control.
- Cross contamination between the layers.
- Drugs with an objectionable odor, bitter taste or drugs that are susceptible to oxygen may require encapsulation or coating.

General properties of bilayer tablet dosage form[5]**:**

i. It should have sufficient strength to withstand mechanical shock during its production, packaging, shipping and dispensing.
ii. The bilayer tablet should release the drug in an expectable and reproducible manner.
iii. It should have physical and chemical stability.
iv. It should have a chemical stability shelf life.
v. It should have attractive product identity free of defects like chips, cracks, discoloration, and contamination.

Various techniques for bi-layer tablet:

OROS® push pull technology:

This system mainly consists of 2 or 3 layers in which one or more layer is essential for the incorporation of the drug or API and the other layer consist of the push layer (Fig.2). The drug layer usually consists of the drug or the API along with two or more different agents. So this drug layer is in poorly soluble form.

Further the suspending agent and osmotic agent are also added. A semi permeable membrane environs the tablet core.[6]

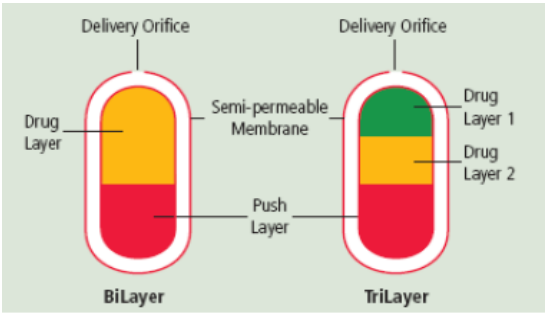

Figure 2: OROS® push pull technology

L-OROS™ technology:

This system is mainly used for the solubility issue. Alza introduced the L-OROS system which is a lipid soft gel product containing the drug present in a dissolved state is initially being manufactured and then it is coated with a barrier membrane, then with osmotic push layer and with a thin semi permeable membrane, finally drilled with an exit orifice.[7]

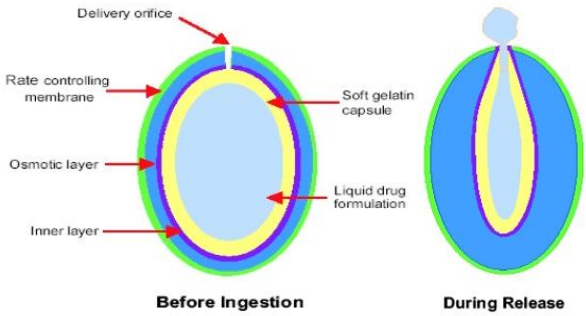

Figure 3: L-OROS™ technology

DUROS Technology:

The system consists of an outer cylindrical titanium alloy reservoir. This reservoir has high impact strength and also protects the drug molecules from enzymes. The DUROS technology is one of the miniature drugs dispensing system which opposes like a miniature syringe and release minute amount of drug in a concentrated, continuous and consistent from over months or year.[8]

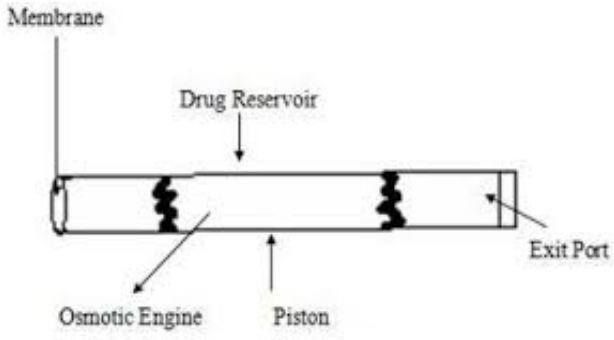

Figure 4: DUROS technology

EN SO TROL Technology:

Shire laboratory use an integrated approach to drug delivery for the improvement of an order of magnitude or to create optimized dosage form that mainly focus on the identification and incorporation of the identified enhancer into controlled release technologies. [9]

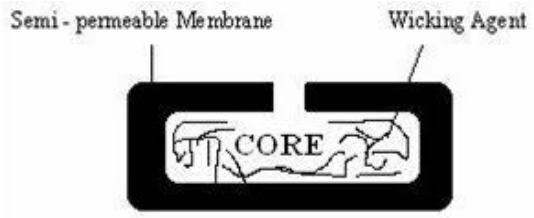

Figure 5: EN SO TROL technology

DUREDAS™ Technology:

"Elan drug technology is a dual release drug delivery system. DUREDAS™ Technology is a bi-layer tablet that provides immediate or sustained release of two drugs with different release rates of the same or different drugs in one dosage form. This method can provide an immediate release granulate and a modified release hydrophilic matrix complex as separate layers within the one tablet. The modified-release properties of the dosage form are provided by a mixture of hydrophilic polymers. [10]

Various Types of Bilayer Tablet Press:

- Single sided tablet press
- Double sided tablet press
- Bi-layer Tablet Presses With Displacement Monitoring

Single sided tablet press:

This is a simplest design consisting of single-sided press with both chambers of the double feeder separated from each other. The chambers having different powders are gravity-or forced-fed and thus produces the two individual layers of the tablet. The die passes under the feeder, which initially compressed the first-layer powder and then the second layer powder is being compressed. Finally the whole tablet is compressed in one or two steps (two = pre- and main-compression). Both the layers in the die mix considerably at their interface and bond sufficiently so that no layer-separation occurs when the tablet is produced. This is one of the easiest ways of producing a bi-layer tablet.

Limitations of the single sided press:

- No weight monitoring/ control of the individual layers.
- No distinct visual separation between the two layers.
- Very short first layer dwell time due to small compression roller, possibly ensuring in poor de-aeration, capping and hardness problems.
- Very difficult first-layer tablet sampling and sample transport to a test unit for in-line quality control and weight recalibration.[11]

Double sided tablet press:

A double-sided press consists of an individual fill station, pre – compression and main compression for each layer. The bi-layer tablet will go through four compression stages before it gets ejected from the press. Most of the double-sided tablet presses with automated production control use compression force to monitor and manage tablet weight. The peak compression force which is exerted on each individual tablet or layer is measured by the control system at main compression of the layer. This calculated peak compression force is the indication used by the control system to refuse out of tolerance tablet and correct the die fill depth when mandatory.

Advantages:

- Displacement weight monitoring for accurate and independent weight control of the individual layer.
- Low compression force exerted on the first layer to avoid capping and separation of the individual layer.
- Increased dwell time at pre compression of both first and second layer to provide sufficient hardness at maximum turret speed.
- Maximum prevention of cross contamination between two layers.
- A clear visual separation between two layers.

Limitations:

- The first layer is compressed at a low compression force to obtain correct bonding, so that this layer can still interact with the second layer during final compression.
- Bonding is too restricted if first layer is compressed at a high compression force.[12]

Bilayer tablet presses with displacement monitoring:

This tablet press worked on principle displacement tablet weight control, which is different from the principle of compression force. As based on standard sensitivity of control system does not depend on the tablet weight but depends on applied pre compression force. In this case the risk of capping and separation increases at higher production speed increases but can be reduced by sufficient dwell time at all four compressions.

Advantages:

- Principle useful for accurate independent weight control of the individual layers.
- Appropriate bonding is possible as there is use of low compression force applied on the first layer avoids capping and separation of the two individual layers.
- Maximum production yield and clear visual separation between layers in tablet.
- Avoid cross contamination between the layers.
- Ability to build adequate hardness to tablet at maximum turret speed by increased dwell time at pre-compression of both first and second layer.
- There is no any effect of stiffness on bilayer tablet. [13]

Evaluation of Bilayer Tablets:

1. General Appearance:

The general appearance of a tablet, its visual uniqueness and overall "elegance" is essential for consumer acceptance. It mainly includes tablet's size, color, shape, presence or absence of an odor, taste, physical flaws, surface structures, consistency and legibility of any identifying marking.

2. Size and Shape:

Dimensionally, the size and shape of the tablet can be described, monitored and controlled.

3. Tablet thickness:

Tablet thickness is one of the important characteristic in reproducing appearance. Some of the filling equipment makes use of uniform thickness of the tablets as a counting mechanism. Ten tablets were taken and their thickness was recorded using micrometer.[14]

4. Weight variation:

Standard procedures are followed as described in the official books.

5. Friability:

Friction and shock are the forces that most common cause for the tablets to chip, break or cap. The friability test is related to tablet hardness and is considered to evaluate the ability of the tablet to resist abrasion during packaging, handling and shipping. It is usually calculated by using the Roche friabilator. A fixed number of tablets are weighed and placed in the apparatus where they fall at a height of 6 inches in each turn within the apparatus. After four minutes or 100 revolutions, the tablets are again weighed and this weight is then compared with the initial weight. The loss of weight due to abrasion is calculated as a measure of the tablet friability. The value is expressed in percentage. A maximum value of not more than 1% is considered generally acceptable and any broken or smashed tablets are not taken. The loss in the weight of tablet is calculated as the measure of friability and is usually expressed in percentage as:

% Friability = 1- (loss in weight/Initial weight) X 100[15]

6. Stability Study (Temperature dependent):

The bi-layer tablets are packed in suitable packaging and should be stored under the following conditions for a period as prescribed by ICH guidelines for accelerated studies.

Study	Storage condition	Minimum time period covered by data at submission
Long – term	25°C ±2°C/40% RH ± 5% RH or 30°C ±2°C/35% RH ± 5% RH	12 months
Intermediate	30°C ±2°C/65% RH ± 5% RH	6 months
Accelerated	40°C ±2°C/not more than (NMT) 25% RH	6 months

After a period of 15 days, the tablets were withdrawn and analyzed for physical characterization (Visual defects, Hardness, Friability and Dissolution etc.) and also for drug content. The data obtained is fitted into first order equations to determine the kinetics of degradation. Accelerated stability data are plotting according Arrhenius equation to determine the shelf life at 25°C.[15]

7. Uniformity of Weight:

Average weight of twenty tablets which was selected at randomly was calculated. Weight Variation of these tablets was calculated and was compared with I. P. standards.

8. Tablet Hardness:

The hardness of a tablet influences the resistance to shipping or breakage under conditions of storage, transportation and handling before usage. Monsanto hardness tester is used to measure the hardness of each tablet. The hardness was measured in kg/cm. [16]

CONCLUSION:

Bi-layer tablet technology is one of the excellent techniques for providing the combined release pattern of drug involving both immediate release and sustained release. Bi-layer tablet technology is one of the important design approaches in which different drugs with different indications, incompatible drugs and same drug with different release rate (e.g. IR and ER) can be designed in a single unit. Bi-layer tablet is enhanced beneficial technology to overcome the limitation of the single layered tablet. Bi-layer tablet is suitable for the gradual or sequential release of two different drugs in combination, also suitable for separating two incompatible substances and also for sustained release tablet in which one layer is for loading or initial dose and second layer is for the maintenance dose. The formulation of tablets in the form of multi layers is used to offer systems for the administration of drugs, which are incompatible and to provide controlled release tablet preparations by providing surrounding or multiple swelling layers. A complete mechanistic understanding must be developed to develop a dynamic bi-layer tablet through the application of scientific and quality risk management tools.

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REVIEW ARTICLE

A Comprehensive Review on Pancreatic Cancer

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ABSTRACT:
Pancreatic cancer is characterized as a fatal disease where the healthy cells of the pancreas stops functioning and undergoes uncontrollable cell division. Three types of pancreatic tumors are in existence. They are pancreatic neuroendocrine tumor, pancreatic ductal adenocarcinoma and exocrine tumor. Pancreatic cancer stands in the 14th position in causing the most general cancer. In India, the pancreatic cancer incidence is measured at a low point i.e. (0.5-2.4 per 100000) in males and (0.2-1.8 per 100000) in females. Diabetes, smoking habit, excessive alcohol consumption for prolonged years, cholecystectomy, gastrectomy and low intake of fruits and vegetables are considered to be the major risk factors. Smoking termination will be the powerful action to decrease the opportunity of pancreatic cancer. Pancreatitis will lead to the pancreatic cancer. Pancreatitis and diabetes acts as a background disease in the pancreatic cancer. To diagnose pancreatic cancer, Computed Tomography, Magnetic Resonance Imaging technique acts as a first-line and second-line techniques respectively. Surgical treatment is the only treatment believed to be producing the potential cure, with the combination of chemotherapy. The medications prescribed as per USAD are Capecitabine, 5-Flurouracil, Irinotecan, Leucovorin, Oxaliplatin, Nab-paclitaxel. Pancreatic cancer is recognized as life threatening disease in both developed and developing countries.

KEYWORDS: Pancreatic cancer, Surgical treatment, Endocrine tumor, Pancreatitis.

INTRODUCTION:
Pancreatic cancer is characterized as a fatal disease where the healthy cells of the pancreas stops functioning and undergoes uncontrollable cell division. Detecting the pancreatic cancer in the early stages may avoid the surgery procedures. It is evident that family members such as first-degree relatives of the individuals will end up with malignant disease of pancreatic cancer. The pancreatic cancer occurrence and the mortality rate have been progressively increasing when it compared with the other disease. Only 4% of patients will survive up to 5 years even though when there is progression in finding and management of pancreatic cancer.(1) From all type of cancer, pancreatic cancer is considered to be in the 5th position in causing death across the globe.

