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RESEARCH ARTICLE

Phytochemical analysis of commercially available *Spirulina*, their activities and Biosynthesis of Nanoparticles

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ABSTRACT:
Spirulina is used as dietary supplement in form of capsules and tablets. It is rich in Bioactive molecules and exhibits various activities. Commercially available *Spirulina* was collected. Extracts were prepared using various polar and non-polar solvents. The extracts were used for qualitative phytochemical analysis and to determine their Anti-Oxidant, Anti-Inflammatory and Anti-Arthritic activity. Phytochemicals like Tannin, Carbohydrates and Glycosides were present in all the extracts, while others were present only in few extracts. Biosynthesis of Nanoparticles was done by using the extract. Chloroform extract and Nanoparticles showed higher Anti-Oxidant activity, Ethanol extract showed higher Anti-Inflammatory activity and Chloroform extract showed higher Anti-Arthritic activity.

KEYWORDS: Nanoparticles, *Spirulina*, Phytochemical, Extracts, Activity.

INTRODUCTION:
Spirulina is a filamentous Cyanobacterium, multicellular and it belongs to Phormidiaceae family which appears as blue green filaments under microscope, composed of cylindrical cells arranged in unbranched trichomes. Blue-green algae are used as a source of B-vitamins, iron and dietary protein which is absorbed when taken orally. Blue-green algae has been used in treatment of precancerous growths which are found in the mouth, it also improves memory, boost the immune system, increase energy, lowers cholesterol and helps to improve metabolism, help in healing wounds, preventing heart disease, improves digestion and bowel health. Nanotechnology is the production and manipulation of materials ranging in size from 1 to 100 nanometre scale. They play a top most role in the field of Nanomedicines like Health care and Medicine, Diagnostic and Screening purposes, Antisense and Gene therapy applications, Drug delivery systems, expectations of Nanorobots configuration and Tissue Engineering. Increasing surface area of Nanoparticles increases their biological effectiveness due to the increase in surface energy.

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Biosynthesis of Nanoparticles using microorganisms, enzymes and plant or plant extracts is eco-friendly alternatives to chemical and physical methods. Different metallic nonmaterial is being produced using Copper, Magnesium, Gold, Zinc, Titanium, Alginate and Silver. Naturally occurring bio molecules play an active role in the formation of Nanoparticles of distinct shapes and sizes thus for the designing of greener, safe and environmental synthesis of Nanoparticles. There is a need for green chemistry synthesis of Nanoparticles using biological systems since it is less toxic, cheap and environmental friendly.

Different Phyto-metabolic contents are present in photosynthetic microalgae which includes various biological activities and chemical structures [1]. Various lethal toxins are produced by freshwater Cyanobacteria like Microcystins and Anatoxins. In the purification of Microcystins from the bloom samples, a toxic Cyanobacterium produced other peptides types [5]. The extraction methods used traditionally have several drawbacks such as laborious, time consuming, have low selectivity and/or low extraction yields; but they make use of large amount of toxic solvents [7].

Drug targeting includes the delivery of drugs to specific receptors or organ which may include specific part of the body to deliver the drug exclusively [8]. Spirulina is

highly nutritious micro salt water plant. The spiral shaped algae were found to be a rich food source [14]. *Spirulina* microalgae is most valuable food which contains macro- and micronutrients including high quality Protein, Gamma-Linolenic Fatty acid, Iron, Carotenoids, Vitamins B1 and B2 [16]. *Spirulina platensis*, a blue-green alga is gaining attention due to its nutritional and medicinal value and it also contains Phycobiliproteins like Phycocyanin and Allophycocyanin [20]. Compounds like diterpenoid and anthraquinone have been isolated from the cyanobacterium cells of the cultured Nostoc commune (EAWAG 122b) by Bio-guided isolation [11].

Nanotechnologies have emerged from Physical, Chemical, Biological and Engineering Sciences as a probe and to manipulate single atoms and molecules [21]. Physical and Chemical methods are available to synthesize Nanoparticles. It contains various drawbacks thus various biological agents are used for the synthesis of Nanoparticles. These methods are nontoxic, safe and eco-friendly [22]. Various methods available for synthesis of Silver Nanoparticles includes Tollens, Polysaccharide, Irradiation (Microwave, UV, Gamma etc.,), Polyoxometalates and Biological [23]. Spirulina platensis (Cyanophyaceae) a one-celled blue green algae that survives in warm alkaline fresh water [24].

Nanotechnology is the new trend in the Pharmacy and Biomedical field [2]. Biosynthesis of Nanoparticles has gained attention due to its eco-friendly benign method of production. Nanoparticles synthesis can be done by the use of parts or whole biological component [4]. The increasing demand for Nanoparticles includes their wide applicability in areas like Electronics, Energy, Catalysis, Chemistry and Medicine [10]. Nanoscience and Nanotechnology includes the study of small nano things and that have applications in all the other Science fields, like Biology, Physics Chemistry, Materials Science and Engineering [19]. Nanomaterials can be synthesized using Chemical methods but now synthesized by the use of biological materials [18]. Microbial source has been used to produce Silver Nanoparticles and its precipitation due to its metabolic activity [12].

Various Modern drugs are isolated from Natural sources which are based on their use in Traditional Medicine. The Traditional Medicinal plants have been used for years to treat disease all over the world [13]. Nanoparticles are excellent Research area since they are bridging the gap between the bulk materials and molecular or atomic structural compounds [26]. Biological method to synthesis Silver Nanoparticles can be done by *Euphorbia* hirta [27]. Microalgal system provides a simple, cheap alternative source for the biosynthesis of silver nanomaterials which have uses in biomedical applications [28]. Characterization of Nanoparticles depends on applications. Characterization can be done by various techniques like those used for determination of parameters like particle size, shape, pore size, crystallinity, fractal dimensions and surface area [(29)]. The advantages are that it increases the absorbance of the Herbal formulation, lowers the dose of formulation and raises its solubility [(30)]. The stabilizing agent that keeps the Silver Nanoparticles stable was identified by FTIR spectrum [31].

Branch of Nanotechnology like Nanomedicine which uses nano-sized tools for the Diagnosis, Prevention and Treatment of disease and also helps to gain understanding on the complex underlying pathophysiology [32]. Nanotechnology is defined as the technology which allows the manipulation, control, study and to manufacture structure and devices of 'nanometer' size range [33].

MATERIALS AND METHODS:

Collection of samples:

100g of commercially available *Spirullina* was purchased from medical shop which was in the form of capsules.

Preparation of crude extract [9]:

10g of dried algal biomass from *Spirullina* capsules was weighed and 30ml of solvent (1:3) was added for extraction. Both polar and non polar solvents were used like Chloroform, Aqueous, Acetone and Ethanol. Extraction was done by maceration, in dark at 30°C for 24 hours. The extraction was done three times to harvest the maximum of compounds. It was finally filtered by Whatmann filter paper and the filtrate was stored at 4°C for further use. These extracts were used for phytochemical analysis and various activity analysis.

Qualitative Phytochemical analysis:

Phytochemical analysis was done by standard procedures as stated by Harborne, 1995 [6].

BIOSYNTHESIS OF NANOPARTICLES [3]:

Biosynthesis requires microalgae extract, which is produced by suspending 5g of *Spirullina* powder from the commercially available capsules in 100ml of double distilled water. It was heated in a water bath at 100°C for 15 minutes. It was then cooled and centrifuged at 10,000 rpm for 15 minutes. The supernatant was collected and stored at 4°C for further use. This extract was used for Biosynthesis of Nanoparticles.5ml of extract was added drop wise into 200ml of 1 milli molar Silver Nitrate solution. It was kept at 60°C for 10 minutes with constant stirring and the colour change was noted. It was finally centrifuged at 15,000 rpm for 20 minutes at 4°C.The pellets were washed with distilled water and 90% Ethanol to get pure Silver Nanoparticles.

ANTI-OXIDANT ACTIVITY [6]:
Test tubes were taken and marked as Blank, Standard, Extracts and Nanoparticles. 40 milli molar solution of Hydrogen Peroxide was prepared in Phosphate buffer.0.1mg/ml of Extract solution was prepared in distilled water. It was mixed with equal amount of Hydrogen Peroxide and left undisturbed for 10 minutes. Absorbance was read using a Colorimeter at 550nm.

ANTI-INFLAMMATORY ACTIVITY [15]:
Different test tubes were taken for Control, Standard, Extracts and Nanoparticles. 0.5ml of Diclofenac Sodium was added to tube labelled as Standard and 0.5ml of extracts were added to the extract tubes. 2ml of distilled water was added to Control and 1.5ml in other tubes. 3ml of Phosphate buffer was added to Control tube and 1 ml to other tubes. 2ml Hyposaline was added to Standard, Extract and Nanoparticles tubes. 0.5ml of HRBC suspension was added to all the tubes. The HRBC suspension was prepared by mixing 5ml of Human Blood and 5ml of Alsever's solution. It was centrifuged at 3000 rpm for 15 minutes. The pellets were taken and washed with Hyposaline.10ml of this suspension was mixed with 10ml of Isosaline. The tubes were incubated at 37°C for 30 minutes. It was then centrifuged at 3000 rpm for 10 minutes. The supernatant was taken for the further Analysis and Absorbance was noted at 550 nm using a Colorimeter.