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Bioactive compounds The risk factor and causes of the pancreatitis differs with the age and gender. Pancreatitis may lead to the development of pancreatic cancer. The alcohol de-dependence and smoking termination may change the growth of the pancreatitis and may have the possibility of reoccurrence. Smoking termination will be the powerful action to decrease the opportunity of pancreatic cancer.(2) High number of pancreatic cancer patients go on asymptomatic upto the time of disease extends to the advance stage. Still now, there is no quality strategy to screen the pancreatic cancer. Pancreatic cancer has 4 primary driving genes they are KRAS, CDKN2A, TP53, and SMAD4.(3) The effective combined radiation and fluorouracil for pancreatic cancer is recommended by the members of gastrointestinal tumor study group.(4)

TYPES:
The organ pancreas is defined as a gland which is mainly consists of two types of cells such as endocrine cells and exocrine cells. These cells may probably progresses to

three main tumors. They are pancreatic neuroendocrine tumor, pancreatic ductal adenocarcinoma and exocrine tumor. Pancreatic neuroendocrine tumor emerges from the pancreatic endocrine part. And on the opposite side pancreatic exocrine neoplasm induce pancreatic ductal adenocarcinoma and acinar cell carcinoma. In the pancreatic cancer, pancreatic ductal adenocarcinoma is considered as the most general type. And this type of cancer causes the more death.[5] Another type of pancreatic cancer is IPMN (Intra-ductal papillary mucinous neoplasm). This IPMN type of cancer is generally diagnosed is most pancreatic cancer patients. IPMN-Intra-ductal papillary mucinous neoplasm will generally develop within the pancreas duct and produces mucin which is a thick fluid portion. This IPMN (Intra-ductal papillary mucinous neoplasm) is not cancerous at the beginning stage, if it is left untreated it gets developed into cancerous stage.[6]

EPIDEMIOLOGY:

Pancreatic cancer stands in the 14th position cancer. Based on the cancer mortality it stands in the 7th position. According to the Globocan database report, there is 458918 cases diagnosed and caused 432242 deaths in the world wide in the year 2018.[7] The pancreatic cancer's incidence rate differs from each countries. Europe and North American Population were highly affected when compared to other countries.[8] In India the pancreatic cancer incidence is measured at a low point i.e (0.5-2.4 per 100000) in males and (0.2-1.8 per 100000) in females.[9]

RISK FACTORS:

The smoking habit and low intake of fruits and vegetables are considered to be the major risk factor for development of carcinoma of the pancreas. Age is considered to be a well known risk factor.[10] Diabetes is also a risk factor probably which will lead to the tumor. Cholecystectomy and gastrectomy is also considered to be a possible risk factor.[11] According to research studies family history also favors the pancreatic cancer.[12] BRCA2 mutations can be seen as a risk factor in utmost cause Pancreatitis and diabetes acts as a background disease in the pancreatic cancer.[13]

SIGNS AND SYMPTOMS:

Pain, Anorexia, Early satiety, Xerostomia, sleeping problems and weight loss are considered to be the general symptoms.[14] The other symptoms are confidence interval, pale stools, Abdominal pain, Jaundice, unusual bleeding unusual bleeding, dark urine, constipation, Fatigue, unusual heart brain, altered ability to sleep, itching, Diarrhea.[15][16]

PATHOLOGY:

Almost 60-70% of pancreatic adenocarcinoma emerge in the pancreas head region and remaining (15%) arise in the body portion and other (15%) found in the tail portion. When diagnosing pancreatic adenocarcinoma it has spreaded earlier beyond the pancreas.[17]

PanIN:

Pancreatic intraepithelial neoplasia plays a key role in the progression of local pancreatitis. In this local pancreatitis men has a chance of 1.5% and women has a chance of 1.3% chance of progressing from panIN to identifiable pancreatic adenocarcinoma in their life span.

IPMN:

Intraductal Papillary Mucinous Neoplasm (IPMN'S) is also well recognized precursor lesions for the pancreatic cancer. These IPMN'S are emerging from the one of the side branches of main pancreatic duct or it arises from the main duct of the pancreas. [18]

DIAGNOSIS:

The techniques involved in diagnosing pancreatic cancer are Computed Tomography and Magnetic Resonance Imaging technique. Computed Tomography acts as the primary line imaging method for diagnosing susceptible pancreatic cancer and Magnetic Resonance Imaging technique acts as the secondary technique. Computed Tomography is highly sensitive in detecting pancreatic cancer up to the range of 96% and Magnetic Resonance Imaging technique is sensitive up to the range of 93.5%.[19]

Endoscopic Ultrasonography, Endoscopic retrograde pancreatography, Angiography, Ultrasonography these techniques are also involved in diagnosing pancreatic cancer. In Endoscopic Ultrasonography, the lesions were detected accurately even when the lesion were below 20mm in diameter. In Ultrasonography technique, pancreatic cancer were easily identified when the tumor size is above 30mm, but it is quite complicated in tumor which is below 20mm in size.[20]

TREATMENT:

The surgical treatment is the only method for treating the pancreatic cancer and it is believed to be producing the potential cure, with the combination of the chemotherapy which will show slow improvement in the survival rate. The various treatment for managing the pancreatic cancer are surgical management and Medical management.[21]

SURGICAL MANAGEMENT:

The surgical management is only possible around one in fifth (20%) of the cases. It is more complicated to remove the tumor successfully from the pancreas.

Because the outer layer of the pancreas still containing the malignant cell which we can find in the CT scan, which it may help in the survival rate of the patients. The process of removing the cancer cell from the pancreas head is known as Pancreaticoduodenectomy.

The pancreaticoduodenectomy contains three stages which involves the removal of the cancer cell. The three stages are Exploration to asses for extra regional spread which is not identified on preoperative imaging where the cells are identified and the resection of the tumor is carried. The reconstruction of the pancreaticobiliary and intestinal tracts are done. These is the process which is done for the pancreaticoduodenectomy. According to the cancer cell, the surgical procedure is done, the various types of the surgical process are the Hemigastrectomy, Pylorus preserving, venous reconstruction.

If the cancer cells are spreads through the spleen and the pancreas the Distal pancreatectomy is used in this process, the spleen and the pancreas is removed and the reconstruction is done and the adjuvant therapy method is used.[22]

MEDICAL MANAGEMENT:

The Adjuvant Therapy indicates that there is decrease of the risk of low regional recurrence and the metastatic recurrence. These therapies are typically given to the patient for 1-2 months after the surgery because to allow the patients to get recover from the complex of the surgery. The drugs used for the treatment are based on the chemotherapy. Mostly Gemcitabine is used in combination with 5-FU(5-Fluro Uracil). These drugs are used for the recovery of the patients form the cancer cells. These drugs are prescribed to the patients for about 6 weeks after the surgery procedures.

MEDICATION:

The mentioned drugs are as per the USAD are Capecitabine, 5-flurouracil, Irinotecan, Lencovorin, Oxaliplatin, Nab-paclitaxel. [23]

CONCLUSION:

Pancreatic cancer is considered as the most general disease in both developed and developing countries. Etiology is not understood clearly, still further more research are required for understanding the pancreatic cancer thereby it will be helpful in treating pancreatic cancer. However Genetic susceptibility acts as a main risk factor. PanIn, IPMN and MCN are considered as well-known precursors to the cancer of pancreas. Detecting the cancer patients with the lesions in the beginning stage it will avoid the unwanted surgery and also lowers the risk to the pancreatic cancer patients. In future, we can do research in finding the potent inhibitor of this pancreatic cancer.

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REVIEW ARTICLE

Foeniculum vulgare, Solanum nigrum and *Cichorium intybus*: A Collectanea of Pharmacological and Clinical uses

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ABSTRACT:

Herbal medicine is the core nucleus of holistic medicine which is gaining rapid popularity all over the world and gradually incorporating and integrating its position in the mainstream health care systems. Herbal medicines include plant and their parts that contain active ingredients alone or in combination to increase the efficacy, prevention and treatment of the disease. Analogous to conventional medicines herbal medicines are employed for various illness like cold, inflammation, diabetes, malaria, tuberculosis, cancer and so on. Indian traditional medicines or medicinal plants are indispensable source of new drugs. *Foeniculum vulgare* also known as Fennel, a member of Apiaceae family is used for various health benefits such as, it helps to regulate blood pressure, reduce water retention, improve eyesight and helps in reducing acne, constipation, arthritis, cough, gingival wound etc. *Solanum nigrum* also known as Makoh, a member of Solanaceae family is used in diverse medical conditions such as used to treat fever and to alleviate pain, used in treating the mouth ulcers, good herbal remedy for asthma, is the main ingredient in various medicines prepared to treat various liver disorders, helps in treating UTI and has excellent hepatoprotective and anti-inflammatory activity. *Cichorium intybus* also known as Kasni, a member of Asteracece family is used in inflammation, hepatomegaly, digestive ailments, malaria, gout, sedative. It is a remedy for high blood pressure, constipation, liver disorders, gallbladder disorders, skin inflammation, loss of appetite, heart failure and cancer. These drugs provide a noteworthy basis in pharmaceutical biology for the development and formulation of new drugs for future clinical uses.

KEYWORDS: *Foeniculum vulgare, Solanum nigrum, Cichorium intybus*, herbal medicine, conventional.

INTRODUCTION:

Herbal medicine means herbs and herbal preparation that contain active ingredients of plants to increase the efficacy, prevention or treatment of the disease. Herbal medicinal products and its supplements has been used since centuries and are extracted for providing maximal benefits to mankind[1]. Herbal medicines are used across the world and continues to amplify rapidly and many people have now resorted to these medicines and its product for various illness[2]. People living in developed countries like Canada and developing countries like India and Pakistan rely on herbal product and phytonutrients and there is an estimate that up to five billion people use it as a traditional medical practice and key source of healthcare[2].

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Indian traditional medicines or medicinal plants are indispensable source of new drugs. Conventional use of medicinal herb is essential for the people.

According to WHO there is a global increase in the use of medicinal herbs for various health problems. It involves the use of different plant parts like roots, stems, leaves, seeds, barks, berries or flower for medicinal purpose for the prophylaxis or treatment of the disease[3]. For Example, Cinchona bark contain various chemical constituents like quinine, quinidine, cinchonine etc. Quinine is used to treat malaria whereas quinidine is used in cardiac arrhythmia.

Herbal treatment is usually aimed at producing persistent improvement in well-being. Plant extracts are shown to have many effects like antioxidant, hepatoprotective, cardioprotective, vasodilatory, anti-inflammatory, anti-microbial, anticonvulsant, antipyretic[4].

Traditional medicine or Herbal products has been widely used to treat various health problems in many developing countries like India, Pakistan, USA, Canada[2].

India is the mother hub for many herb-based systems like Ayurveda, Unani, Siddha and Homeopathy[5].

Traditional Medicinal System involve plants as drug source in treatment therapy. One such system is Unani System of medicine.It isa well-known traditional system of medicine and draws its origin from ancient traditional system of medicine of Egypt, Iraq, Persia, India, Syria and China[6-7]. Unani system of medicine use theories of Hippocrates, known as the Father of Unani Medicine. It works on the principle of four humors black bile, yellow bile, phlegm and blood and treats person as a whole and not as a group of individuals parts[7-9].

According to Unani System of medicine there exist seven compartments (Umoor-e-Tabiya) that works as a single unit[7][10]. Here, the body is built up of four basic elements i.e. Air, Earth, Fire and Water and these four elements have different temperaments i.e. Hot, Cold, Dry and Wet respectively[7][11]. When these four temperaments are interacted and mixed together a new compound is formed having new temperamenti.e. hot wet, hot dry, cold wet, cold dry[7][10][11]. For Example, Unani formulation Majoon-e-sandal contain *Santalum album, Tamarindus indica, Punica granatus, Bambusa bambos and Crocus sativus*is used as a stomachic, antibilious, psychoneurosis and in vomiting and nausea[12]. In this review we will see the pharmacological and clinical uses of fennel, black nightshade and chicory.

Fennel:

Botanical name *Foeniculum vulgare*, a member of Apiaceae family. *Foeniculum vulgare* also known as fennel (English) saunf (Hindi) and badyan (Urdu) is a biennial medicinal and aromatic plant[13-14]. It is a flavourful and culinary herb with many medicinal properties like carminative, stomachic, etc.

Fennel is cultivated in Argentina, America, Germany, China, Indonesia, Russia, Japan and Pakistan.

In India the fennel cultivating states are Haryana, Punjab, Himachal Pradesh, Maharashtra and Uttar Pradesh[15].

Phytochemistry:

Foeniculum vulgare contain 6.4% of moisture, 9.50% protein, 10% fat, 13.40% minerals, 18.50% fibre and 42.32% carbohydrates and 3-8% of volatile oil. It contains high concentration of minerals and vitamins like sodium, potassium, calcium, phosphorous, iron, vitamin B1, vitamin B2, vitamin B3 and vitamin C[16].

The most abundant macronutrient is carbohydrate and proteins, reducing sugar and fats are the least abundant macronutrient. Stems and leaves have the highest while inflorescence exhibit the lowest moisture content[14].

The main volatile oils are fenchone, ketone, estragol, phenolic ether anethole. The oil is pale yellow liquid[17]. It is reported that there is a decrease in essential oil content as the fruit matures and its pharmacological effect is due to its essential oils[16].

Traditional Uses of Fennel

Country	Preparation	Condition	Reference
Italy	Tender leaves, chewed and stuck on ulcer	Mouth Ulcer	[18]
India and South Europe	Seeds, decoction seeds mixed with sugar	Constipation	[19], [20]
USA	Root and seed, decoction	Diuresis	[21]
North Iran	Seed, leaf and stem, infusion and edible	Hypnotic	[22]
Brazil	Leaf and fruit, infusion	Colic in children	[23]
Pakistan	Seed, infusion and edible	Laxative	[24]
South Africa	Leaf, an infusion is drunk which is made from the leaves	Arthritis	[25]
Mexico	Whole plant, oral infusion	Cough	[26]
Jordan	Leaf and seed, infusion	Flatulence	[27]
Spain	Whole plant, decoction	Gingival wound	[28]

Pharmacological and Clinical Observations of Fennel (*Foeniculum vulgare*)

S. No	Study Type	Part Used	Extract Type/Essential Oil	Study Subject	Reference
1	Antioxidant	Fruits	Water, methanol, acetone and ethanol extract	*In Vitro*	[30], [31], [32]
2	Antioxidant	Fruits	Water infusion	Rats	[33]
3	Antioxidant	Fruits	Essential oil	*In Vitro*	[34], [35]
4	Antioxidant	Fruits	Ethanol Extract	*In Vitro*	[36]
5	Neuroprotective	Seeds	Ethanol, Methanol, n-hexane Extract	Mice	[37]
6	Gastric Antiulcer	Fruits	Water Extract	Rats	[38]
7	Gastro- protective	Fruits	Distilled water	Rats	[39]
8	Hepatoprotective	Seeds	Hexane and Methanol Extract	Rats	[40]
9	Anti-	Fruits	Essential oil	Rats	[41]

	inflammatory				
10	Analgesic	Fruits	Essential oil	Mice	[42]
11	Antihypertensive	Fruits	Water Extract	SHR (Spontaneously Hypertensive Rats	[43]
12	Antihypertensive	Leaves	Water Extract (IV)	Rats	[44]
13	Anti-thrombotic	Fruits	Essential oil	Mice, Rats	[45]
14	Antimicrobial	Fruits	Hot Water and Acetone Extracts	*E. faecalis, S. aureus, E. coli, P. aeruginosa, S. typhi, S. typhimurium, S. flexneri*	[46]
15	Antimicrobial	Fruits	Essential oil	*E. coli, S. aureus, P. aeruginosa, B. subtilis, B. megaterium, B. cereus, S. enterica, L. monocytogenes, C. albicans, A. niger, T. rub rum, T. tonsurans, S. enteriditis*, strains of *K. pneumonia, T. mentagrophytes, M. gypseum, A. baumannii, S. typhimurim, E. faecalis, P. mirabilis*	[47], [48], [49]
16	Antimicrobial	Seeds	Methanol Extracts	15 strains of *H. pylori*	[50]
17	Antimicrobial	Fruits	Hydro-ethanol Extract	*C. jejuni*, MDR *M. tuberculosis*	[51], [52]
18	Antidiabetic	Aerial parts	n-hexane	Rats	[53]
19	Infantile colic	Seeds	Oil Emulsion	Infants	[54]
20	Antispasmodic	Fruits	Oil Emulsion	Young Women with Primary Dysmenorrhoea	[55], [56], [57], [58]
21	Anorexigenic	Fruits	Tea	Overweight Women	[59]
22	Vaginal Symptoms	Fruits	Vaginal Cream	Postmenopausal Women	[60]
23	Idiopathic Hirsutism	Fruits	Cream and Gels	Women	[61], [62]

Formulations:

1. Arq Badiyan (Unani Formulation)
2. Saunf Ark (Ayurvedic Formulation)

Makoh

Botanical name *Solanum nigrum*, a member of Solanaceae family. *Solanum nigrum* commonly known as black nightshade (English), mokoya (Hindi) and makoh (Urdu) is an established medicinal plant which grows wild in India as a weed.

Makoh is cultivated throughout the Temperate and Tropical areas of the world.

Phytochemistry:

Solanum nigrum contain alkaloid, flavonoid, tannins, saponins, proteins, coumarins, carbohydrate, glycoside and phytosterols as the chief chemical constituents. Solasodine concentration is high in small unripe fruits. Saturated steroidal genin found in the berries is known as tigogenin. The oil of *Solanum nigrum*containslinoleic acid. The presence of ascorbic acid was reported by some researcher and its concentration was found to be more in fruits than roots. Many new steroidal saponins and known saponins are reported like solanigrosides C-H and degalactotigonin from the whole plant. The mineral element include magnesium in large amount and protein content are present in considerable amount. According to some researcher *Solanum nigrum* acts as an antioxidant against reactive oxygen species, this is due to the presence of glycoprotein. The lipid content is found to be high in *Solanum nigrum*[63].

Traditional Uses of Makoh

State, Country	Part Used	Preparation	Conditions	References
Tanzania, Africa	Leaf	Leaves are pounded and applied topically.	Treatment of ringworm	[64]
Tunisia, Africa	Sap		Erysipelas (acute Streptococcus bacterial infection)	[65]
United Republic of Congo, Africa	Whole plant	Maceration	Snake bite/sting by a venomous animal	[66]
Tamil Nadu, India	Leaf	Fresh leaves cooked with cumin seeds and onion bulbs or can be taken orally in the form of leaf juice	Stomach ulcer	[67], [68]
Himalayan region, India	Leaf	-	Liver tonic, indigestion	[69]
Thar Desert, India	Roots	Roots boiled by adding small amount of sugar.	Increase women fertility	[70]
Assam, India	Roots	Roots are extracted in the form of its juice	Whooping cough and asthma	[71]

Pharmacological and Clinical Observations of Makoh (Solanum nigrum)

S. No	Study Type	Part Used	Extract Type	Study Subjects	Reference
1	Antioxidant	Leaves	Methanolic/Water Extract	*In Vitro*	[72]
2	Antioxidant	Leaves	Aqueous Extract	Rats	[73]
3	Antioxidant	Berries	Methanolic Extract	Rats	[74]
4	Gastric Antiulcer	Fruits	Methanol Extract	Rats	[75]
5	Anti-Gastritis and Antiulcer	Berries and Leaves	Aqueous and Hydroalcoholic Extract	Rats	[76]
6	Gastroprotective and Antioxidant	Leaves	Methanolic Extract	Rats	[77]
7	Anti-inflammatory	Berries	Methanolic Extract	Rats	[78]
8	Anti-inflammatory	Whole Plant	Methanolic Extract	Rats	[79]
9	Antidiabetic	Berries	Aqueous Extract	Rats	[80]
10	Antidiabetic	Leaves	Water, Ethanol Extract	Rats	[81]
11	Antidiabetic	Fruits	Ethanol Extract	Rats	[82]
12	Hepatoprotective	Aerial parts bearing ripe fruits	Ethanol, Water Extract	Mice	[83]
13	Hepatoprotective	Aerial parts	Aqueous -Ethanolic Extract	Rat	[84]
14	Antibacterial	Stem, Berries and whole Plant	Methanol Extract	*B. subtilis, E. coli, K. pneumonia, P. aeruginosa*	[85]
15	Antimicrobial	Leaves	Methanol Extract	*E. coli, B. subtilis, S. aureus, P. multocida, A. niger, A. flavus, R. solani*	[86]
16	Antibacterial	Root Tissues	Ethanol Extract	*S. aureus, P. aeruginosa, E. coli*	[87]
17	Anti-seizure	Leaves	Aqueous Extract	Rats	[88]
18	Pelvic inflammatory disease	Berries	Distillate	Female patients 18-25 years of age.	[89]
19	Nephroprotective	Berries	Distillate	Female patient	[90]

Formulations:

1. Arq Makoh (Unani Formulation)

Chicory:

Botanical name *Cichorium intybus*, a member of Asteraceae family. *Chicorium intybus* isknown as chicory (English) and kasni (Hindi and Urdu). It is an established medicinal plant which grows wild in India and has medicinal properties.

Chicory is cultivated in Europe, USA, Canada and Asia.

Phytochemistry:

Cichorium intybus contain chicoric acid as the chief chemical constituent. Derivatives of aliphatic compound comprise the major fraction while minor fraction comprise of terpenoids. The flowers contain saccharides, methoxy coumarins cichorine, flavonoids and essential oils. The principle volatile component include octane, hexadecane, n-nonadecane and pentadecanone[91]. According to many researchers the insulin concentration is reported to be 68%, 5% cellulose, 14% sucrose, 6% proteins and 3% other compounds. Chicory root extract contains fatty acids, volatile oil, alkaloids, triterpenes, latex tannins, saponins and flavonoids. Benzo-isochromenes like cichorin A, B and C are also isolated from roots. Tannins, pectin, fatty acid, α lactucerol, sugar, cichorin, choline, fixed oils, etc are also present in roots. Chicory seeds are rich in essential amino acid like lysine, leucine, methionine, phenylalanine, isoleucine, etc and contain high concentration of crude protein. The seeds also contain essential linoleic acid, saturated and unsaturated fatty acid, demulcent oil, palmitic acid and stearic acid. Calcium, magnesium, potassium, selenium and zinc are also found in chicory seeds[92].

Traditional Uses of Chicory:

Different plant parts or preparations of chicory is a therapy for different ailments of the body[91]. It is conventionally used as a liver tonic, ininflamed throat, acne, etc. The plant finds immense use as a cardiotonic and in the treatment of gout, hepatomegaly, jaundice, asthma.

In Afghanistan, the aqueous extract of chicory roots is used customarily for the treatment of malaria[93]. Leaf decoction is used for treating high blood pressure in Italy[94]. In Morocco decoction of whole chicory plant is used in kidney disorders and diabetes[95]. Decoction of roots and aerial parts is used conventionally in Bulgaria as cholagogue stimulant for gastric secretions and has hypoglycaemic potential[96]. In Turkey root decoction is used traditionally in cancer and in kidney stones[97]. In Lithuania the flowers are used for the treatment of cuts, gallstone, gastroenteritis and bruises[98]. In Pakistan root decoction is used for the management of diabetes [99]. In India root decoction is used as a therapy for liver enlargement, gout, jaundice and rheumatism [100].

Pharmacological and Clinical Observations of Chicory (*Cichorium intybus*)

S.No	Study Type	Part Used	Extract Type	Study Subjects	Reference
1	Hepatoprotective	Whole Plant	Ethanol, Water, Ethyl Acetate Extract	Rats	[101]
2	Hepatoprotective	Leaves	Ethanol-water Extract,	Rats	[102]
3	Hepatoprotective	Roots	Ethanol Extract	Rats	[103]
4	Hepatoprotective	Seeds	Ethanol Extract	Rats	[104]
5	Hepatoprotective	Leaves	Chloride Extract	Ross Chicken Broilers	[105]
6	Anti-inflammatory	Roots	Aqueous and Ethanol Extract	Rats	[106]
7	Anti-Ulcer	Leaves	Water-Ethanol Extract	Rats	[107]
8	Antimicrobial	Seeds	Water, Ethanol and Ethyl Acetate	*S. aureus, P. aeruginosa, C. albicans.*	[108]
9	Antimicrobial	Roots	Ethyl Acetate or Hexane	*S. aureus, B. subtilis, P. fluorescens, R. leguminosarum, E. coli, V. cholerae, S. cerevisiae, A. niger`3*	[109]
10	Antibacterial	Roots and Aerial parts	Water, Ethanol and Ethyl Acetate	*P. fluorescens, P. aeruginosa, A. radiobactor, E. carotovora*	[110]
11	Antibacterial	Rootsand Leaves	Methanol, Distilled Water, chloroform, Petroleum Ether and Acetone	*P. aeruginosa, E. coli*	[111]
12	Nephroprotective and Diuretic	Seeds	Ethanol Extract	Rats	[112]
13	Antidiabetic	Whole Plant	Ethanol Extract	Rats	[113]
14	Anthelmintic	Leaves	Methanol Extract	*In-Vitro*	[114]
15	Antiseizure	Root, Stem and Leaves	Hydro alcoholic Extract	Mice	[115]
16	Cardioprotective	Leaves	Aqueous Extract	Rats	[116]
17	Antithrombotic	Roots	Coffee	Healthy men and women	117]
18	Osteoarthritis	Roots	Capsule	Elderly (50 years or more)	[118]

Formulations:

1. Arq Kasni (Unani Formulation)

CONCLUSION:

Herbal medicines have been conventionally used since ages and provide the basis for medical treatments and is widely practiced even today.Thus, from the vast literature study and experimental result analysis we can conclude that fennel, makohand chicory can be used in wide range of ethnomedical treatments like conjunctivitis, laxative, constipation, jaundice, stomach-ache, flatulence, gastritis, gout, rheumatism, diuretic, etc. The extract of plantfindsvarious pharmacological uses like hepatoprotective, antioxidant, cardioprotective, neuroprotective, antidiabetic, antihypertensive, antithrombotic, gastroprotective, anti-inflammatory, analgesic, etc. It also finds immense utility in various microbial infections and nephrological problems.

The bioactive molecule in fennel, makoh and chicory can be developed as novel pharmacological lead molecules provided their bioavailability, pharmacokinetics, physiological pathways and importance to human health are known with sufficient detail.

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CONFLICT OF INTEREST:

The authors declare no conflict of interest.

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REVIEW ARTICLE

3D Printing: A Review on Technology, Role in Novel Dosage Forms and Regulatory Perspective

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ABSTRACT:

The existence of 3D printing (3DP) ways back to 1992, and its sound presence in the pharmaceutical industry was made in 2015 by the launch of 1st 3D printed drug, Spritam was manufactured by Aprecia Pharmaceuticals. Implementation of 3DP is escalating in the number of industries, including the pharmaceutical industry. The purpose of this review paper is to briefly discuss types of 3DP and their role in formulating novel dosage forms. Binder jet printing, VAT polymerization, powder bed fusion, and material extrusion are briefly explained along with an example of their implementation in the formulation of the dosage form. A few novel dosage forms which can bypass the first-pass metabolism and how 3D printing is useful in formulating them as been discussed. It also includes a comparison of the process of 3D printed tablets and conventional methods of manufacturing. The significance of 3D printing in novel dosage form and augmenting 3DP with hot-melt extrusion (HME) method is discussed. The regulatory concerns in adopting this technology on a large-scale are addressed. 3DP technology could rapidly print transdermal needles, buccal patches, and different shapes of vaginal rings and proved it can be a versatile tool in formulation technology. As the pharmaceutical industry involves stringent regulations, certain aspects need to be considered by regulatory authorities before implementing this tool into commercial-scale manufacturing.

KEYWORDS: 3D printing; additive manufacturing; 3D scanner; Novel Dosage Forms; HME; Regulatory perspective.

INTRODUCTION:

"What you were taught 10-20 years ago is fast becoming obsolete. Upskill yourself and recreate your world," as said by Nicky Verd, our society has followed to evolve. Starting with the first industrial revolution being "The Age of Mechanical Production," the second industrial revolution as "The Age of Science and Mass Production," followed by the third industrial revolution "The Digital Revolution." Each of the revolutions had a significant change in the industry. Now it's time for the fourth industrial revolution, i.e., the era of 3D printing that will revolutionize the manufacturing industry, not limiting to the pharmaceutical sector [1].