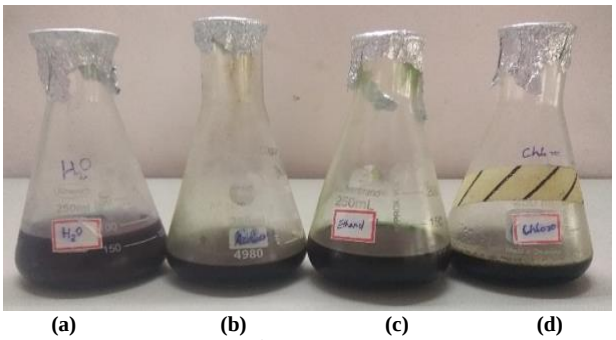
Anti-Arthritic Activity [17, 25]:
Tubes were taken for control, standard, extract and Nanoparticles and labelled respectively.0.5ml of BSA was added to all the tubes. 0.5ml of extract and Nanoparticles were added to the respective tubes.1ml of isosaline was added to all the tubes.0.5ml of Diclofenac Sodium was added to standard tube. Incubate all the tubes at 37°C for 20 minutes. Heat all the tubes at 51°C for 3 minutes and cool the tubes to room temperature. 2.5ml of Phosphate buffer was added to all the tubes.

RESULTS:

Phytochemical Analysis:

Table 1. Phytochemical analysis of various extracts of *Spirulina*.

S.No.	Test	Aqueous Extract	Acetone Extract	Ethanol Extract	Chloroform Extract
1	Triterpenes	-	-	-	-
2	Terpenoids	+	+	-	+
3	Triterpenoids	-	-	-	-
4	Phenols	+	+	-	+
5	Quinones	+	-	-	-
6	Flavonoids	+	+	+	-
7	Tannin	+	+	+	+
8	Phlobatannins	+	-	-	+
9	Alkaloids	+	+	+	+
10	Saponin	-	-	-	-
11	Carbohydrate	+	+	+	+
12	Glycosides	+	+	-	+
13	Anthraquinone	-	-	-	+
14	Coumarins	+	-	-	+
15	Proteins/amino acids	+	-	+	-



(a) (b) (c) (d)

Figure 1. Various extracts of *Spirulina*.
*A-Aqueous Extract, B-Acetone Extract, C-Ethanol Extract and D-Chloroform Extract

ANTI-OXIDANT ACTIVITY:
The activity was determined using the formula-

$$\text{Anti-Oxidant Activity} = \frac{\text{Absorbance of Control - Absorbance of Test}}{\text{Absorbance of Control}}$$

Table 2: Anti-oxidant activity of various extracts

Extract	Od value (550 nm)	% Activity
Control	0.50	-
Ethanol	0.22	56
Acetone	0.31	38
Aqueous	0.23	54
Chloroform	0.05	90
Nanoparticles	0.05	90

Chloroform extract and Nanoparticles showed higher anti-oxidant activity followed by Ethanol, Aqueous and Acetone extract respectively.

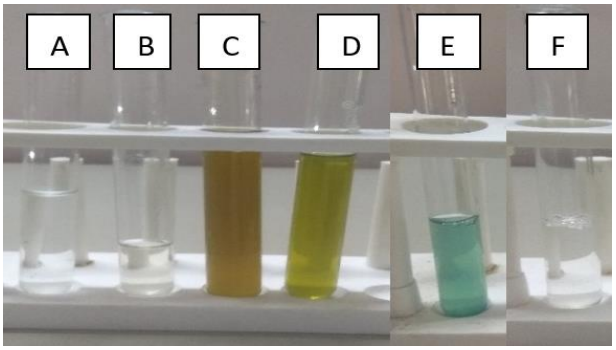


Figure 2. Anti-Oxidant activity
*A-Control, B- Nanoparticles, C-Acetone Extract, D-Ethanol Extract, E- Aqueous Extract and F- Chloroform Extract.

Anti-Inflammatory Activity

The activity was determined using the formula –

$$\text{Anti-inflammatory Activity} = \frac{\text{Absorbance of Test- Absorbance of Control}}{\text{Absorbance of Test}}$$

Table 3. Anti-Inflammatory activity of various extracts

Extract	OD Value (550 nm)	% Activity
Control	0.22	-
Standard	0.48	**54.1**
Ethanol	1.52	**85.5**
Acetone	1.15	**80.8**
Aqueous	0.36	**38.8**
Chloroform	0.38	**42.1**
Nanoparticles	**0.69**	**68.1**

The activity was found to be higher than the standard in Ethanol extract followed by Acetone extract and Nanoparticles. Aqueous extract and Chloroform extract showed comparatively lesser activity than the Standard.

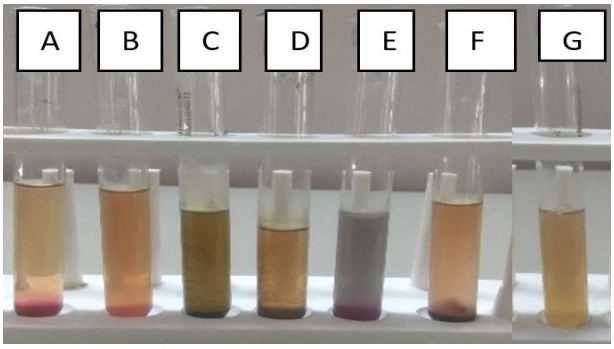


Figure 3. Anti-Inflammatory Activity
*A-Control, B-Standard, C, Ethanol Extract, D-Acetone Extract, E-Aqueous Extract, F-Chloroform Extract and G-Nanoparticles

Anti-Arthritic Activity:

The activity was determined using the formula –

$$\text{Anti-Arthritic Activity} = \frac{\text{Absorbance of Control - Absorbance of Test}}{\text{Absorbance of Control}}$$

Table 4. Anti-Arthritic activity of various extracts

Extract	OD Value (660 nm)	% Activity
Control	0.56	-
Standard	0.04	92.8
Ethanol	0.35	37.5
Acetone	0.28	50
Aqueous	0.46	17.8
Chloroform	0.10	82.1
Nanoparticles	76.7	76.7

The Chloroform extract had highest activity nearer to standard followed by Nanoparticles, Acetone, Ethanol and Aqueous extracts.

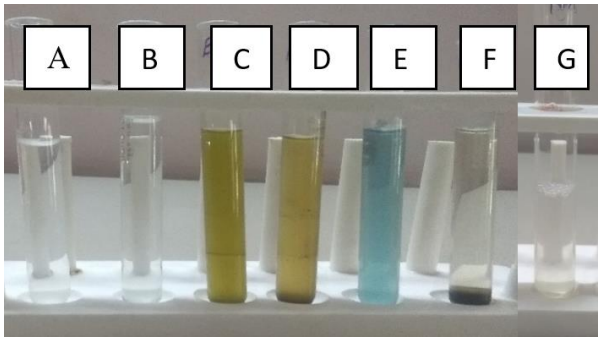


Figure 4. Anti-Arthritic Activity
*A-Control, B-Standard, C-Ethanol Extract, D-Acetone Extract, E-Aqueous Extract, F-Chloroform Extract and G-Nanoparticles

Biosynthesis of Nanoparticles

Nanoparticles characterization was done by colorimeter. Optical density between 400 to 700 nm was checked for Nanoparticles suspension. Nanoparticles have maximum absorption between 400nm to 480nm. Thus, the production of nanoparticles was confirmed.

Table 5: Absorbance of Nanoparticle suspension at various Nanometers.

Nanometer (nm)	Optical Density(OD)
430	0.69
470	0.68
490	0.60
520	0.53
550	0.48
580	0.38
610	0.32
700	0.26

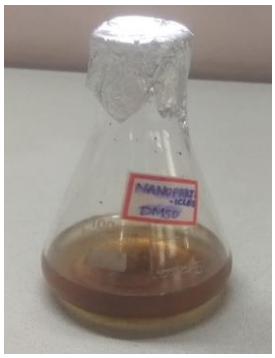


Figure 5. Nanoparticles.

DISCUSSION:

The extracts were produced by using commercially available *Spirulina* capsules and were analyzed for qualitative phytochemicals. It was used for the biosynthesis of Nanoparticles. Anti-oxidant activity was found to be higher in Chloroform extract and Nanoparticles. Anti-inflammatory activity was highest in Ethanol and Acetone extracts. Anti-arthritic activity was seen maximum in Chloroform extract and Nanoparticles. The study indicates the presence of phytochemicals in different solvent extracts of commercially available *Spirulina* tablets. It showed Anti-oxidant, Anti- inflammatory and Anti-arthritic activity higher than the standards. Biosynthesis of Nanoparticles was done and it also showed the activities similar to the extracts.

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RESEARCH ARTICLE

Evaluation of Oxidative Stress potential of some Nsaıds against Hydrogen Peroxide in experimental animal

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ABSTRACT:

Background: Oxidative stress is imbalance between aggressive and defensive system. Overproduction of oxidative stress contribute in pathogenesis of many diseasesincluding Parkinsonism, Alzheimer diseases, apoptosis, hepatic fibrosis ,chronic kidney failure and liver steatosis etc . There are several OTC drugs including NSAIDs that generate oxidative stress when administered. So there is a need to explore about these drugs. Therefore this study was designed to evaluate the oxidative stress potential of Acetaminophen, acetyl salicylic acid and Celecoxib NSAIDs. **Objective:** The present study is design to investigate the oxidative stress of NSAIDs of acetaminophen, aspirin and Celecoxib drug with reference to the hydrogen peroxide. **Material and method:** The Experimental protocol was designed for estimate the level of oxidative stress in NSAIDs treated animals against hydrogen peroxides. Animal of control group received only vehicle throughout experimental protocol. Rats of AAP group, ASA group ,CX group were exposed to acetaminophen (150mg/kg; orally) acetyl salicylic acid (300mg/kg ;orally) and Celecoxib (50mg/kg; orally) for forty two days . Rodent of HP group were challenged with Hydrogen peroxides (0.5%) with same schedule as above. At end of experimental protocols, all the animals were sacrificed and their organ were identified and collected for oxidative stress estimation and histological examination. **Result:** NSAIDs administration caused increase in oxidative stress measured in terms of SOD, CAT, MDA, GSH and GPx. HP administration produced maximum oxidative stress compare to all other groups. Oxidative parameter i.e. SOD, CAT, GSH and GPx were found to be decreased as compare to control rats. However MDA were found to be increased as compare to control rats. Additionally, CX produced less oxidative stress compare to other NDAIDs. Further, histological examinations support the biochemical results. **Conclusion:** From the above observations it can be concluded that NSAIDs have oxidative stress potential and generate oxidative stress and damage the organs when administrated chronically. Thus, these drugs should be used judiciously.