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Charles W. Hull invented 3D Printing, also known as additive manufacturing (AM), rapid prototyping (RP), or solid-freeform (SFF) technology [2,3]. He was also the pioneer of one of the 3D printing method, stereolithography [4]. The technology was first executed by the Massachusetts Institute of Technology in 1992. 3D printing is based on computer-aided design (CAD) models created by the user and manipulated by a terminal computer [5]. It is an additive manufacturing process from which the digital files, created by a 3D scanner or CAD software, are converted into physical objects.

The printing method has been tailored to a wide spectrum of applications in automotive and aerospace industries (Rolls-Royce, a British supplier, and provider of power systems for the aircraft and automobile sectors, has announced plans for 3D printing of aerospace components using quad laser technology from SLM Solutions) [6,7], transistor circuits [8], biological material

including protein and cells[9], drug delivery systems [10], architectural world [11] and food industry (3D printed food like pizza)[12,13]. Researchers have 3D printed artificial corneas that mimic the human eye using the stem cell bio-ink, a breakthrough that can significantly decrease the need for eye donations[14]. However, forming, machining, casting, and molding remain as traditional methods of industrial manufacturing.

The pharmaceutical industry is conservative, regulated, and prefers well-developed production procedures and designs to guarantee product stability. But the pharmaceutical sector can revolutionize how drugs are being manufactured for individual patients by leveraging innovative techniques and help in manufacturing procedures[15]. 3DP of pharmaceutical products is a potential game-changer for the pharmaceutical industry. This opens up unique opportunities to adopt the innovative principle of product design. For instance, the size and shape of the product can be tailored because of the ability of 3DP, which fetches an option to design the inner structure of the product. Mass customization and patient-centered medicine strategy can be achieved by implementing this innovative 3D printing technology [16]. 3DP plays a very important role in serving the small-scale supply chain. For instance, in the manufacturing of orphan drugs [17].

We live in a technological age; we are on the verge of the next industrial revolution, were living in a cloud-based globe of computing, NFC payments, and 3DP, once a fantasy, have now become a reality [15].

3DP process:
A typical 3DP process starts with creating a design of the intended final product. This can be either achieved by creating the product design using CAD software or by scanning the pre-existing object by 3D scanners, which is intended to be 3D printed.

1. CAD software:
a) Open-source software (OSS) relates to the program that allows free use of the technology accessible on the Internet. Many users and organizations can edit, change, or remove the file. Since the app is available to the public, this helps in continually upgrading, changing, and growing as more individuals are encouraged to focus on its development [18]. E.g. Blender, SketchUp, OpenSCAD, TinkerCAD etc. [19].

b) Closed source software (CSS) is contrasted to OSS, which means the program that utilizes the proprietary technology is secured. Only the original program developers can view, duplicate, and change the information. In a case of proprietary source applications, you won't purchase the program but just pay to access it [18]. E.g., Adobe suite, Autodesk inventor, Catia, PTC creo, Rhinoceros, Maya, Solid works, etc. [19].

2. 3D Scanner
The CAD design can also be created using the 3D scanner. We create the digital file of the pre-existing three-dimensional physical object, which intends to be recreated. This method captures detailed information about the texture, color, and shape of the scanned object [20]. 3D scanners are classified based on their design, technical specifications, and field of application.

3. Desktop 3D scanners:
These types of scanners get accommodated on a desktop. They are of two types, closed-frame desktop 3D scanner in which the user can place the object to scan in a closed environment, and the other type is open-frame desktop 3D scanner where the user places the object to be printed on the rotatable platform on which the camera is focused. Desktop 3D scanners are usually accompanied by a turntable. Turntable facilitates by rotating the object to be scanned, and contemporarily the camera scans and captures the 3D structure of an object.

4. Handheld 3D scanners:
These are portable scanners that are used when it is difficult to reach the scanning areas. These may be wired by connecting to the computer via USB or wireless. These can capture 3D objects of various sizes [21].

5. 3D scanning mobile phone apps:
Application installed on the mobile phone is used to scan the object. Here the camera and inertial sensors of the mobile phone are the hardware that decides the overall performance of a 3D scan [21].

Based on the application, the feasible 3D scanner is used to create a 3D design. Once the 3D design is constructed, it is saved in specific file formats. The various file formats used for saving 3D designs are .stl (Stereolithography), .obj (Wavefront OBJ), .mb (Maya Scene), .ply (Stanford PLY), .u3d (Universal 3D) etc. [22]. This 3D design file is loaded into a slicing software that cuts the model into many horizontal layers. Later this data is communicated with the 3D printer, which prints as per the design created [23].

CFD and 3D printing:
The creation of the 3D printed model depends on the flow of extruded material from the printer nozzle. CFD can solve fluid mechanical problems involved in 3D printing. It is a branch of fluid mechanics that involves broad-spectrum numerical problems solving complex three dimensional and time-dependent flow problems [24,25]. It can be employed to prevent overfill and underfill of 3D printed models. It can leverage the simulation of

material extrusion through a printer nozzle and provide an optimized deposition strategy, thereby achieving the accuracy of the printed model [26].

Types of 3D Printing:

The taxonomy is implemented by the American Society for Testing and Materials (ASTM), an organization that sets technical standards for materials, products, and systems. The classification of 3D printers relies on the type of material used, the technology of disposition, and the process of layering of material, which results in the characterized formation of the product. Based on the additive process ASTM classifies 3DP technologies like material extrusion, powder bed fusion, material jetting, binder jetting, vat polymerization, sheet lamination, and directed energy deposition and is depicted in Table 1. [2].

Table 1: Classification of 3DP by ASTM

ASTM taxonomy	3D printing technologies	Substrate material
Binder jetting	Powder bed inkjet printing Theriform™ ZipDose® S-printing M-printing	Solid particles (plaster, metal, sand, polymer)
Vat polymerization	Stereolithography (SLA) Digital light projection (DLP) Continuous layer interface production (CLIP)	Liquid (photopolymer)
Powder bed fusion	Selective laser sintering (SLS) Electron beam melting (EBM) Concept Laser Direct metal laser sintering (DLSM) Selective metal sintering (SLM)	Solid particles (metal, plastic, polymer)
Material extrusion	Fused deposition modelling (FDM) Gel/paste extrusion	Filament (thermoplastic polymers e.g. ABS; PLA; PC ULTEM)
Material jetting	Ink-jet printing Polyjet Thermojet	Liquid (acrylic-based photopolymers, elastomeric photopolymers, wax-like materials)
Directed energy deposition	Be additive manufacturing (BeAM) Electron beam direct Manufacturing Direct metal tooling (DMT)	Metal wire
Sheet lamination	Laminated object manufacturing	Sheets

1. Binder jet printing:

It is the 1st patented 3D printing method, which also finds its commercial application in the manufacturing of fast disintegrating tablet-Spritam by Aprecia pharmaceuticals which is US-FDA approved and patented as Zipdose technology [27].

The platform serves as the base for the pre-determined 3D design of formulation. As shown in Fig. 1 and Fig. 2, the design is built up by the addition of a layer-by-layer of powder with the help of roller and subsequent addition of binder from the inkjet nozzle. The deposition of binder can occur by two mechanisms: through the thermal head, the head is comprised of the cartridge, which acts as a reservoir of ink (binder liquid), and a resistive element. When the current pulse is passed through the element, there is internal temperature rise and drop binder is ejected by vaporization, nucleation, and expansion[2,28]. The other mechanism is by a piezoelectric system, which comprises an electromechanical device where the piezo crystal produces pressure oscillations in fluid when current is passed and thereby ejecting the ink droplet through the nozzle. The advantage of the piezoelectric system is that it doesn't depend on the thermal properties of the ink, and it aids in the life of print head since it doesn't create thermal stress on fluid and the working system [2,29]. The binder serves its cause of addition, thereby hardening the system. This procedure is repeated successively to build up the predetermined 3D model. The downstream process includes drying for removal of volatile solvent residuals and removes excess powder deposited during printing, thereby signifying the wastage of powder. The overall process of Binder jet 3D printing is shown in Fig. 3.

Yu DG *et al.* [30] formulated paracetamol fast disintegrating tablets (FDT'S) using binder jet printing. Using colloidal silicon dioxide as a fast disintegrating agent, they were able to achieve average disintegration time of 21.8 ± 5.4 seconds and cumulative drug release of 97.7% in initial 2 minutes hence thereby concluded that 3DP has potential application in the formulation of FDT'S.

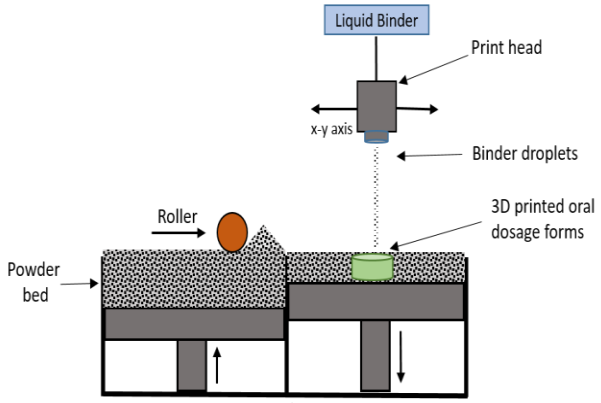

Fig 1: Schematic representation of Binder jet printing

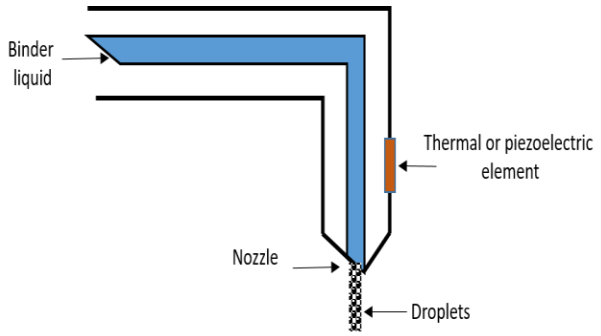

Fig 2 Schematic representation of print head with ink deposition system

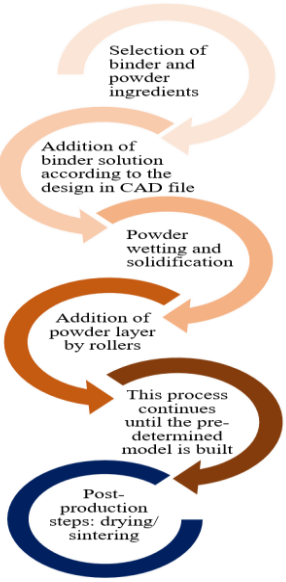

Fig 3 Schematic representation of overall Binder jet 3DP process

2. VAT Polymerization:

Vat polymerization is a familiar term for a range of 3D printing techniques. The basic principle involved in VAT polymerization is selective solidification of the liquid photopolymer present in vat or container by an appropriate type of radiation or LASER beam. The major types of vat polymerization processes include SLA, DLP, CLIP, lithography-based ceramic manufacturing (LCM), and two-photon polymerization (2PP) [31]. Here we discuss the most frequently used type of VAT polymerization method, i.e., SLA.

SLA: The method uses liquid photopolymers, which are low molecular weight acrylates, epoxy macromers, or monomers, which results in the formation of very rigid, glassy, and brittle 3D printed designs. The principle involved is the solidification of liquid resin is by photopolymerization, where a cross-linked polymer structure is formed by exposing the liquid resin to visible or UV light.

The laser beam is projected for a specific depth in a pre-designed pattern. As a result of this liquid resin solidifies due to polymerization and adheres to supporting platform. In the subsequent step, the build platform moves vertically into the vat as much as the thickness of the first layer. The platform continues to move downwards, and layer by layer polymerization of liquid resin takes place until the 3D structure in the CAD file is formed [31,32]. This solidification can take place by two methods based on the illumination of light and orientation of build platform: Bottom-up and top-down [32].

In the top-down method, the build platform moves vertically down into the vat, and the light is illuminated from the top, where layer by layer solidification of the liquid resin occurs on top of the platform. Whereas in the bottom-up method, the light source is illuminated from the bottom of the VAT and moving platform on the top [2].

Economidou SN *et al.* [10] fabricated novel microneedle arrays of insulin using SLA. Microneedles were printed by polymerization of biocompatible resin in consecutive layers. Inkjet printing was also used to form thin layers of insulin and sugar alcohol, which were used as carriers. In-vivo animal trials were conducted and achieved lower glucose levels within 60mins, demonstrating excellent hypoglycemic activity.

3. Powder bed fusion (PBF):

PBF is a birds-eye view term that covers SLS, DMLS (Direct metal laser sintering), and SLM. These methods have a similar printing process where thermal energy generated by printer heating system and laser beam fuses the powder particles and differ in the starting powder material used. SLM needs the highest thermal energy supply, and SLS needs the lowest thermal energy [2].

SLS: uses a laser to selectively combine the powder particles to create a 3D structure in a predefined design. SLS printing setup consists of a powder bed and reservoir, a roller, and a radiation source (laser) [33].

The initial stage of printing involves a thin layer of powder on the building platform, followed by laser irradiation. As the sintering takes place, the building platform moves downwards, and the reservoir platform moves upwards while the roller transfers a thin layer of powder to the building platform. This process of transfer of powder takes place until the predefined 3D structure is formed. Excess powder on the structure is removed by using compressed air or brushing [3].

Fina F *et al.* [33] constructed paracetamol loaded cylindrical, gyroid lattices, and bi-layer tablets using Eudragit (L100-55 and RL) (EUD L and EUD RL), polyethylene oxide (PEO) and ethyl cellulose (EC) as polymers. They demonstrated that SLS is also a 3DP technology that is feasible in customizing the drug release profile, which in conventional practice could avoid changing the formulation composition instead it could be achieved by changing the 3D design.

4. Material extrusion:

The material extrusion method involves layer by layer fabrication of a 3D model using FDM, 3D plotting, multiphase jet solidification (MJS), and precise extrusion manufacturing (PEM). FDM involves the melting of materials, and other methods are assisted by pressurized micro-syringe for non-melted liquid materials through the printer nozzle[34]. This method of 3D printing extrudes the material filaments through the heated nozzle of the 3D printer. Initially, polymer filaments made of acrylonitrile butadiene styrene (ABS) and polylactic acid (PLA) were used, but due to the expansion of research in 3D printing, polyvinyl alcohol (PVA), ethylene-vinyl acetate and hydroxypropyl cellulose (HPC), etc. are also explored. The materials extruded are melted when passed through the nozzle and hardened on cooling [35].

FDM: is a form of material extrusion 3D printing technique focused on the deposition of successive layers of softened / molten thermoplastic materials [35]. In this method, the pre-determined 3D model is printed by moving the printer head through specific directions and keeping the nozzle tip at a specific temperature, which melts the polymer filament. Fig. 4., gives an overview of material extrusion 3D printing.

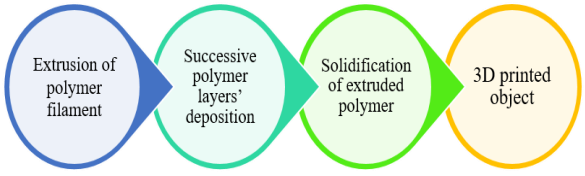

Fig 4: Overview of material extrusion 3D printing method

Two methods can achieve the presence of the drug in polymer filament. The first method is by impregnation of the polymer matrix in a highly concentrated drug solution. The drug then gets loaded by passive diffusion into the tedious polymer matrix. Thereby this method is expensive in drug loading and inefficient because an inadequate amount of drug is being trapped by passive diffusion. Another method involves the application of HME. The raw materials are loaded through the hopper of HME, and polymer filaments are extruded as products. The shape and diameter of filaments can be altered by changing the nozzle of HME. The polymer filaments soaked in drugs can be printed into different dosage forms [36,37]. This 3D printing technique is versatile and alters the dose of the drug. Varying the 3D design and altering the in-fill density leads to achieving the altered drug dose [31,38].

Chai X *et al.*[39] printed floating sustained-release tablets of domperidone using fused deposition modeling 3D printing technique. Since domperidone is an insoluble weak base, it is formulated as a floating sustained-release tablet, which would increase bioavailability and decrease the frequency of administration. Hydroxypropyl cellulose filaments along with domperidone were prepared by the hot-melt extrusion process, thereby achieving the drug loading. The optimized formulation containing 10% domperidone with 0% infill showed sustained release and achieved 10 hours in vitro floating. The results showed that FDM is a promising technology to manufacture hollow tablets utilized as floating drug delivery systems.

3DP in Bypassing the first-pass Metabolism of The Drug:

With the emerging trend of 3D printing utilized in the manufacture of tablets [40], it also has potential in the manufacturing of novel dosage forms which can bypass the first-pass metabolism; for instance, transdermal patches, ophthalmic inserts, rectal and vaginal implants, buccal patches, parenteral injections, etc.

1. Transdermal patches:

Transdermal delivery is a great alternative to the enteral route of drug administration. It is advantageous as it bypasses the first-pass metabolism, pain caused by hypodermic injections, disease transmission by needle re-use, patient compliance, and treatment can be terminated at any time by the patient. Microneedles are third-generation transdermal drug delivery systems that pierce into the skin painlessly and give extended-release ranging from small molecules, proteins, and nanoparticles. And microneedles are coated or encapsulated by drug formulations, which provide rapid or controlled release of proteins and vaccines [41].

Caudill CL *et al.* [42] printed microneedles on a transdermal patch using CAD files, which were developed SolidWorks 2014 for designing the structure of the needle. The pyramidal microneedles were composed using polyethylene glycol dimethacrylate (PEG) with 2.5 wt% TPO as a photoinitiator. The dimension of needles was 1000µm tall and 333µm wide at the base. The CAD files were designed delicately before fabrication to print 1000µm tall microneedles. Post creation, IPA was used to wash-off the residual resin on microneedles and was cured for 5 minutes using a Phoseon FireJet™ FJ800 UV LED lamp. They used CLIP, a novel additive manufacturing technique, were UV light aids to photo polymerize the liquid resin. The microneedles were made of polyethylene glycol and coated by model proteins such as ovalbumin, bovine serum albumin, and lysozyme. CLIP microneedles quickly released protein-coated cargo in solution on penetration into the porcine skin. They concluded that there was sustained retention of protein in the skin over 72hr when applied to live mice. The conventional methods of microneedle formulation are limited to the efficiency to quickly create microneedles along the high degree of control over their design parameters, which can be achieved by using 3DP.

2. Buccal patches:

The buccal drug delivery system is a novel drug delivery system which is aimed to increase the bioavailability of drugs, which are affected by hepatic first-pass metabolism. The drug delivery system consists of API, permeation enhancers, and adhesive. The conventional methods of manufacturing include solvent evaporation and HME[43]. There is a time lag in the conventional manufacturing process due to multistep process involvement such as drug loading, drying, adding backing layers, etc. In this case, there is a potential implication of a 3D printer, which is versatile and timesaving [44].

Eleftheriadis GK *et al.* [44] used FMD 3D (Maker Bot. Inc) printer to manufacture poly (vinyl alcohol) based diclofenac sodium mucoadhesive buccal films. Xylitol was added into the polymer blend to exhibit its plasticizing character. The potential FDM printer was utilized to develop buccal films. The CAD design of films was created using AutoCAD 2019 (Autodesk Inc., USA), which was a 20x20 mm rectangle of 0.2mm thickness. The extrusion temperature through printer nozzle was set to 200°C and 190°C. The physicochemical characterization was done by scanning electron microscopy (SEM), differential scanning calorimetry (DSC), thermogravimetric analysis (TGA), and X-ray diffraction (XRD). The results of SEM found that the surface of polymer filaments was smooth without porous nature, indicating homogeneity of polymer filaments extruded. The printing temperature did not affect the thermal degradation of polymer or drug. XRD diffractogram confirmed the successful molecular dispersion into the polymer matrix. The buccal films printed after the addition of chitosan to PVA filaments showed three times an increase in permeation parameters. The results of the overall study confirm the potential of implementing 3D printing in formulating buccal films.

3. Vaginal rings:

These are polymeric drug delivery systems that are used to administer the drug, which exhibits local or systemic effects [45]. Vaginal rings are devices majorly used for delivering hormones to overcome their bioavailability issues. Though there are several marketed vaginal rings, e.g., Estring, Femring, NuvaRing, and Progering, they lack the customization of dose and shape in compliance with the patient's need. This need can be addressed by 3D printing technology [36].

Fu *et al.*[36] printed different shapes of progesterone vaginal rings using the FDM 3D printing technique. The polymer matrix was made of PLA and polycaprolactone (PCL) and progesterone. The polymer filaments were formed by passing the polymer matrix through the single screw extruder. The CAD design of vaginal rings was developed in AutoCAD 2007. The filaments were passed through the 3D printer nozzle, and rings were printed according to the CAD design. Different shapes such as 'O,' 'Y,' 'M' were printed, and the drug release profile was studied. All the formulated shapes exhibited sustained drug release were 'O' shaped formulation exhibited maximum drug release. This shows that AM is a future technology that can be used in marketed formulations to solve the unmet need.

3D Printing in tablet Manufacturing:

Tablet manufacturing is one of the well-established solid unit dosage form process [46]. 3D printing of tablets can be explored as an initial step in adopting this new technology in the pharmaceutical industry. Fig. 5. gives an overview of conventional tablet manufacturing vs. 3D printing tablet manufacturing process.

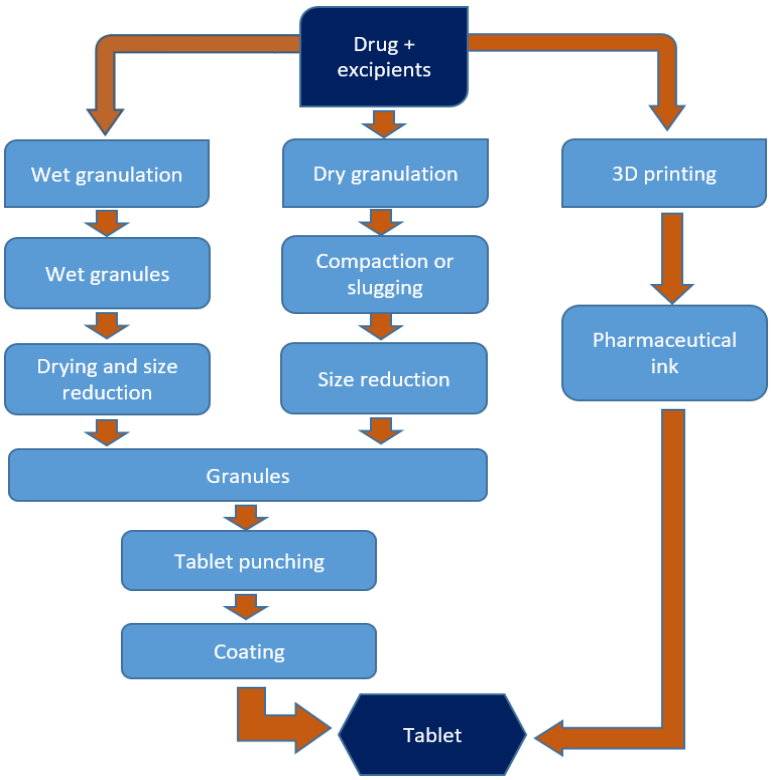

Fig 5: Conventional tablet manufacturing process vs 3D printing assisted tablet manufacturing process

Disadvantages of conventional tablet manufacturing:

- Involves time-consuming unit operations, wastage of raw materials, and require human resources in each step [47].
- The minimum batch size for large scale tablet manufacturing is one lakh as recommended by the regulatory agency [48].
- Since various critical process parameters have to be optimized, continuous batch manufacturing is a challenge.
- The shape of tablets is restricted to dies used in tablet punching [49].

Advantages of 3D tablet manufacturing:

- Involves pharmaceutical ink or drug immersed polymer filament preparation, which is fed further into a 3D printer, thereby overcoming various unit operations and reduce resource investment.
- 3D printing can be used in the formulation of dosage forms in FIH trials, which demands a need for inexpensive, rapid small scale production with flexible-dose of drug [2].
- 3D printer combined with or without HME can leverage continuous batch manufacturing [50].
- The shape of tablets can be varied by changing the CAD design based on the patient's age and preference [51,52].
- The 3D printer needs a small place to accommodate, which brings the potential to integrate with the healthcare professionals or pharmacists. They can tailor the dose of the drug according to the patient's needs and provide seamless supply and experience [27].

Augmented 3d Printing in the Pharmaceutical Industry

HME technology dates back to 1930. It is used widely in the processing technology of plastic, rubber, and food industry. Currently, its application is not limited to the pharmaceutical industry. It is also employed to increase the solubility and bioavailability of thermostable drugs [53].

The major component of HME is an extruder. As shown in Fig. 6 and Fig. 7, it consists of motor, extrusion barrel with one screw (single) or two (twin) screws and die or orifice at the end. The extruder also comprises heaters that facilitate heat for softening or melting of raw materials (polymers). The screws in the extruder are capable of exerting shear pressure and cause extreme material mixing. The friction caused by the screws in the barrel and the heat supplied results in the melting of polymers. Later the screw conveys the molten polymers down the barrel while pressure and temperature are the major process parameters. The shape of the extrudate is dependent on the type of exit dies used in the HME set-up [50]. The types of exit die to include sheet and film, pellets, granules, and strands, and various auxiliary components are used in the downstream process [53].

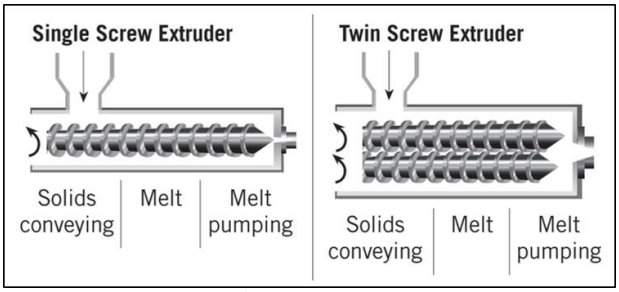

Fig 6: Cross-section view of single and twin screw extruder barrel

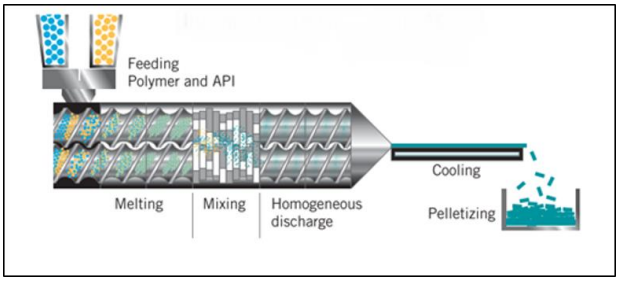

Fig 7: Hot melt extrusion process

Due to availability and low printing costs, FDM is one of the 3DP methods that is widely used in pharmaceutical sciences [54,55]. FDM involves extrusion of polymer filament through the heated nozzle, and the melted product is printed according to the CAD file design. The drawback of the FDM method is there are no readymade polymer filaments with the desired active pharmaceutical ingredients where in this case, the strands of polymer and active pharmaceutical ingredients produced by HME play a very potential role and serves the need [56].

Regulatory Perspective:

Compliance with regulatory requirements is a key requirement for the market launch of a 3D printed product [10]. 3D printing's ability to revolutionize manufacturing and drug development has attracted the attention of regulatory bodies across the globe. Hence the challenge of following traditional regulations for introducing 3D printed products as raised. The FDA released final guidance on technical considerations for Additive manufactured devices, which considers the design, manufacturing, and testing of devices [57].

Aprecia Pharmaceuticals is the pioneer company that has received FDA approval for the manufacturing of orally fast disintegrating tablets (Spritam) containing Levetiracetam used in the treatment of epilepsy. This is considered a milestone in the Pharma sector for the implementation of 3D printing. ZipDose® technology is a 3D printing method employed in the manufacturing of SPRITAM and is similar to conventional powder compaction of mass manufacturing of pharmaceutical tablets [58,59].

For 3D printed dosage forms, regulatory bodies can draw insights from the path of setting up the regulatory framework for approval of AM medical devices, which was issued in December 2017 [57,60].