KEYWORDS: Oxidative Stress; Nsaids; Superoxide Anion, Hydroxyl Radical, Hydrogen Peroxide.

INTRODUCTION:

Oxidative stress occurs when the production of oxidizing agents, free radicals and reactive oxygen species (ROS), exceeds the antioxidant capacity of cellular antioxidants in a biological system[1]. A free radical is defined as any species capable of independent existence that contains one or more unpaired electrons. Free radicals can react with and cause damage to DNA, lipids and proteins.3 Examples of free radicals and ROS include: superoxide anion ($O^{-}2$), hydroxyl radical ($OH\cdot$), hydrogen peroxide (H2O2), nitric oxide and nitric

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dioxide, and peroxynitrite.[2] This imbalance leads to tissue injuries and to the progression of degenerative diseases in humans, such asvarieties of neurodegenerative diseases,including Alzheimer's, Parkinson's, Hunting-ton's, tardive dyskinesia ,epilepsy and acute diseases of the central nervous system, such as spinal cord injuries and/or brain traumatic.The acetaminophen has antipyretic and analgesic property.[3,4] That is increase the pain threshold by inhibiting two isoform of cyclooxygenase, COX-1 and COX-2, which are involved in prostaglandin (PG) synthesis,PG is responsible for pain sensation. Acetaminophen is metabolized in the liver by first-order kinetics and its metabolism via the cytochrome P450 enzyme pathway and by conjugation process,Overdose of paracetamol causes hepatotoxicity, which can in severe cases lead to liver failure in experimental animals and humans . Although paracetamol directly conjugated with glucoronic acid and significant amount of acetaminophen metabolized by the cytochrome P450 system[5]. This leads to the formation of a reactivemetabolite, presumably N-acetyl-pbenzoquinoneimine (NAPQI) and react with glutathione (GSH).[5] Thus, paracetamol metabolism causes dramatic depletion of cellular glutathione levels in the liver. If the formation of the reactive metabolite exceeds the capacity of hepatocellular glutathione, NAPQI will covalently bind to cellular proteins.[6] Paracetamol challenge (300mg/kg, i.p) for 7 days caused a significant increase in the levels of bilirubin, liver enzymes, TBARS, and iron, while catalase activity and total protein level were reduced significantly in the serum and liver homogenate.[7]

Acetylsalicylic acid is also termed as aspirin and widely used medication for the treatment of pain and fever. This has anti inflammatory as well as antipyretic. This is also prevents stroke from blood clots and platelets aggregation used for myocardial infarction (MI). The acetylsalicylic acid (ASA) inhibits the synthesis of prostaglandin.This is non-selective for the enzymes (Flower, 2003) COX-1 and COX-2. The main metabolites of acetylsalicylic acid are salicylic acid ether or phenol glucoronide and ester or acyl glucoronide**.** Long term use of low dose aspirin (ASA) has been recommended to reduce the risk of heart attack but gastric bleeding and formation of gastrointestinal ulcers are the most common adverse effects. It has been shown that, ASA damages gastric mucosa by inhibiting the synthesis of protective prostaglandins and by having direct action on the mucosa.[8] This results in enhancing acid back-diffusion and microvascular injury accompanied by the activation of neutrophils that produce excessive oxygen-derived free radicals which cause lipid peroxidation and tissue damage.[9]

Celecoxib inhibit both forms of cyclooxygenase (COX-1 and COX-2) which is a selective, non-competitive cyclooxygenaqse-2 (COX-2) enzyme inhibitor. Their uses minimize inflammation and pain.Celecoxib a nonselective COX inhibitor were studied in rat intestine. The study showed a significant decrease in the lipid peroxide levels as TBA reactive substances as well as the conjugated diene. H2O2, are more stable, less reactive and may act as second intracellular However; they can alter protein conformation after oxidation of cysteine and methionine residues. Moreover, in the presence of Fe2+ or Cu+, via the so-called Fenton reaction, H2O2 is converted to •OH However, although the iron-dependent formation of •OH occurs in vivo, the physiological significance of the copper-dependent formation of •OH is still debated. The amount of ROS formed is not negligible because of the high amount of O2 consumed by aerobic organisms. Indeed, approximately 2–4% of oxygen consumed in mitochondria is converted to the superoxide ion by iron-sulfur proteins. Thus, to maintain homeostasis, accumulation of excess ROS is prevented by multiple enzymatic and non-enzymatic systems that receive the generic name of 'host antioxidant defense systems·

MATERIAL AND METHODS:

Reagents and chemicals:

Acetylsalicylic acid purchased from High purity laboratory chemical Mumbai. Acetaminophen and Celecoxib were procured from Sigma-Aldrich chemical corporation India. Hydrogen peroxide solution (6%), potassium dihydrogen orthophosphate and sodium hydrogen pellets purchased from Thermo Fischer scientific India Pvt Ltd.

Animals and experimental protocol:

The study was carried out thirty male Wistar rats (200±20g; 8-10 week old) were procured from central animal house of Sambhunath Institute of pharmacy Allahabad and used the present study. Previously acclimatized (for 7 days) were randomly divided into equal-sized five groups. All the animals were housed in poly acrylic cage having clean paddy husk at standard condition (12:12 light/dark cycle; humidity 50±5% and temperature 26±1° C) with food and water *ad libitum* throughout the experimental study. All the experimental protocol was approved by IAEA. All the experiments were performed under the guideline of CPCSEA. after acclimatization thirty male rats were randomly divided into five groups (n=6) namely control, AAP, ASA, CX and HP. Experimental protocol was designed for 42 days to estimate the level of oxidative stress in NSAIDs treated animals against hydrogen peroxides. Details of groups and their treatment were as follows:

Group I (Control Group): Only vehicle (distilled water, 1ml and via stomach tube) was administered to the animals of this group for 42 days
Group II (AAP Group): AAP (150mg/kg; *via* stomach tube) was administered to the animals of this group for 42 days
Group III (ASA Group):ASA (300mg/kg; *via* stomach tube) was administered to the animals of this group for 42 days
Group IV (CX Group):CX (50mg/kg; *via* stomach tube) was administered to the animals of this group for 42 days
Group V (HP Group): Animals of thiswas exposed to H_2O_2 (0.5%) as drinking water for 42 days

In this study we did not seen any physiological sign of toxicity in treated animal group. Different route of drugs administration (intragastrically via stomach tube or with drinking water *ad libitum*) required three distinct group control, standard and treatment group. Acetaminophen, acetylsalicylic acid and celecoxib solution freshly prepared daily, hydrogen peroxide is used as standard in drinking water for standard group. Water and chow utilization was estimate daily; body weight was controlled once a week. Body weights of individual animals were recorded before the experimental procedure on day (D1) - (D42). After six week exposure the animal were sacrificed and sample of blood, kidney, liver and brain were collectedThe blood samples were collected by retro-orbital sinus and heart puncture, under chloroform anesthesia using a fine capillary tube. Blood was collected into K2EDTA coated tubes for hematology estimation and tubes without anticoagulant for clinical chemistry. The blood Serum was separated by centrifugation process at 3000 rpm for 10 min at 4℃. Sample of serum were frozen at -80 ˚C for further biochemical estimations. The liver, brain, and kidney was quickly removed , washed in cooled 0.9% NaCl and then homogenized in ice- cold buffer.Then, homogenates were centrifuged at 1500rpm for 15 mints at 4℃ and supernatant was collected. Their contents were examined for abnormalities and placed in 10% formal saline. The five rats that died during the course of the study were noticed after autolysis had set in. Hence, though the rats were necropsies, three organs were collected for examination.[10]

Analytical procedure:

Determination of lipid peroxidation:

Thiobarbituric acid regent, (50mM Dissolve 185mg TBA in 1ml DMSO and then make up volume up to 50 ml distilled water.) TCA in distilled water. 100µl of sample were combined with 500µl TCA (8%w/v) and mixed thoroughly. Sample was centrifuged at 2500rpm for 5 minute to remove the protein precipitation and supernatant was collected in fresh tube. 250µl of supernatant was mixed with 1.8ml of TBA regent and the solution was heated for fifteen minutes in a boiling heating mental water bath The absorbance was analyzed at 532nm against a blank than contains all selected regent except the sample The malondialdehyde concentration was estimated using an extinction coefficient of 1.56×10^5 M^{-1} cm^{-1}

Determination of catalase activity

Hydrogen Peroxide (0.065 M): Amount of 6.639ml of H2O2 (30%) was diluted to a final volume of 1 liter by Na-K-phosphate buffer. Sodium-Potassium Phosphate Buffer, pH 7.4 (60mM): Taken a weight of 11g of disodium monohydrogen phosphate (Na2HPO4) and 2 g of potassium dihydrogen phosphate (KH2PO4) were dissolved in 1 liter of DW. The pH was adjusted to 7.4 by the addition of 1M of HCl. Ammonium Molybdate (32.4mM):Taken a weight of 40.04g of ammonium molybdate was dissolved in 1 liter of DW. All chemical compound and serum were brought to 37°C. Sample, blank, standard, and control tubes were prepared then pipette into test tubes. Tubes were incubated for 4 min second at 37°C.

Determination of superoxide dismutase activity

SOD was assayed as described by The assay mixture contained 0.5ml brain, kidney, liver and blood homogenate, 1ml 50mM sodium carbonate, 0.4mi of 25µm nitroblue tetrazolium (NBT) and 0.2ml freshly prepared 0.1mM hydroxylamine hydrochloride.The changes in the absorbance were calculated at 560 nm and recorded.

Determination of reduced glutathione (GSH) activity:

In this method, an aliquot of 1.0ml of supernatant of the homogenatefor measurement of glutathione activity was treated with 0.5ml of Ellman's reagent [19.8mg of 5, 5'-dithiobisnitro benzoic acid (DTNB) in 100ml of 0.1% sodium nitrate] and 3.0ml of phosphate buffer (0.2M, pH 8.0). The absorbance was calculated at 412nm. The percentage inhibition of reduced glutathione was calculated using the following equation.

Estimation of glutathione peroxidase (GPx) activity:

The activity of glutathione peroxidase (GPx) was measured by the method gunzler and flohe-clariborne (1985) method. Take 100µL of tissue supernatant of liver kidney, brain and blood was put on 10µL of glutathione reductase and 290µL of buffer solution (50mM K2HPO4), (50mM KH2PO4) ph7.0, 3.5mM reduced glutathione, 1mM sodium azide and 0.12mM NADPH and 100µLH202 of 0.8mM their absorbance was read at 340 nm at 0 and 60 s .

Histopathology examination:
Histological estimation was go through on the selected organs of representative rat of each group. The tissues were processed for routine paraffin embedding and sections were stain with Haematoxylin and Eosin stain.

Statistical analysis:
All values were expressed as mean± standard error of mean (SEM). All the statistical analysis of data was done using one way analysis of variance (ANOVA) followed by student – Newman –Keuls post hoc test to monitor significance among group $p<0.05$ was considered as significant.

RESULT:

Effect of ASA, AAP and CX treatment on catalase activity in discrete organs of rat:
Effect of ASA, AAP and CX treatment on catalase activity in discrete organ [(brain; A) (liver; B) (kidney; C)] of rat are depicted in figure (1). Statistical analysis revealed that there was significant difference in catalase activity in brain [$F(4, 25) = 26.34$, $P < 0.05$], liver [$F(4, 25) = 46.55$, $P < 0.005$] and kidney [$F(4, 25) = 25.91$, $P < 0.05$] between the group. Post hoc study illustrated that HP significantly attenuated the catalase activity in above organs of rats as compare to control rat. Further, ASA, AAP and CX also significantly abolished the catalase activity in all above organs as compare to control rat decrement in the activity was found to be less as compare to HP rat. However, CX showed less decrement in catalase activity as compare to ASA and AAP.

Effect of ASA, AAP and CX treatment on SOD activity in discrete organs of rat:
Effect of ASA, AAP and CX treatment on SOD activity in discrete organ [(brain; A) (liver; B) (kidney; C)] of rat are depicted in figure (4). Statistical analysis revealed that there was significant difference in SOD activity in brain [$F(4, 25) = 24.88$, $P < 0.05$], liver [$F(4, 25) = 22.85$, $P < 0.005$] and kidney [$F(4, 25) = 18.62$, $P < 0.05$] between the group. Post hoc study illustrated that HP significantly attenuated the SOD activity in above organs of rats as compare to control rat. Further, ASA, AAP and CX also significantly abolished the SOD activity in all above organs as compare to control rat decrement in the activity was found to be less as compare to HP rat. However, CX showed less decrement in SOD activity as compare to ASA and AAP.

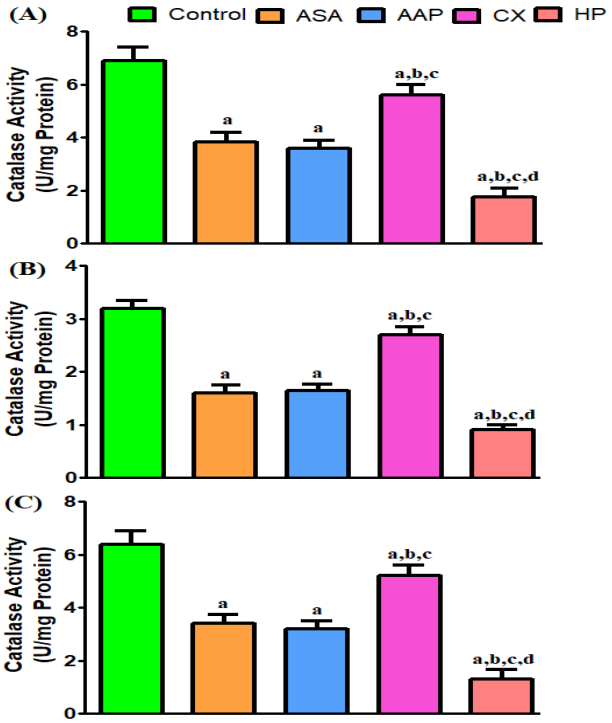

Figure 1:- Effect of treatment on oxidative stress marker in terms of catalase activity in brain (A) liver (B) and kidney (C). All values are Mean ± standard Error of Mean (SEM, n=6). aP < 0.005 compared to control, bP < 0.005 compared to ASA, Cp < 0.005 compared to AAP and dP < 0.005 compared to CX. (One way ANOVA followed by student- Newman – kelus post –hoc test).

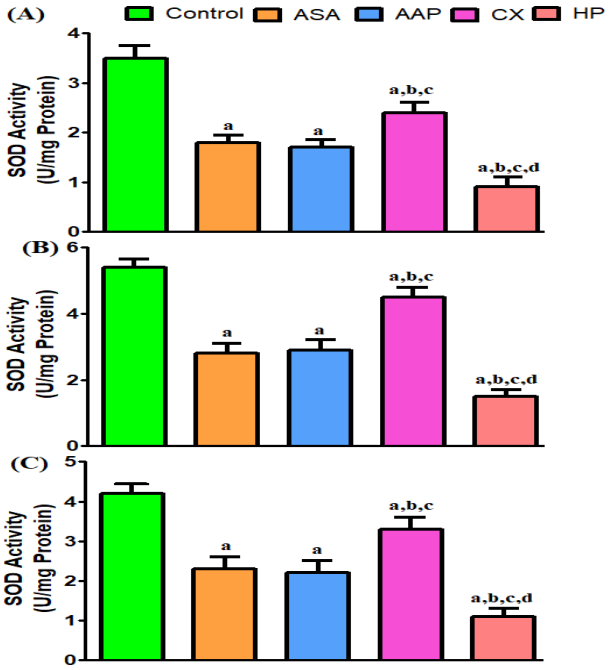

Figure 2:- Effect of treatment on oxidative stress marker in terms of SOD activity in brain (A) liver (B) and kidney (C). All values are Mean ± standard Error of Mean (SEM, n=6). aP < 0.005 compared to control, bP < 0.005 compared to ASA, Cp < 0.005 compared to AAP and dP < 0.005 compared to CX. (One way ANOVA followed by student- Newman – kelus post –hoc test)

Effect of ASA, AAP and CX treatment on MDA activity in discrete organs of rat:

Effect of ASA, AAP and CX treatment on MDA activity in discrete organ [(brain; A) (liver; B) (kidney; C)] of rat are depicted in figure (3) . Statistical analysis revealed that there was significant difference in MDA activity in brain [F (4, 25) =14.74, P < 0.05], liver [F (4, 25) = 17.41, P <0.005] and kidney [F (4, 25) = 15.25, P < 0.05] between the group . Post hoc study illustrated that HP significantly increased the MDA activity in above organs of rats as compare to control rat. Further, ASA, AAP and CX also significantly increased the MDA activity in all above organs as compare to control rat but increment in the activity was found to be less as compare to HP rat. However, CX showed less increment in MDA activity as compare to ASA and AAP.

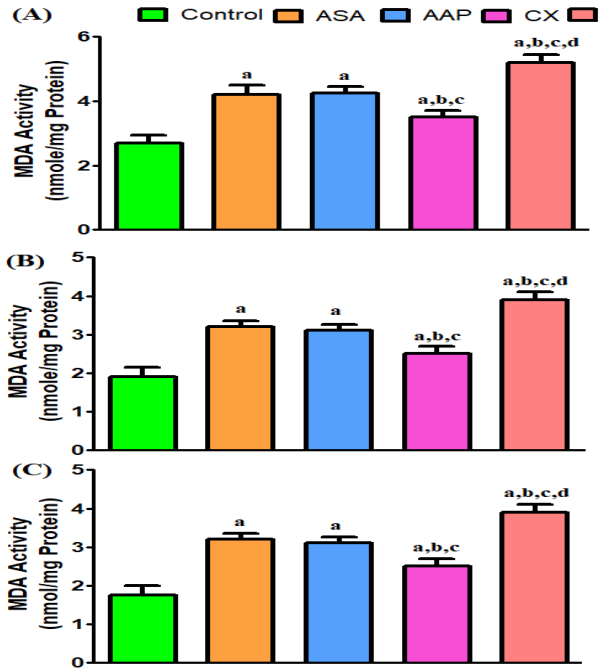

Figure 3:- Effect of treatment on oxidative stress marker in terms of MDA activity in brain (A) liver (B) and kidney (C). All values are Mean ± standard Error of Mean (SEM, n=6). aP < 0.005 compared to control, bP < 0.005 compared to ASA, Cp < 0.005 compared to AAP and dP < 0.005 compared to CX. (One way ANOVA followed by student- Newman – kelus post –hoc test).

Effect of ASA, AAP and CX treatment on GSH activity in discrete organs of rat:

Effect of ASA, AAP and CX treatment on GSH activity in discrete organ [(brain; A) (liver; B) (kidney; C)] of rat are depicted in figure (4). Statistical analysis revealed that there was significant difference in GSH activity in brain [F (4, 25) =18.62, P < 0.05] , liver [F (4,25) = 22.85, P <0.005] and kidney [F (4, 25) = 16.21, P < 0.05] between the group . Post hoc study illustrated that HP significantly attenuated the GSH activity in above organs of rats as compare to control rat. Further, ASA, AAP and CX also significantly abolished the GSH activity in all above organs as compare to control rat decrement in the activity was found to be less as compare to HP rat. However, CX showed less decrement in GSH activity as compare to ASA and AAP.