1. Anticipated questions to be addressed by regulatory agencies for setting guidance for 3D printing of pharmaceutical dosage forms:

a) Will the law cover the original "Pharmaceutical ink," 3D printer, and end product? If yes, there are few initial pharmaceutical inks which are toxic to humans, but the product is non-toxic as in case of dosage forms manufactured by vat polymerization

b) Can the FDA control all the various AM technologies? This can be complicated since the materials used in each AM technology are quite distinct from each other

c) Will there be a need for additional data from clinical trials other than safety and efficacy details followed in conventional clinical trials of drugs? [60]

d) Should the CAD software be included under Schedule M2 of the D&C act, as per the regulations of medical devices?

2. Assuming the implementation of 3D printing, the following are the topics to be considered by the regulatory body while developing a framework for 3D Printing process

a) 3D printed product design and process: Small scale or large scale has to be defined, which influences the design and process considerations. If it is large scale manufacturing, CMC related regulations have to be followed, thus addressing the safety and efficacy specifications [2].

b) State of Control: 3D printing is less explored in pharmaceutical manufacturing. Hence the process variables such as print head voltage gap, printer nozzle clog, the pace of roller, powder bed alignment, print head alignment, laser power, and temperature need to be well studied to ensure product homogeneity and quality. Throughout implementation, correct process attributes or ranges must be identified. A detailed risk analysis can be utilized to ensure that any effect on product quality in all operating states is identified and handled adequately [2].

c) Raw Material and Intermediate Control: 3D printing involves the layer-by-layer distribution of material; which might affect finished product due to variations in the printability and physicochemical properties of API and excipients in the process of printing. Micromeritics (Surface area, porosity, etc.) is one of the properties of raw material that can affect 3D printing. Some of the other properties of raw materials are flow properties, viscoelastic properties, glass transition point, thermal conductivity, melting point, impurities, degradation properties, solid-state, and moisture. Based on product and process understanding, critical product attributes

have to be established, and Process analytical techniques (PAT) monitoring may be included [2].

d) Equipment and Software Workflow: It is essential to take into account the aspects of equipment control in 3D printing; however, most of the 3D printers have their operating principles varying from one another. The key software attributes to consider are software control, predetermined design parameter range, and file format conversions [2].

e) Print Cartridges: They are the filaments loaded with drugs or excipients, which runs through the heated nozzle of a 3D printer, and the dosage form is created. The filaments should comply with standard Chemistry, manufacturing, and controls (CMC) and the manufacturing process with the standard GMP [2].

f) Product Collection and Rejection: Post FDA approval of Spritam, 3D printing, has become the platform for commercial bulk manufacturing. Similar to conventional pharmaceutical manufacturing methods, a state of control for the AM process has to be well established to deal with the scenarios of malfunctioning of printer or software. In the future, there would be a need to optimize the standards in the collection of the product, rejection of product, or rejection of a whole batch [2].

g) Post-processing and Packaging: Post-processing unit operations may include drying, harvesting of unused / unbound powder content, recycling, and packaging when using the powder bed inkjet 3D printer. Identification of the post-processing steps involved in the particular use of 3D printers is a critical research area. Such post-processing operations that affect the quality of the final drug product may not be directly related to the type of 3D printer used. It may be resolved with a thorough understanding of the root cause [2].

h) Equipment Cleaning: Some parts of the 3D printers may be disposable or may be used for one time. The process of cleaning should consider material build, degradation, microbial growth, cleaning agent removal from the 3D printing equipment parts keeping equipment complexity, and product changeover in mind. Also, the frequency, length, and quantity of the cleaning agent should be considered. Establishing the cleaning validation procedure for 3DP equipment and systems could be the same as traditional pharmaceutical manufacturing techniques [2].

CONCLUSION:

Many innovative technologies are trying to signify their potential in the pharmaceutical industry. This review article demonstrated the capability of 3D printing and gave an overview starting from its history to a regulatory perspective in the pharmaceutical industry. The results of novel dosage forms like transdermal patches, buccal patches, and vaginal rings indicate the potential of 3DP in formulation technology. The regulatory approval of 3D printed Spritam tablets by Aprecia Pharmaceuticals has led to a thunderstorm of research in 3D printing for pharmaceutical applications. As FDA is probing research in 3D printing and trying to establish the standards for its implementation in the pharmaceutical industry, the future of 3D printing is not so far. However, FDA has to address questions related to CAD software implementation, standards of polymeric filaments, SOP's for equipment cleaning, process workflow, etc. 3D printing has been employed in various sectors like aerospace, automobile, food, and architecture, etc. its role in the pharmaceutical industry is not an exception.

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CONFLICT OF INTEREST:

The authors confirm that this article content has no conflict of interest.

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REVIEW ARTICLE

Exploring intention to use Asma Droid app of Indonesian Asthmatics using Technology acceptance model (TAM): A Descriptive Quantitative Study

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ABSTRACT:

Education on asthma self-management has shown to be effective in improving asthma control levels and quality of life while reducing exacerbations and healthcare utilization. Many available healthcare smart phone apps could potentially help asthmatics in improving asthma self-management and outcomes. Asma Droid is a new smart phone app intended for asthmatics in Indonesia. It has many contents and features related to asthma self-management, including: asthma education, a list of asthma medications, asthmatic daily journal, peak flow meter, asthma control test, asthma action plan, asthma asked question, and a map of nearest local hospitals or community health centers. This study primarily aimed to explore intention to use Asma Droid app of Indonesian asthmatics using Technology Acceptance Model. The study applied a quantitative descriptive approach and was conducted in July to September 2019 at Yogyakarta Indonesia with 100 participants through a purposive sampling method. To test the hypotheses, the study applied a multiple linear regression analysis. The results of research showed that the calculated F value of study was 31.622 with a Sig. of 0.000, meaning that Perceived Usefulness and Perceived Ease of Use together significantly affected Intention to Use. The study also showed that t-test Sig. value of Perceived Usefulness was 0.000while Perceived Ease of Use was 0.003, meaning that Perceived Usefulness and Perceived Ease of Use each significantly affected Intention to Use. The study concluded that end-users will more easily accept Asma Droid if they feel that the app is easy to use and has benefits for their asthma self-management.

KEYWORDS: Technology Acceptance Model, Perceived Usefulness, Perceived Ease of Use, Intention to Use, Asma Droid.

INTRODUCTION:

Asthma is a chronic respiratory disease commonly characterized episodes of wheezing, dyspnea, chest tightness, shortness of breath, coughing, and bronchial hyperresponsiveness[1,2]. The disease is very common among children and teens, though in some cases it can arise at any age.

Estimates vary, but a review on the pathophysiology of asthma remission published in 2019 confirmed that more than 300 million people around the world have asthma[3,4,5]. In Indonesia, it has reportedly reached 1.77% of total death with population reaching the 19th over the world[6,7]. The primary causes of this rapid increase were still unclear, though a number of studies suggested that lifestyle changes to be a possible explanation[8,9]. Asthma self-management education, according to some evidences, has shown to be effective in improving asthma control levels and quality of life in asthmatics[10]. Additionally, the education also helped asthmatics in reducing exacerbations and healthcare utilization. The

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bad news, however, was that adolescent asthmatics aged 10 –19 years old tend to have lower asthma control levels when compared to adult asthmatics[11]. Many smartphone apps, according to a review published in 2017, performed well across all applied review frameworks[12]. The apps are most often developed to monitor a certain medical condition, to inform users about their general health and wellness, or to make it easier to manage individual medications. It means that asthma apps could potentially help asthmatics in improving asthma self-management[13].

Asma Droid is an Operating System (OS) Google Android-based asthma self-management app, which was created by the authors in the previous study. This app was designed, developed, and implemented in accordance with the five steps of User Centered Design approach. The design, development, and implementation process of the app involved end-users including asthmatics and medical professionals (general practitioner, pharmacist, and nurse). The app has eight features: basic asthma education, a list of personalized asthma medications, asthma action plan, peak flow meter, asthmatic daily journal, asthma asked question, asthma control test, and a map of nearest local hospitals or community health centers. The app itself was commercially available for download at http://umy-asthma.com/app/app-umy-asthma-v03.0.apk[14].

The use of smart phones app for health management is now growing rapidly[15]. Due to this phenomenon, there has been a growing interest in determining the purpose of health apps in education regarding self-management of certain medical condition and the factors affecting its acceptance[16]. The World Health Organization stated that mHealth potentially transformed the face of health service delivery around the world. A powerful combination of factors is driving this disruption, including rapid advances in mobile technologies and applications, a rise in new opportunities for the integration of mobile health into existing mHealth services, and the continued growth in coverage of mobile cellular networks[17]. A number of studies were always trying to explore the factors affecting the Intention to Use to ensure its successfulness. There are many Information Systems (IS) evolving to accomplish the exploration, one of them is Technology Acceptance Model (TAM)[18]. TAM was developed by Fred D. Davis in 1989[19,20]. It is a theory that defines how end-users accept a new information system or technology. Literally, this theory explains that Perceived Usefulness and Perceived Ease of Use are the principal determinants of end-user's Intention to Use or User Acceptance of any new information system or technology. The main goal of this theory is to understand better why end-users accept or reject a new technology or information system, and how end-users can be improved through technology design. The term "Perceived Usefulness" is that a user believes a particular information system helps improve their job performance, while "Perceived Ease of Use" refers to the degree to which a user believes that using a particular information system will be easy to use and free of effort. The last term, "Intention to Use", describes to user's motivation to strive to complete all the tasks. In healthcare, the TAM was able to predict 30 – 70% of variance of Behavioral Intention to Use, considered reasonably high[21].

In Indonesia, where the study took place, studies on Intention to Use asthma self-management app have not been conducted yet. The study, therefore, primarily aimed to examine Intention to Use Asma Droid app of Indonesian asthmatics, using Technology Acceptance Model. The study was approved by the Research Ethics Committee of the Faculty of Medicine and Health Sciences of Universitas Muhammadiyah Yogyakarta Indonesia. Fig. 1showed the relationship of Perceived Usefulness and Perceived Ease of Use to Intention to Use.

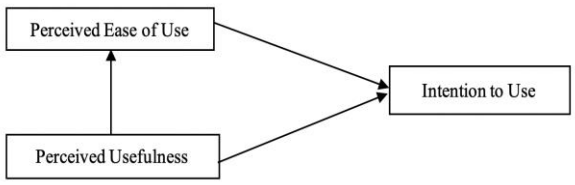

Fig. 1: Relationship of perceived ease of use and perceived usefulness to intention to use[22]

METHODS

This study applied a quantitative descriptive approach and was conducted in July to September 2019 at Yogyakarta Indonesia with 100 participants through a purposive sampling method. The study applied a 4-point Likert scale consisting of 4 items, e.g. strongly disagree, disagree, agree, and strongly agree[23]. The scale items were used to assess the following variables: Perceived Usefulness, Perceived Ease of Use, and Intention to Use of Asma Droid users and to define the effect of Perceived Usefulness and Perceived Ease of Use on Intention to Use. To describe characteristics of study participants, a descriptive analysis was conducted through IBM® SPSS® Statistics 25.

RESULTS:

1. Sample Characteristic:

The study involved asthmatics of different age groups, genders, educational levels, mHealth user experience, and asthma control levels. According to, almost half of study participants (n=48) were aged between 18 – 22 years old, 22% (n=22) were 23 – 27 years old, 21% (n=21) were 28 – 32 years old, and 9% (n=9) were equal

or more than 33 years old. The gender distribution was 56% (n=56) female and 44% (n=44) male. In the terms of educational background, more than half of participants (n=60) were undergraduate, 17% (n=17) were bachelor, 11% (n=11) were master, and 12% (n=12) were doctoral degree. Of all respondents, 60% (n=60) were "on-target" asthma control levels while 40% (n=40) were "well-done". All participants (n=100) had mHealth user experience.

Demographics	Value (n)
Age	
· - 22	48
· - 27	22
· - 32	21
· 33	9
Gender	
· Male	44
· Female	56
Education	
· Undergraduate Degree	60
· Bachelor Degree	17
· Master Degree	11
· Doctoral Degree	12
Health User Experience	
· Yes	100
· No	0
Asthma Control Levels	
· Off Target	0
· On Target	60
· Well-done	40

Table 1: A summary of study participant background and demographic information (n=100)

Demographics	Value (n)
Age	
• 18 - 22	48
• 23 - 27	22
• 28 - 32	21
• ≥ 33	9
Gender	
• Male	44
• Female	56
Education	
• Undergraduate Degree	60
• Bachelor Degree	17
• Master Degree	11
• Doctoral Degree	12
Health User Experience	
• Yes	100
• No	0
Asthma Control Levels	
• Off Target	0
• On Target	60
• Well-done	40

2. Validity and Reliability:

Table 2 represented the results of a questionnaire validity test of Perceived Usefulness (n=5), Perceived Ease of Use (n=5), and Intention to Use (n=3). A valid question item has a validity score greater than r-table[24]. According to the table, Pearson correlation values of question items on Perceived Usefulness (PU1 – PU5), Perceived Ease of Use (PEU1 – PEU5), and Intention to Use (IU1 – IU3) were greater than r-table (0.361), meaning that all the question items of this study were valid.

Table 2: The results of questionnaire validity test

Items	Pearson Correlation	r-Table	Result
PU*1	0.786	0.361	Valid
PU*2	0.739	0.361	Valid
PU*3	0.717	0.361	Valid
PU*4	0.803	0.361	Valid
PU*5	0.717	0.361	Valid
PEU1**	0.667	0.361	Valid
PEU2**	0.677	0.361	Valid
PEU3**	0.805	0.361	Valid
PEU4**	0.635	0.361	Valid
PEU5**	0.826	0.361	Valid
IU*1**	0.834	0.361	Valid
IU*2**	0.828	0.361	Valid
IU*3**	0.872	0.361	Valid

*PU = Perceived Usefulness; ** PEU = Perceived Ease of Use; *** IU = Intention to Use

A reliability test defines the consistency of a measurement. A questionnaire was considered reliable when its value was greater than Cronbach's alpha coefficient[25]. In this study, the coefficient was greater than 0.6. Table 3 showed that all variables (n=3) has values greater than Cronbach's alpha, meaning that they were reliable.

Table 3: The results of questionnaire reliability test

Variables	R-alpha	Cronbach's Alpha	Result
Perceived Usefulness	0.834	0.6	Reliable
Perceived Ease of Use	0.828	0.6	Reliable
Intention to Use	0.872	0.6	Reliable

3. Data Normality Test:

A normality test can be described as a statistical test determining whether the data are normally distributed or not[26]. Data were normally distributed if Asymp. Sig (2 tailed) value was greater than 0.05. In this study, the authors applied Kolmogorov-Smirnov to determine normality distribution of the data. Table 4 confirmed that Asymp. Sig (2 tailed) values of the variables were higher than 0.05, meaning that the data were normally distributed.

Table 4: Results of Kolmogorov-Smirnov data normality test

Variables	Asymp. Sig (2 tailed)	Information
Perceived Usefulness	0.064	Normally distributed
Perceived Ease of Use	0.211	Normally distributed
Intention to Use	0.050	Normally distributed

4. Multiple Linear Regression Analysis:

A multiple linear regression analysis can be described as a mathematical method for modelling the relationship between variables, while a linear regression means that

the relationship between a dependent variable and one or more independent variables is linear[27].

Table 5represented the results of multiple linear regression analysis of this research.

Table 5: Results of multiple linear regression analysis

Model	***Unstandardized Coefficients***		***Standardized Coefficients***	t	Sig.
	B	Std. Error	Beta	B	Std. Error
(Constant)	.512	1.048		.489	.626
Perceived Usefulness	.341	.073	.435	4.681	.000
Perceived Ease of Use	.198	.066	.280	3.011	.003
R^2	**Adjusted R^2**		**F**		**Sig.**
.395	.382		31.662		.000

$Y = 0.512 + 0.341X_1 + 0{,}198X_2 + e$

According to the Table 5 above, the linear regression equation of this study was as follow:

A multiple regression analysis contains F-test (general linear test) and t-test (simple linear test). The analysis was done by determining the results of multiple regression analysis, i.e. determination coefficient of R^2 (Goodness of Fit Model), to test the relationship between one dependent variable and two or more independent variables. The F test was conducted to determine whether independent variables affect dependent variables or not, meaning that the test will prove both Perceived Usefulness and Perceived Ease of Use together significantly influence Intention to Use. Meanwhile, t-testis a statistical method used to test the null hypothesis that the means of two populations are equal. It indicates that the test will determine whether Perceived Usefulness and Perceived Ease of Use each significantly affects Intention to Use or not.

DISCUSSION:

This study mainly aimed to examine Intention to Use Asma Droid of Indonesian asthmatics using Technology Acceptance Model with three variables, e.g. Perceived Usefulness (PU), Perceived Ease of Use (PEU), and Intention to Use (IU). TAM hypothesizes that two variables, i.e. Perceived Usefulness and Perceived Ease of Use, are of primarily relevance for intention to use or user acceptance. In this study, the authors applied TAM approach asit reportedly has high validity, reliability, and robustness. Asma Droid is an OS Google Android-based asthma self-management smart phone app intended for Indonesian asthmatics. The app has many contents and features related to asthma self-management, including: asthma education, a list of asthma medications, asthmatic daily journal, peak flow meter, asthma control test, asthma action plan, asthma asked question, and a map of nearest local hospitals or community health centers. Fig. 2below is a screenshot of Asma Droid app examined in this study.

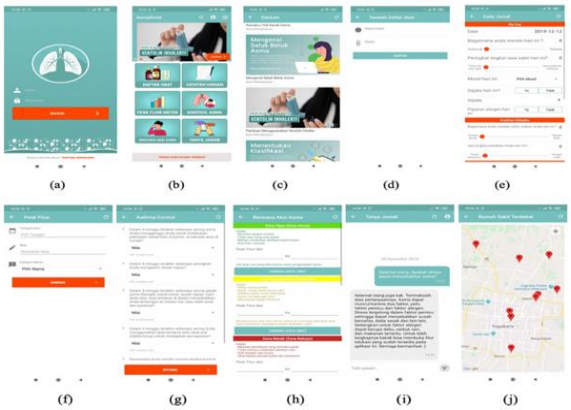

Fig. 2: Screenshot of Asma Droid app

1. Perceived Usefulness (PU):

Table 6was a summary of descriptive analysis of Perceived Usefulness (PU), containing 5 question items. Overall minimum value was 1, while maximum was 4, and average mean was 3.096 with an average standard deviation of 0.409. It represents that the average mean value of PU was greater than the average standard deviation, which indicates good. Of the table below, the modus of all indicators (PU1 to PU5) was 3, meaning that the most popular choice was “agree”. In the term of perceived usefulness, it can be described that almost all study participants agreed that Asma Droid app gives benefits for them regarding asthma self-management.

Table 6: Descriptive statistic of perceived usefulness

Question Items	**n**	**Min**	**Max**	**Mean**	**SD**
AsmaDroid app makes monitoringasthma symptoms easier.	100	3	4	3.11	0.314
AsmaDroid app helps my daily asthma symptom monitoring.	100	1	4	3.03	0.437
AsmaDroid app is my alternative solution in controlling asthma symptoms.	100	2	4	3.06	0.467
The information in AsmaDroid app isaccurate.	100	2	4	2.92	0.485
AsmaDroid app is very useful for me.	100	2	4	3.36	0.522
Average				**3.096**	**0.409**

2. Perceived Ease of Use (PEU):

Table 7summarized the descriptive analysis of Perceived Ease of Use (PEU), consisting of 5 question items. According to the table below, overall minimum value was 1, while maximum was 4, and average mean was 3.052 with an average standard deviation of 0.5562. It explained that the average mean value of PEU was greater than the average standard deviation, which indicates good. Based on the Table 7, the modus of all indicators (PEU1 – PEU2) was 3, meaning that the most popular choice was "agree". It the term of perceived ease of use, it can be described that almost all study participants agreed that the app was easy to use.

Table 7: Descriptive statistic of perceived ease of use

Question Items	n	Min	Max	Mean	SD
I have no problem in operating Asma Droid app.	100	2	4	2.78	0.612
Asma Droid features are easily operated and understood.	100	3	4	2.75	0.687
"Searching for information" in Asma Droid can easily be accessed.	100	1	4	3.04	0.549
Information on symptom control can be easily and rapidly obtained.	100	2	4	3.05	0.411
Asma Droid app is easily accessed; anywhere and anytime.	100	2	4	3.64	0.522
Average				**3.052**	**0.5562**

3. Intention to Use (IU):

Table 8below showed a summary of descriptive statistic of Intention to Use (IU), containing 3 question items. According to the table below, the overall minimum value was 2, while the maximum was 4, and average mean was 2.93 with an average standard deviation of 0.558. It represents that the average mean of the question items was greater than the standard deviation, which indicates good. It the term of intention to use, it can be defined that almost all study participants committed to use, or adopt, the technology of Asma Droid.

Table 8: Descriptive statistic of intention to use

Question Items	n	Min	Max	Mean	SD
I intend to use Asma Droid continuously.	100	2	4	3.15	0.538
I accept Asma Droid because it helps me.	100	2	4	3.24	0.514
Asma Droid features have met the needs of my asthma self-management.	100	2	4	2.42	0.622
Average				**2.93**	**0.558**

4. Hypothesis Test:

F-test

The F-test explains a statistical test that assigns a probability value to each of two hypotheses concerning samples taken from a parent population: a hypothesis assumes that line components are present while the other hypotheses assume that they are not[28]. A hypothesis was accepted when the probability value was equal or greater than 0.05 ($p \geq 0.05$), while rejected when the value was less than 0.05 ($p < 0.05$). According to the

Table 5, it was found that the calculated F value of the study was 31.622 with a Sig. of 0.000, meaning that Perceived Usefulness and Perceived Ease of Use together significantly affect Intention to Use.

t-test:

The t-test means a type of inferential statistical test applied to determine if there is a significant difference between the means of two groups, which might be related in certain features. In t-test, a hypothesis was accepted when the probability was equal or greater than 0.05 ($p \geq 0.05$) while rejected when the value was less than 0.05 ($p < 0.05$).

Table 5showed that the t-value of Perceived Usefulness has a sig. number of 0.000 (<0.05), while Perceived Ease of Use was 0.003 (<0.05). It means that Perceived Usefulness and Perceived Ease of Use each significantly affect Intention to Use.

Adjusted R^2

The adjusted R^2test is used to compare two or more regression models having the same dependent variable but different number of independent variables[29].

Table 5showed that R^2 value of the study was 0.395 while adjusted R^2 was 0.382. It means that the ability of Perceived Usefulness and Perceived Ease of Use to Intention to Use was 38.2% while 61.8% was influenced by external factors not included in this study.

LIMITATION:

This research needs to be evaluated in the context of study limitations. First, the research only involved a small number of participants (100 asthmatics) with high educational background (undergraduate to doctoral degree) and aged more than 18 (18 to≥ 33 years old). As a consequence, the results of the study might not reflect the opinion of asthmatics with lower educational background and aged less than 18 years old thus leading to sampling bias. Another limitation was the study did not include the external variables. This is why, further studies need to include the external variables to determine the influence of Perceived Usefulness and Perceived Ease of Use in which the variables indirectly influenced Intention to Use.

CONCLUSION:

The study employed a descriptive quantitative method and primarily aimed to explore intention to use Asma Droid app of Indonesian asthmatics, using Technology Acceptance Model involving the following variables: Perceived Usefulness, Perceived Ease of Use, and Intention to Use. The results of this study had confirmed that the calculated F-test value of both Perceived Usefulness and Perceived Ease of Use was 31.622 with a Sig. of 0.000. Since the Sig. value of the test was less than 0.05, it could be explained that both Perceived Usefulness and Perceived Ease of Use together significantly affected Intention to Use. Meanwhile, the t-test Sig. value of Perceived Usefulness was 0.000, while Perceived Ease of Use was 0.003. It explains that Perceived Usefulness and Perceived Ease of Use each significantly affect Intention to Use. In this case, as a conclusion, study participants will more easily accept Asma Droid app if they feel that the app has benefits for their asthma self-management and is easy to use.

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CONFLICTS OF INTEREST:

The authors declare that there are no conflicts of interests.

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RESEARCH ARTICLE

Solubility of Salicylamide in (n-propanol, water) and their binary solvent mixtures at T= (288.15 to 313.15) K

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ABSTRACT:
The solubility of salicylamide in pure water, n-propanol, and also in water-n-propanol binary mixtures were experimentally measured using a gravimetric method at temperatures (288.15, 291.15, 293.15, 296.15, 298.15, 301.15, 303.15, 306.15, 308.15, 311.15 and 313.15) K. Solubility values was correlated by the modified Apelblat equation. Thermodynamic properties including $\Delta H_{soln}°$, ΔGsoln°, and ΔSsoln° of salicylamide in pure and mixed solvents were calculated according to the van't Hoff equation.

KEYWORDS: n-Propanol, Solubility, Binary solvents, Thermodynamics, Apelblat equation.

INTRODUCTION:
Solubility data in aqueous and non-aqueous solvents are providing crucial information for the solid phase properties, preparation of drug formulation and development of pharmaceutical analysis in the drug discovery and development[1-3]. Salicylamide is a slight analgesic with antipyretic and anti-inflammatory properties[4].

Fredrik L. Nordstrom and Ake C. Rosmuson determined solubility of salicylamide in methanol, acetic acid, acetonitrile, acetone, ethyl acetate and water from 10 to 50°C[5]. However there is very little data available for solubility of salicylamide in pure n-propanol and water-n-propanol solvent mixtures.

In the present study, solubility of salicylamide in pure n-propanol, water and water-n-propanol solvent mixtures at 288.15 to 313.15 K are reported.

MATERIAL AND METHODS:
Material:
Salicylamide was provided by Loba chemie with purity 99%. n-propanol Provided by Spectrochem with purity 99.8%.

Apparatus and Procedure:
The solubility of acetanilide was measured using an apparatus similar to that described as in the literature[6-8]. An excess amount of salicylamide was added to the binary solvent mixture prepared by weight (Scale-Tec) with an accuracy of ± 0.0001g, in a specially designed 100 ml double jacketed flask. Water was circulated at constant temperature between the outer and inner walls of the flask. The temperature of the circulating water was controlled by thermostat within (±0.1) K. The solution was continuously stirred using a magnetic stirrer for long time so that equilibrium is assured, and the temperature of solution is same as that of circulating water; the stirrer was switched off; and the solution was allowed to stand for 2 hour to ensure complete settlement of un-dissolved suspended substance. Then a fixed quantity of the supernatant liquid was withdrawn from the flask in a weighing bottle with the help of pipette which is hotter than the solution. The mass of solution were weighed by electronic analytical balance, and kept in an oven at 343 K until constant sample weight. The solubility has been calculated using weight of solute and weight of solution. The experiment was repeated three times and the average value were used to calculate the mole fraction solubility(x_B) of salicylamide.

RESULTS AND DISCUSSION:
Solubility data:
Table 1 reports the experimental and calculated (using Apelblat equation) values of solubility (x_B) of salicylamide at 288.15 to 313.15 K in water + n-propanol. Variation of solubility with x_C^0 is visually shown in Figures 1.

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Table 1: Experimental $x_{B(\text{exp.})}$ and calculated $x_{B(cal.)}$ mole fraction solubility of salicylamide for various initial mole fractions, x_C^0, of n-propanol at temperatures (288.15 to 313.15) K.