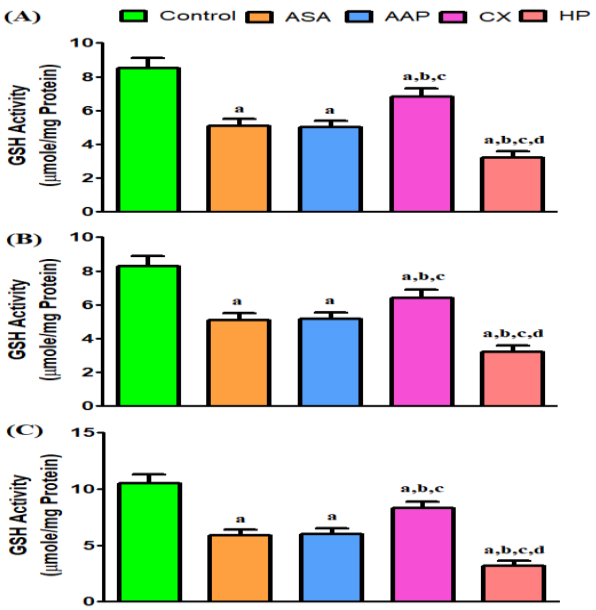

Figure 4:- Effect of treatment on oxidative stress marker in terms of GSH activity in brain (A) liver (B) and kidney (C). All values are Mean ± standard Error of Mean (SEM, n=6). aP < 0.005 compared to control, bP < 0.005 compared to ASA, Cp < 0.005 compared to AAP and dP < 0.005 compared to CX. (One way ANOVA followed by student- Newman – kelus post –hoc test).

Histological studies on liver, brain and kidney

Figure (5), and (6) illustrated the effect of treatment on rat brain (A), kidney (B) and liver (C). Histological examination suggested that hydrogen peroxide caused maximum damage to the all above organs compare to all other organs. Further, ASA and AAP damaged the all selected organs more as compare to control animal but less as compare to HP group rats. Additionally, CX also damaged the organ but less than all other treatment group except control.

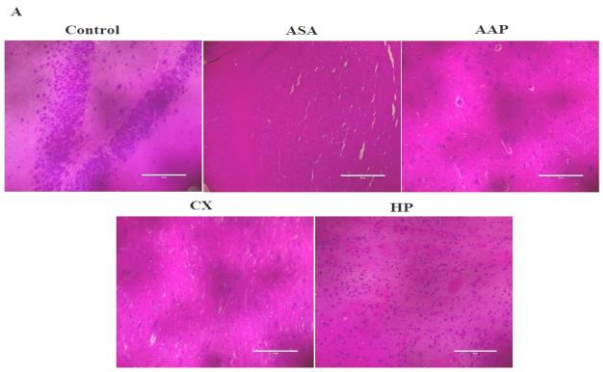

Figure 5: histological representative of brain (control, ASA, AAP, CX and HP)

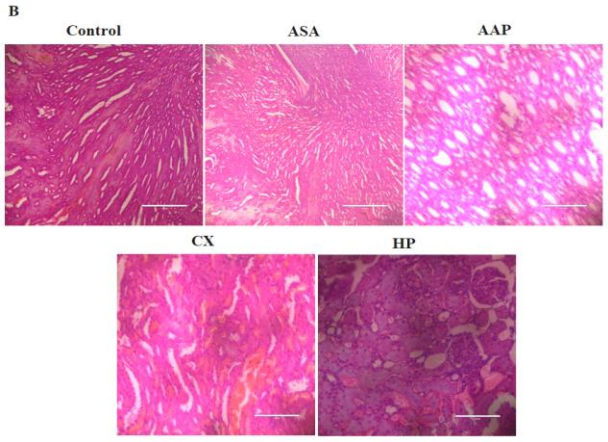

Figure 6 :- histological representative of kidney (control, ASA, AAP, CX and HP)

DISCUSSION:

The present study was designed to demonstrate the oxidative stress potential of NSAIDs against hydrogen peroxide. ASA, AAP and CX were selected as treatment drugs and administered successively for 42 days to evaluate the oxidative stress potential. This study reports for first time about comparative study of ASA, AAP and CX. All these drugs generate oxidative stress when administered. Further, ASA and AAP produced more oxidative stress as compare to CX. The oxidative stresses were evaluated in terms of, SOD, MDA, GSH, GPx, and catalase. Body weight of each animal was recorded every week. At last animals were sacrificed and brain, kidney and liver were collected and applied for histological examination.

Free radical and reactive oxygen species are toxic to biological system.The toxicity is concern in particular to hydroxyl radical, which in turn, may react with the molecule component of the cell and produce second radical that interact with other molecule which is continue to the radical chain reaction.[11] Oxidative stress associated with the formation of ROS, play an important role in the pathogenesis of various deleterious processes and diseases in human, such as brain related diseases[12], amyotrophic lateral diseases.[13] and Huntington diseases,[14] renal fibrogenosis (Masyuk et al., 2008) and liver fibrosis, Apoptosis, liver aging etc. ROS are particularly active in the brain and neuronal tissue as the excitatory amino acid and neurotransmitter, whose metabolism is a factory of ROS which are unique to the brain serve as source of oxidative stress.[15]

In recent year mitochondria have been gradually recognized as the main source of oxidative stress after an overdose of acetaminophen due to excessively formation of NAPQI which was reduce the amount of GSH and attach on the cellular protein According to researcher AAP excess used caused direct molecular changes in mitochondrial electron transport chain (ETC) which was damage to chain of electron caused free radical formation in renal and liver function. According to Hayrettin ozturk Celecoxib responsible for liver injury due to oxidative stress, and other researcher reported that the chronic administration of Celecoxib may have damaging effect in kidney and this damage by oxidants. The evidences of non selective NSAIDs show that they cause more liver toxicity and brain toxicity due to known COX1/COX2 relationship which is responsible for oxidative stress.

SOD superoxide dismutase is the first detoxification enzyme. It is act like first line defense against free radicals. Superoxide anion constantly generated by endogenously and exogenously and produces hydroxyl radical and indirectly from hydrogen peroxide (Tarrunet al., 2003). Further, researcher reported that SOD deficiency causes neurodegenerative disease, myocardial disease and (Strange et al., 2003) death, and deficiency of SOD isoenzyme SOD1 cause amyotrophic lateral sclerosis's (Roberts et al., 2007). Recently (Dayal et al.2017) says that SOD deficiency cause cerebral vascular hypertrophy. In the current study we estimated the SOD level in the different organ such as liver, brain, kidney and blood exposed to NSAIDs against hydrogen peroxide. We found that NSAIDs administration decreased the in SOD level in selected rodent tissue compare to control group.

Malondialdehyde is one of the final products of polyunsaturated fatty acid peroxidation in the cells. It increases due to oxidant species attack lipids containing products and those lipids can oxidize by enzyme cyclooxygenase, cytochrome P450 and lipoxygenase in response to cell death.[16] In the current study we evaluated the MDA level in the different organ such as liver, brain, kidney and blood exposed to NSAIDs against hydrogen peroxide. NSAIDs treatment caused increase in MDA level in selected tissue of rats compare to control group.

Glutathione reductase is an enzyme which catalyzes the reduction of oxidized glutathione (GSSG) to glutathione (GSH). GSH serve as an antioxidant, reacting with free radical and organic peroxides. GSH play a central role in cell death .In the current study we estimated the GSH level in the different organ such as liver, brain, kidney and blood exposed to NSAIDs against hydrogen peroxide. We found that NSAIDs administration caused decrease in GSH level in all the tissue of rodents compare to control group.

Glutathione peroxidase is similar to catalase enzyme which is breakdown the hydrogen peroxide to water and lipid peroxides.[18] These GPx is important role in the

blocking lipid peroxidation and prevent for oxidative damage.[19] The deficiency of GPx can cause neurotoxic damage cancer and cardiovascular disease.[20] In the current study we evaluated the GPx in the different organ such as liver, brain, kidney and blood exposed to NSAIDs against hydrogen peroxide. Result indicated that NSAIDs administration caused decrease in GPx level in selected tissue of rats compare to control group.

Catalase is a tetrameric protein which has four subunits. Each subunit has contained single ferri protoporphyrin . The ability of catalase reduces the concentration of hydrogen peroxide which is important for physiological process. The reduction of this catalase enzyme can cause various disease and abnormalities in the cell.[21] Previous study reported that reduction of catalase can cause risk of cancer and mental disorder.[22] In the present study we evaluated the CAT level in the different organ such as liver brain kidney and blood exposed to NSAIDs against to hydrogen peroxide. We found that NDAIDs administration caused significant decrease in CAT level in the all above tissue of animals compare to control group. Tissues of all the selected organs were implied for histological examinations that confirm the biochemical results.[23] Histological examination suggested that hydrogen peroxide caused maximum damage to the all above organs compare to all other organs. Further, ASA and AAP damaged the all selected organs more as compare to control animal but less as compare to HP group rats. Additionally, CX also damaged the organ but less than other NSAIDs treated groups.

CONCLUSION:

The obtained results indicated the NSAIDs have oxidative stress potential and generate oxidative. NSAIDs administration caused increase in oxidative stress measured in terms of SOD, CAT, MDA, GSH and GPx. HP administration produced maximum oxidative stress compare to all other groups. Oxidative parameter i.e. SOD, CAT, GSH and GPx were found to be decreased as compare to control rats. However MDA were found to be increased as compare to control rats. Additionally, CX produced less oxidative stress compare to other NDAIDs. Further, histological examinations support the biochemical results. From the above observations it can be concluded that NSAIDs have oxidative stress potential and generate oxidative stress and damage the organs when administrated chronically. These drugs are OTC but should be administered with precaution and should be used judiciously

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RESEARCH ARTICLE

In vitro Antioxidant activity and Phytochemical composition of *Syringodium isoetifolium*

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ABSTRACT:

Seagrass are the marine flowering plants found mainly in clear, shallow estuaries and coastal waters. In all temperate and trophical region seagrasses grow both internally and subtidally. One such seagrass namely *Syringodium isoetifolium* has many medicinal properities. This seagrass have most promising pharmacological activities which may include anti-inflammatory, anticancer, antidiarrheal, antihaemorrhoidal activities. This study is focussed on the phytochemical evaluation and *in vitro* antioxidant activity of aqueous, ethanol and hydroalcoholic extract of *Syringodium isoetifolium.* The qualitative analysis of *Syringodium isoetifolium* shows the presence of tannin, saponin, flavonoids, steroids, terpenoids, alkaloids, anthraquinone, polyphenol and coumarin. In all the three extracts only ethanol shows the high concentration of phytocompounds. Emodins, glycoside and anthocyanin were found to be absent in all the three extracts. Quantitative analysis of total phenol, flavonoid, saponin and tannin were found to be 193.10 ±13.52, 106.11 ± 7.42, 52.96 ± 3.64 and 81.30 ± 5.69. Superoxide anion radical, Nitric oxide and Hydroxy radical scavenging assay showed that *Syringodium isoetifolium* was an excellent scavenger of these radicals. These results are an indication of the potent antioxidant property of the extract and may be responsible for some of the therapeutic uses of *Syringodium isoetifolium.*

KEYWORDS: *Syringodium isoetifolium*, ethanol extract, Phytochemical, antioxidant property, free radical scavenger, anticancer activity.

INTRODUCTION:

Many developing countries, including India cure various diseases by these medicinal herbs[1]. These herbs were considered to be a folkoric medicine from the ancient times which has ability to treat neurological disorders, acts as an antidiabetic agent, also shows anti-inflammatory activity[2].

Nowadays herbs play an important role due to its high medicinal value and around 2500 herbs were found all over India[3]. Herbal plants naturally cures the liver diseases due to its antioxidant activity, hence it has ability to heal the damaged liver[4]. Since herbs have high medicinal value, it may cure diseases without any side effects; hence they found to be safe and effective.

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Traditional plants have the medicinal plants as its backbone[5]. Recent investigation shows that the medicinal plants also used in treating asthma, high blood pressure, heart diseases and other health issues[6]. Marine plants are the natural products which show high anticancer activity[7]. A unique group of marine plants namely seagrass located fully in submerged sea. Seagrass exist as a food source for sea animals which may include manatees, green sea turtles and dugongs[8]. In ecosystem, seagrass plays an important role in the biogeochemical process. This seagrass distribution declines nowadays because of natural calamities[9]. If the concentration of sea level increases, it may greatly affects the growth of seagrass. Seawater pollution because of human being is another cause for seagrass decline[10]. Seagrass productivity may be controlled by the abiotic and biotic factors; probably seagrass growth can be controlled by the effect of light, inorganic nutrients, temperature of species[11]. Seagrass can be used as potential drugs, since it has medicinal importance[12].

One such seagrass namely *Syringodium isoetifolium* shows maximum antioxidant activity also exhibit multiple function in cell signaling pathway and protects from oxidative stress[13]. The present study is used to investigate the phytochemical constituents of *Syringodium isoetifolium* by qualitative and quantitative analysis and antioxidant assay was analysed. The leaves were cylindrical and brittle. The plant has slender and shoot rhizome[14]. Cancer is a dreadful disease all over the world, which is a non communicable diseases[15] and the major problem in case of chemotherapy is the drug toxicity[16]. Medicinal herbs play a vital role from the ancient period[17]. Since phytochemical acts as the antibiotic which has ability to cure cancer[18].

MATERIALS AND METHODS:

Collection and Authentication of Sea Grass:

Sample has been collected from Devipattinam, Ramanadhapurm District on June, 2019. The Samples were washed with tap water, shadow dried and powdered by Herbal grinder. And Authenticated in Plant anatomy Research Centre by Dr. P.Jeyaraman, PhD., Director, Retd Professor, Presidency College.

Preparation of Plant extract:

20 grams of powder sample were transferred separately in to three different conical flask. The conical flask containing 1000ml of different solvents (aqueous, ethanol and hydro-alcohol (70%)) were added and vigorously shaking for one hour using rotatory shaker. After 24 hours incubation of mixture, the different extracts were filtered with Whatman No.1 filter paper. For phytochemical screening the filtrate was stored at 4°C.

Preliminary Qualitative phytochemicals screening and Quantitative analysis of phytochemicals:

Chemical tests were carried out different extracts using standard procedures to identify the preliminary phytochemicals following the methodology of Sofowara (1993)[19], Trease and Evans (1989)[20] and Harborne (1973)[21]. The total phenolic contents of the plant were determined by the spectrophotometric method of Kim *et al*., (2003)[22] with slight modification. According to Katasani, the total flavonoids assay was conducted (2011)[23]. Total flavonoids content was determined by using Aluminium chloride colorimetric method. The total Tannins assay was conducted according to Bajaj and Devsharma (1977)[24] method. By colorimetric methods the total saponins contents in different plant materials were estimated (Hiai *et al.,* 1976)[25].

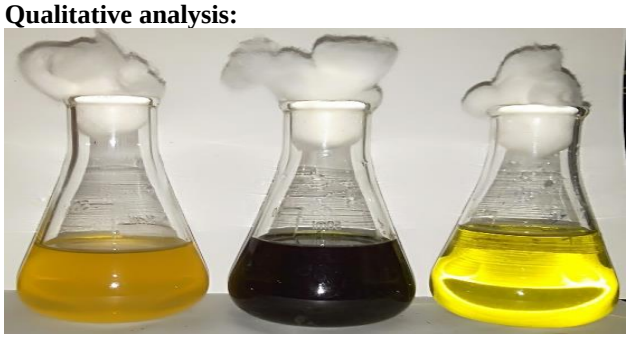
Invitro antioxidant assay:

DPPH radical-scavenging activity was determined by the method of Shimada, *et al.,* (1992)[26]. The superoxide anion radicals scavenging activity was measured by the method of Liu *et al*., (1997)[27]. The scavenging activity for hydroxyl radicals was measured with Fenton reaction by the method of Yu *et al*. (2004)[28]. According to the method reported by Garrat (1964)[29], Nitric oxide radical scavenging activity was determined.

RESULTS AND DISCUSSION:

Phytochemical analysis of leaves extract of *Syringodium isoetifolium*:

Qualitative analysis:



Aqueous extract Ethanol extract Hydro-alcoholic extract

Figure 1: Aqueous, ethanol and Hydro-alcoholic extract of *Syringodium isoetifolium*

Figure 1 shows the aqueous, ethanol and hydroalcoholic extract of *Syringodium isoetifolium*. Phytocompunds are highly present in the ethanol extract rather than the other two extracts. Among all the phytocompounds tannin, saponin, flavonoids, steroids, alkaloids, polyphenols and coumarins shows higher concentration in the ethanol extract.

Table 1: Qualitative Phytochemical analysis of *Syringodium isoetifolium*

S. No	Phytochemicals	Aqueous extract (100%)	Ethanol extract (100%)	Hydro-alcoholic (70%)
1	Tannin	+	++	+
2	Saponin	+	++	+
3	Flavonoids	+	++	++
4	Steroids	+	++	+
5	Terpenoids	+	+	+
6	Triterpenoids	-	+	-
7	Alkaloids	+	++	+
8	Anthroquinone	+	+	+
9	Polyphenol	+	++	+
10	Glycoside	-	-	-
11	Coumarins	+	++	++
12	Emodins	-	-	-
13	Anthocyanins	-	-	-

("+" indicates presence of the compounds; "-" indicates absence of the compounds, "++" indicates the high concentration).

Emodins, Anthocyanin, Glycoside compounds are absent in all the three extracts. Titerpenoids is absent in the Aqueous and Hydroethanolic extract. A bitter plant polyphenols that either bind or precipitates are the tannin compound, since they acts as an astringent. The medicinal important of the tannin compound are haemostatic, antidiarrheal, antihaemorrhoidal, they also used for the treatment of gastritis, enteritis, irritating

bowel disorders and esophagitis[30]. They also exhibit antiparasitic, antibacterial and antiviral activity. The other medicinal uses of tannin includes, they acts as the immediate pain relief of sore throats, dysentery, fatigue, skin ulcers and also protect kidney from various disorders[31].Saponin are responsible for biological effects, it shows more effect on inflammation[32]. In Plants flavonoids acts as a feeding repellant, photoreceptor, visual attractors and also has biological activities which may include anti-inflammatory, antioxidant, antiallergenic, antiviral, antimicrobial activity[33]. Flavonoids acts as an anticancer agent because they involve in chemoprevention of cancer by inactivating carcinogens, also arrest the cell cycle mechanism and induce apoptosis[34]. Flavonoids are the important modulator of cell signalling and also act as Cardioprotectants[35]. Flavonoid has ability to scavenge superoxide anions and has antithrombotic effect by limiting the formation of isoprostanes[36]. Terpenoids act as an allopathic agent. Monoterpenes and diterpenes show maximum antioxidant activity and acts as an anticancer agent[37]. Among all the three extracts only ethanol extract shows high concentration of phytocompounds.

Quantitative analysis of Phytochemicals:

Quantitative ananysis of total phenol, flavnoid tannin and saponin were interpreted in table 2. Total phenol shows higher value 193.10±13.52 than the other phytocompounds. Total Tannin shows lower value 81.30 ±5.69 than favonoids saponin and phenol.