T/K	x_C^0	$x_{B(exp.)} \times 10^{-2}$	$x_{B(cal.)} \times 10^{-2}$	$RD \times 10^{-2}$	T/K	$x_{B(exp.)} \times 10^{-2}$	$x_{B(cal.)} \times 10^{-2}$	$RD \times 10^{-2}$
288.15	0.0000	0.0029	0.0037	-0.2827	303.15	0.0361	0.0394	-0.0910
	0.0322	0.0194	0.0183	0.0594		0.0858	0.0862	-0.0047
	0.0697	0.1147	0.1131	0.0133		0.3156	0.3116	0.0126
	0.1139	0.3569	0.3586	-0.0048		0.7602	0.7659	-0.0076
	0.1666	0.7201	0.7197	0.0006		1.3673	1.3645	0.0020
	0.2306	1.1593	1.1539	0.0047		2.0807	2.0877	-0.0034
	0.3102	1.6634	1.6792	-0.0095		2.9147	2.9294	-0.0051
	0.4116	2.2963	2.2933	0.0013		3.8246	3.8320	-0.0019
	0.5453	2.8803	2.8793	0.0003		4.6647	4.6694	-0.0010
	0.7296	3.1069	3.1476	-0.0131		4.9407	4.9803	-0.0080
	1.0000	2.3997	2.3639	0.0149		4.2163	4.0894	0.0301
291.15	0.0000	0.0069	0.0073	-0.0563	306.15	0.0394	0.0468	-0.1882
	0.0322	0.0231	0.0266	-0.1511		0.1037	0.1072	-0.0338
	0.0697	0.1386	0.1407	-0.0150		0.3730	0.3732	-0.0005
	0.1139	0.4192	0.4210	-0.0044		0.8673	0.8806	-0.0153
	0.1666	0.8160	0.8172	-0.0016		1.5493	1.5524	-0.0020
	0.2306	1.2895	1.2990	-0.0073		2.3406	2.3507	-0.0043
	0.3102	1.8706	1.8698	0.0004		3.2887	3.2912	-0.0008
	0.4116	2.5318	2.5365	-0.0019		4.2651	4.2571	0.0019
	0.5453	3.1706	3.1719	-0.0004		5.1409	5.1424	-0.0003
	0.7296	3.4013	3.4035	-0.0006		5.4321	5.5636	-0.0242
	1.0000	2.6460	2.6641	-0.0069		4.4042	4.4995	-0.0216
293.15	0.0000	0.0176	0.0109	0.3811	308.15	0.0458	0.0500	-0.0902
	0.0322	0.0340	0.0335	0.0158		0.1239	0.1221	0.0147
	0.0697	0.1591	0.1620	-0.0180		0.4134	0.4194	-0.0144
	0.1139	0.4685	0.4674	0.0024		0.9611	0.9644	-0.0034
	0.1666	0.8920	0.8897	0.0026		1.6942	1.6922	0.0012
	0.2306	1.4064	1.4058	0.0004		2.5401	2.5442	-0.0016
	0.3102	2.0318	2.0111	0.0102		3.5561	3.5599	-0.0010
	0.4116	2.7045	2.7144	-0.0036		4.5773	4.5683	0.0020
	0.5453	3.3737	3.3833	-0.0028		5.4722	5.4837	-0.0021
	0.7296	3.6366	3.5998	0.0101		6.0108	6.0092	0.0003
	1.0000	2.8597	2.8769	-0.0060		4.7134	4.7839	-0.0150
296.15	0.0000	0.0198	0.0180	0.0893	311.15	0.0519	0.0511	0.0148
	0.0322	0.0504	0.0461	0.0846		0.1414	0.1451	-0.0261
	0.0697	0.2019	0.1989	0.0153		0.4956	0.4968	-0.0022
	0.1139	0.5511	0.5447	0.0116		1.1011	1.1018	-0.0006
	0.1666	1.0112	1.0110	0.0002		1.9268	1.9261	0.0004
	0.2306	1.5810	1.5828	-0.0012		2.8724	2.8645	0.0028
	0.3102	2.2714	2.2467	0.0109		3.9368	4.0094	-0.0184
	0.4116	3.0233	3.0071	0.0054		5.0701	5.0811	-0.0022
	0.5453	3.7332	3.7269	0.0017		6.0232	6.0380	-0.0025
	0.7296	3.9978	3.9381	0.0149		6.7201	6.7764	-0.0084
	1.0000	3.1333	3.2149	-0.0260		5.1961	5.2263	-0.0058
298.15	0.0000	0.0230	0.0238	-0.0364	313.15	0.0602	0.0495	0.1771
	0.0322	0.0558	0.0561	-0.0060		0.1641	0.1604	0.0224
	0.0697	0.2277	0.2270	0.0029		0.5573	0.5542	0.0056
	0.1139	0.6077	0.6018	0.0098		1.2141	1.2018	0.0101
	0.1666	1.0951	1.1012	-0.0056		2.0982	2.1000	-0.0008
	0.2306	1.7086	1.7131	-0.0026		3.0974	3.1000	-0.0009
	0.3102	2.4216	2.4213	0.0001		4.4248	4.3436	0.0184
	0.4116	3.2332	3.2212	0.0037		5.4608	5.4565	0.0008
	0.5453	3.9887	3.9750	0.0034		6.4588	6.4378	0.0032
	0.7296	4.2442	4.1966	0.0112		7.5073	7.3635	0.0192
	1.0000	3.3686	3.4527	-0.0250		5.5702	5.5312	0.0070
301.15	0.0000	0.0321	0.0333	-0.0366				
	0.0322	0.0739	0.0733	0.0076				
	0.0697	0.2750	0.2752	-0.0004				
	0.1139	0.6977	0.6964	0.0019				
	0.1666	1.2562	1.2522	0.0032				
	0.2306	1.9550	1.9289	0.0133				
	0.3102	2.6977	2.7130	-0.0057				

	0.4116	3.5546	3.5739	-0.0054	
	0.5453	4.3801	4.3783	0.0004	
	0.7296	4.6310	4.6410	-0.0022	
	1.0000	4.0342	3.8276	0.0512	

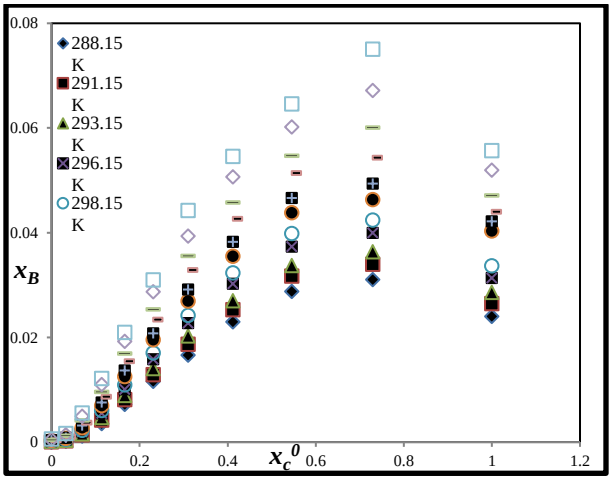

Fig. 1: Plot of mole fraction solubility (x_B) of salicylamide versus initial mole fraction (x_c^0) of n-propanol at temperatures (288.15 to 313.15) K.

The solubility of salicylamide increases with increasing temperature. Salicylamide is an organic compound; naturally the solubility is more in n-propanol as compared with water. In mixed solvents with water as the proportion of organic solvent increases, as expected the solubility also increases.

Modified Apelblat Equation:

The modified Apelblat equation can be expressed as equation 1[9, 10] which is semi-empirical equation; it describes the relation between solubility and temperature.

$$lnx_{B=} A + \frac{B}{T} + ClnT \qquad (1)$$

A, *B*, and *C* are the parameters of the equation and T is temperature in Kelvin. The value A and B represents the variation in the solution coefficient and the C value represents the effect of temperature on the fusion enthalpy. Relative deviation (*RD*) [11] was calculated using equation 2.

$$RD = \frac{x_B^{exp.} - x_B^{cal.}}{x_B^{exp.}} \qquad (2)$$

From table 1 it can be seen that there is an excellent agreement between experimental and calculated values of mole fraction solubility. The values of parameters *A*, *B* and *C* along with co-relation coefficient (R^2) are listed in table 2.

Table 2: Parameters and correlation coefficient (R^2) of modified Apelblat equation

Solvents	x_c^0	*A*	*B*	*C*	R^2
n-Propanol + Water	0.0000	6223.62	-287097	-924.782	0.9521
	0.0322	1690.09	-82698.7	-249.264	0.9934
	0.0697	243.925	-16071.3	-34.4196	0.9995
	0.1139	85.4697	-7766.18	-11.3266	0.9995
	0.1666	-187.589	4915.71	29.2393	0.9999
	0.2306	-152.204	3603.72	23.8786	0.9997
	0.3102	-258.085	8477.81	39.6536	0.9989
	0.4116	-187.867	5601.94	29.0724	0.9998
	0.5453	-117.941	2670.10	18.5623	0.9999
	0.7296	-547.644	21779.7	82.7407	0.9978
	1.0000	180.237	-10830.7	-25.8488	0.9924

Thermodynamic functions of dissolution:

According to the van't Hoff equation, the standard molar enthalpy change of solution ΔH_{sol}^0 is generally obtained from the slope of the lnx_B versus ($1/T - 1/T_{hm}$) plot. Average temperature T_{hm} is introduced to obtain a single value of ΔG_{sol}^0 and ΔS_{sol}^0 in the temperature range studied

$$T_{hm} = \frac{n}{\sum_{i=1}^{n}\left(\frac{1}{T}\right)} \qquad (3)$$

Where n is the number of experimental points. In the present work, T_{hm} = 300.8773 K and the temperature range is (288.15 to 313.15) K in both pure solvents and binary solvent mixtures.

Table 3: Slope (*m*) and intercept (*c*) of the lnx_B versus ($1/T - 1/T_{hm}$) plot along with R^2.

Salicylamide + n-Propanol + Water			
x_c^0	*m*	*c*	R^2
0.0000	-9276	-8.348	0.850
0.0322	-7799	-7.327	0.981
0.0697	-5728	-5.924	0.999
0.1139	-4362	-4.984	0.999
0.1666	-3870	-4.381	0.999
0.2306	-3571	-3.950	0.999
0.3102	-3437	-3.603	0.997
0.4116	-3133	-3.330	0.998
0.5453	-2907	-3.130	0.999
0.7296	-3082	-3.051	0.989
1.0000	-3063	-3.281	0.991

The values of slope and intercept of lnx_B versus ($1/T - 1/T_{hm}$) plot for different solutions including pure solvents and binary solvent mixtures are listed in table 3, form which enthalpy, entropy and standard Gibbs energies of dissolution can be obtained [12].

Table 4: Thermodynamic parameters for salicylamide at mean harmonic temperature T_{hm} = 300.8773 K

x_C^0	ΔH_{sol}^0/kJ ·K^{-1}·mol^{-1}	ΔG_{sol}^0/kJ ·K^{-1}·mol^{-1}	ΔS_{sol}^0/kJ ·K^{-1}·mol^{-1}	$T\Delta S_{sol}^0$/kJ ·K^{-1}·mol^{-1}	% ζH	% ζTS
Salicylamide + n-Propanol + Water						
0.0000	77.1265	20.8830	0.1869	56.2435	57.8290	42.1710
0.0322	64.8475	18.3300	0.1546	46.5176	58.2297	41.7703
0.0697	47.6301	14.8206	0.1090	32.8095	59.2123	40.7877
0.1139	36.2715	12.4674	0.0791	23.8040	60.3765	39.6235
0.1666	32.1760	10.9610	0.0705	21.2150	60.2649	39.7351
0.2306	29.6918	9.8829	0.0658	19.8089	59.9826	40.0174
0.3102	28.5778	9.0149	0.0650	19.5628	59.3631	40.6369
0.4116	26.0544	8.3322	0.0589	17.7222	59.5168	40.4832
0.5453	24.1730	7.8319	0.0543	16.3410	59.6657	40.3343
0.7296	25.6254	7.6323	0.0598	17.9931	58.7489	41.2511
1.0000	25.4716	8.2074	0.0574	17.2642	59.6025	40.3975

The relative contribution of enthalpy (ζH) and entropy (ζTS) to Gibbs energy of solution process is calculated by equation 4 and 5 respectively. The results are shown in table 4,

$$\zeta_H = \frac{|\Delta H_{Sol}^0|}{|\Delta H_{Sol}^0|+|T\Delta S_{Sol}^0|} * 100 \quad (4)$$

$$\zeta_{TS} = \frac{|T\Delta S_{Sol}^0|}{|\Delta H_{Sol}^0|+|T\Delta S_{Sol}^0|} * 100 \quad (5)$$

The values of ΔH_{sol}^0 and ΔS_{sol}^0 for all the solutions are positive indicating the solution process as endothermic. The contribution of enthalpy to positive molar Gibbs energy is more as compared to entropy for all solutions.

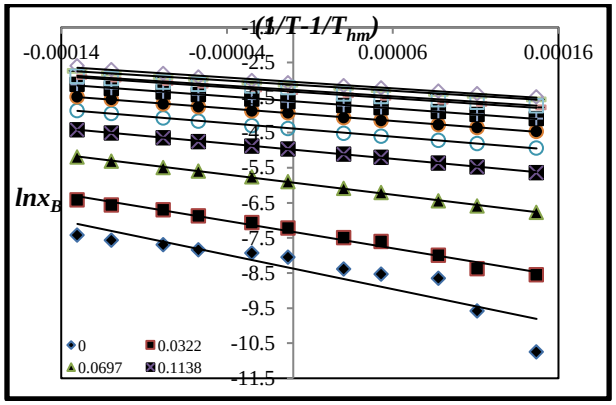

Fig. 2: Plot of lnx_B versus ($1/T - 1/T_{hm}$) for Salicylamide + water + n-propanol system

CONCLUSION:

This study presents experimental data for the solubility of salicylamide in pure water, n-propanol and binary mixtures of water-n-propanol from (288.15-313.15) K. the solubility of salicylamide in pure and binary solvents increases with upward temperature. The modified Apelblat equation is use to correlate the measured solubility data. The calculated values are satisfactory agreement with the experimental data. The thermodynamic aspects of the solubility process of salicylamide in binary mixtures were studied in order to select the best solvent and optimize its solubility.

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