Table 2: Quantitative analysis of Phenol, Flavonoids, Saponin and Tannin content of *Syringodium isoetifolium*

Name of Sample	Total phenol (Milligrams of Gallic acid (GAE) equivalents per gram)	Flavonoids (Milligrams of quercetin equivalents per gram)	Saponin (Milligrams of Quillaja saponin equivalents per gram)	Tannin (Milligrams of tannic acid equivalents per gram
Syringodium isoetifolium	193.10 ±13.52	106.11 ± 7.42	52.96 ± 3.64	81.30 ± 5.69

Values are expressed as Mean ± SD for triplicates

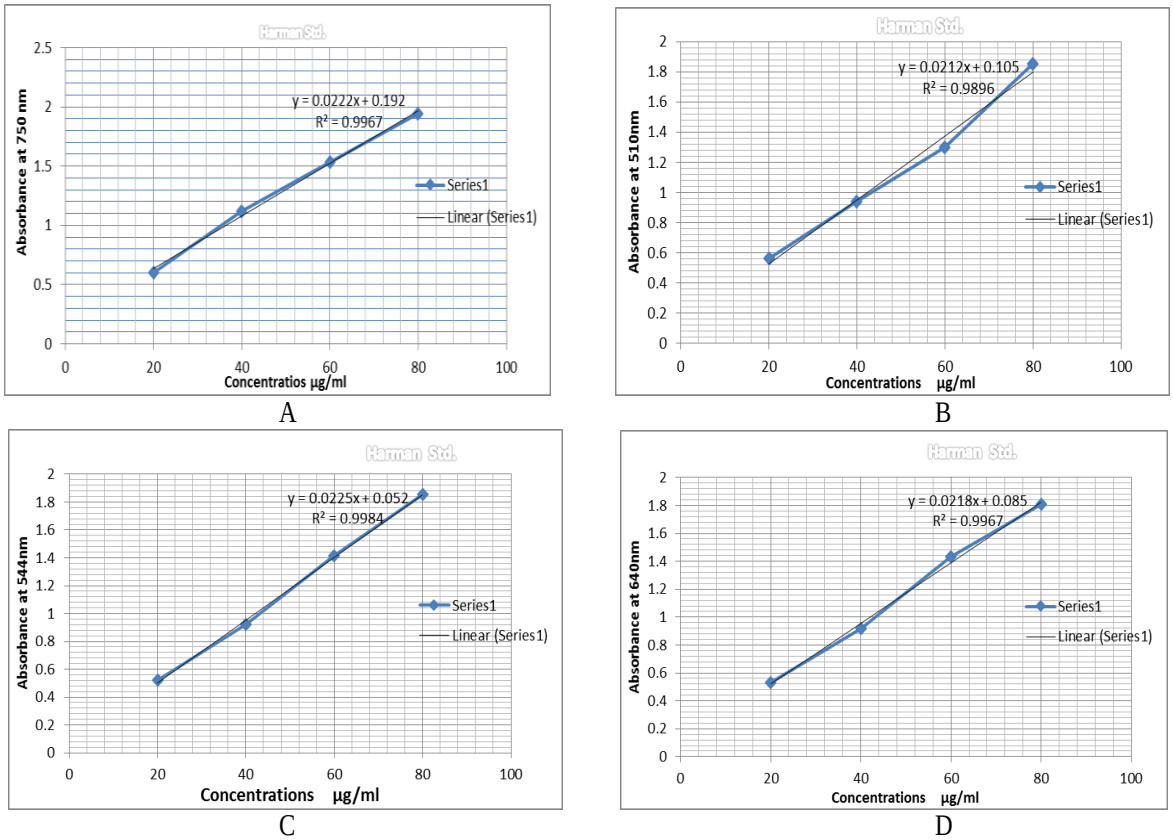


Figure 2: Standard Curve for Phenol, Flavonoids, Saponin and Tannin

A. Standard Curve for Phenol using Gallic acid, B. Standard Curve for Flavonoids using Quercetin, C.Standard Curve for Saponin using Quillaja saponin, D. Standard Curve for tannin using tannic acid.

Major group of antioxidant photochemical are the phenols. The presence of phenol compound is important because they posses free radical scavenging and biological activities[38]. The presence of butylated hydroxytoluene has ability to develop cancer. Due to antioxidant activity the phenols scavenges the free radical and prevent the formation of cancer cells[39]. Figure 2 shows the standard curve of Phenol using Gallic acid, Flavonoids using Quercetin, Total Tannin and Total Saponin.For the treatment of inflammation and Piles tannin is important and also exhibit antiviral, antiulcer, antibacterial activity[40].

***Invitro* Antioxidant Assay:**

DPPH assay was done with the hydroalcoholic extract of *Syringodium isoetifolium* which shows free radical scavenging activity increases with increasing concentration. With the standard ascorbic acid the IC_{50} value of the extract shows 47.46 in the table 3 and the results were interpreted in figure 3. The best method to investigate free radical scavenging activity is the DPPH assay because it shows the hydrogen donating ability[41].

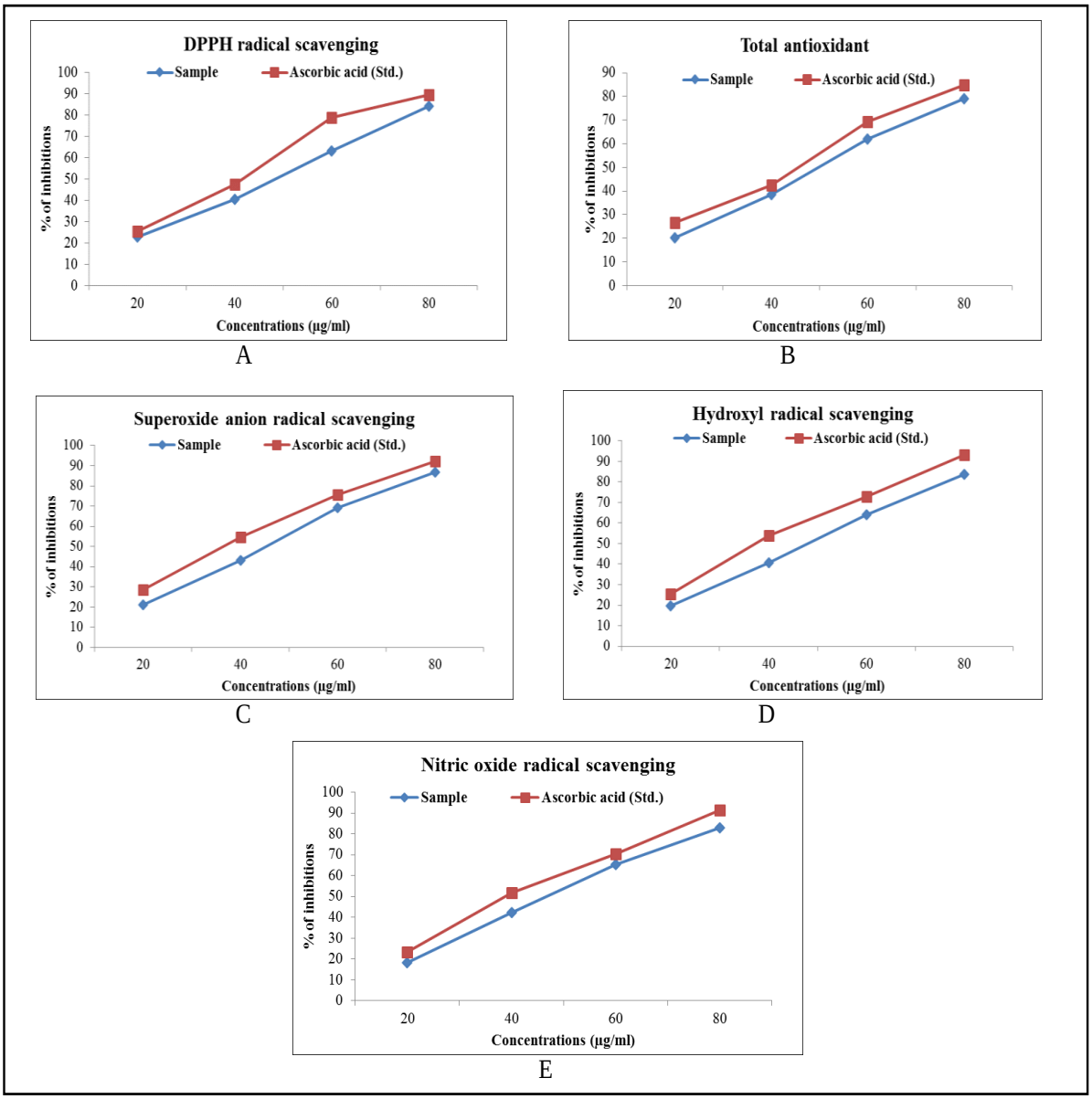


Figure 3: *Invitro* Antioxidant activity of *Syringodium isoetifolium* and standard as ascorbic acid at different concentrations

Table 3: *Invitro* Antioxidant activity of *Syringodium isoetifolium* and standard as ascorbic acid at different concentrations

Samples		Concentrations (µg/ml)				IC_{50} value (µg/ml)
		20 µg/ml	40 µg/ml	60 µg/ml	80 µg/ml	
DPPH radical scavenging	*Syringodium isoetifolium* (% of inhibitions)	22.73±1.59	40.46±2.83	63.19±4.42	84.10±5.88	47.46
	Std. (Ascorbic acid) (% of inhibitions)	25.46±1.78	47.28±3.30	78.64±5.50	89.55±6.26	40.84
Total antioxidant activity	*Syringodium isoetifolium* (% of inhibitions)	20.31±1.42	38.43±2.69	61.87±4.33	79.06±5.53	50.08
	Std. (Ascorbic acid) (% of inhibitions)	26.56±1.85	42.50±2.97	69.37±4.85	84.68±5.92	44.25
Superoxide anion radical scavenging activity	*Syringodium isoetifolium* (% of inhibitions)	21.07±1.47	43.21±3.02	69.28±4.84	86.78±6.07	45.44
	Std. (Ascorbic acid) (% of inhibitions)	28.57±1.99	54.64±3.82	75.71±5.29	92.14±6.44	37.94
Hydroxyl radical scavenging activity	*Syringodium isoetifolium* (% of inhibitions)	19.58±1.37	40.83±2.85	64.16±4.49	83.75±5.86	48.07
	Std. (Ascorbic acid) (% of inhibitions)	25.41±1.77	53.75±3.76	72.91±5.10	93.33±6.53	39.81
Nitric oxide scavenging activity	*Syringodium isoetifolium* (% of inhibitions)	18.09±1.26	42.38±2.96	65.23±4.56	82.85±5.79	48.02
	Std. (Ascorbic acid) (% of inhibitions)	23.33±1.63	51.90±3.63	70.47±4.93	91.42±6.39	41.67

Values are expressed as Mean± SD for triplicates

A. DPPH radical scavenging activity, B. Total antioxidant activity C. Superoxide anion radical scavenging activity, D. Hydroxyl radical scavenging activity, E. Nitric oxide scavenging activity

With increasing concentration the total antioxidant activity also increases and the half inhibition value of extract was found to be 50.08 when compared to the standard ascorbic acid. Increase in Total antioxidant may reduce the oxidative stress which has ability to cure neurological disorders, hypertension, diabetes, atherosclerosis, acute respiratory distress; including cancer[42].Superoxide anion has ability to scavenge O_2^- radicals. Indirectly superoxide anion initiates lipid oxidation and hence it serves as a precursor of singlet oxygen. In biological system the reactive species can be formed which has ability to damage all living cells and hence this radical should be scavenged to avoid carcinogenesis, cytotoxicity and mutagenesis. Hydroxyradical can be estimated by using standard ascorbic acid[43]. In Pathology NO play an important role and the potent NO scavengers are the Flavonoids. They were used for the treatment of vascular endothelial damage[44]. At different concentrations Nitric oxide shows IC_{50} value of 48.02 as shown in Table 7 and the results with standard ascorbic acid were interpreted in Figure 10. The most important molecular species are the NO. They play an important neurotransmitter in Central Nervous System. During microbial infections the free radical species are produced which has ability to cause cell damage. And these cells were found to be scavenged due its increase in antioxidant activity[45].

CONCLUSIONS:

From the present findings it was concluded that the seagrass *Syringodium isoetifolium* has many phytocompounds by qualitative analysis. And the total phenol, flavonoid , tannin and saponin was determined. It shows high strong antioxidant properties due to the high value of total phenolic content. Various studies shows that phenols are the major contributor for the antioxidant capacities of seagrass. *Invitro* antioxidant activity was determined and interpreted which shows that *Syringodium isoetifolium* has high antioxidant activity and has ability to scavenge free radicals. Since this seagrass has various pharmacological activities which may include antibacterial, antiviral, anti-inflammatory effects, further research studies are needed to study the Compounds present in this hydroalcoholic extract of *Syringodium isoetifolium* and the biological activity will be determined for each compound by GCMS analysis.

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RESEARCH ARTICLE

A Flavonoid compound of *Turbinaria decurrens* Bory with The Potential Antioxidant and Anticancer Activity

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ABSTRACT:

Isolation and characterization of quercetin flavonoid compound from Dutungan Island, South Sulawesi Province, Indonesia has been successfully done from ethyl acetate extract. Extraction method used maceration, isolation used chromatography, anticancer activity with MTT method and antioxidant test used DPPH radical. Structure was discussed with the FT-IR, NMR spectrophotometer and compared with the literature. Total flavonoids from ethyl acetate extract were 4.8 mgEQ/g, IC_{50} value of antioxidant activity was 4.23 µg/ml using the DPPH method (2,2-diphenyl-1-picrylhydrazyl), and anticancer activity of H460 cells IC_{50} value was 10.95 µg/ml. The quercetin compound is potential as an anticancer and was first report in the *T. decurrens* Bory species.

KEYWORDS: *Turbinaria decurrens* Bory, Antioxidant, Quercetin, Anticancer, Total flavonoids.

INTRODUCTION:

Turbinaria is a species of seaweed that is included in the class phaeophyceae or brown algae. Some of Indonesian waters have various type of seaweed, including species *T. ornata*, *T. conoides*, and *T. decurrens* Bory. Dutungan Island is a coral island in South Sulawesi which is found of brown algae, one of which is *T. decurrens* Bory. Phytochemical screening of this species contains phenolics, flavonoids, terpenoids and steroids[1], however the research for chemical compounds is still very limited for this species.

Information on the chemical content of the genus *Turbinaria* that have been reported are (22E)-3β-hydroxycholesta-5,22-dien-24-on, 24-ketocholesterol, Saringosterol and fucoxanthin in *T. ornate*[2]. Sterol also found in *T. conoides* including 3,6,17-trihydroxy-stigmasta-4,7,24-triene, 14,15,18,20-diepoxiturbinarine, and fucosterol[3]. Turbinaric acid was found in *T. conoides*[4], as for the phenol group, myricetin was found in *T. ornata*[5]. Besides other phenolic compounds that are also found in brown algae, namely tannins, phlorotannins are found in *Turbinaria ornata*[6].

Ethyl acetate extract was previously reported to has higher antioxidant activity than other extracts to inhibiting DPPH radicals, this extract was also toxic to shrimp larvae *Artemia salina.*[7] The anticancer test begins with a toxicity test against *Artemia salina* Leach as a preliminary test. Flavonoids are the main ingredients and are found in almost all medicinal plants and have a very good pharmacological effect.[8] Flavonoid compounds can be efficacious as an antioxidant or anticancer. Antioxidants are compounds that are able to inactivate the development of oxidation reactions by inhibiting the formation of free radicals[9,10]. Therefore, in our study were interested to isolating antioxidant compounds from ethyl acetate extract. In this paper we also report structure elucidation, total flavonoids, antioxidant activity and anticancer activity on H460 cells from isolate. The structure was determined using FT IR, NMR spectroscopy and compare the reported data.

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MATERIAL AND METHODS:

Chemicals and Reagents:

H460 cell ATCC HTB-177, Roswell Park Memorial Institute (RPMI 1640), MTT (3-(4,5-dimethylthiazolyl-2)-2,5 diphenyltetrazolium bromide), Phosphate Buffer Saline, Fetal Bovine Serum, DMSO Trypsin EDTA, cisplatin, thin layer chromatography, silica gel 60 Merck F_{245}. 2,2-diphenyl-l-picrylhydrazyl (DPPH), H460 cell line, aluminum chloride, and all solvents were analytical reagent grade.

Instrumentation:

FT IR Shimadzu, ^{1}H, ^{13}C, HMBC, HSQC, and COSY (Bruker 500 MHz) spectrophotometers, ultra violet lamps radiation (254 and 365 nm), ELISA reader, and UV VIS spectrophotometers.

Collection and Preparation of seaweed:

Brown seaweeds, *T. decurrens* Bory was collected freshly from Dutungan Island, South Sulawesi Province, Indonesia. Algae had been taken by pulling from the substrate where the brown algae are attached, then put in a bag. After collected, the sample was sorted to remove of impurities. After sorting and cleaning, then washed with water until clean and put in a cool box that has been labeled. The next sample was dried in the oven simplisia, before the extraction process the dried sample was mashed with a blender. Furthermore, the sample powder was stored in a dry condition, for the next process extraction.

Extraction and Isolation:

Dried powder 1 kg was extracted by maceration method with step gradient polarity solvent (n-hexane, ethyl acetate, and methanol). The combined extracts were evaporated to produce crude extracts of n-hexane (3g), ethyl acetate (10g), and methanol (8g).

The dried ethyl acetate extract (10g) was separated by liquid vacuum chromatography (LVC) using silica gel as the stationary phase, and eluted with n-hexane: ethyl acetate: methanol gradient method as mobile phase. LVC results obtained 5 combined fractions (T1-T5), T4 fraction was separated by flash column chromatography eluent n-hexane: ethyl acetate (8:2 - 2:8) and obtained 12 sub-fractions (T1-T12). T10 sub-fraction was re-purified by flash column chromatography and obtained 8 sub-fractions. T8 sub-fraction was recrystallized with ethyl acetate and a white solid of 15mg was obtained. This solid was tested for antioxidant and anticancer activity against H460 cells.

Determination of Total Flavonoid Content[11]

Total flavonoid content in ethyl acetate extract was carried out by colorimetric method and use quercetin as standard solution.[12] Preparation of quercetin series by pipetted from 100µg/mL stock solution of 0.1mL, 0.2 mL, 0.3mL, 0.4mL, and 0.5mL. Each inserted into a 5 mL volumetric flask, from each concentration added 0.2 mL $AlCl_3$, and 0.1mL sodium acetate, then added volume up to 5ml with 70% alcohol to obtain a concentration of 2, 4, 6, 8, and 10µg/mL, shake until homogeneous. Incubated for 30 minutes at room temperature, the quercetin concentration was measured at the wavelength of 420nm. Furthermore, the relationship curve between absorption and concentration was made.

Flavonoid content analysis of 10mg ethyl acetate extract dissolved with 10mL ethanol with 1000µg/mL solution then pipette 1mL with 100µg/mL stock solution. Pipette as much as 0.2mL into a 5 mL volumetric flask, added 0.2mL $AlCl_3$, 0.1mL sodium acetate, then sufficient volume up to 5mL with 70% alcohol. Homogenized and incubated for 30 minutes at room temperature, measured sample absorption at a wavelength of 420 nm.

$$\text{Total Flavonoids} = \frac{(a \times v) \times 1000}{G} \times Df$$

Description:
a = Quercetin concentration in the test sample (mg/L)
V =Total volume of test solution (mL)
G = Weight of extract used (g)
Df = Dilution factor
1000= conversion factor for total solution volume

Measurement of Antioxidant Activity:

The antioxidant activity was carried out by Garg and Mittal (2018), with slight modification. The isolate was made with serial concentration of 2, 4, 6, 8, and 10 µg/mL. Then each concentration was pipette as much as 1 mL and added with 1 mL of 0,4 mM DPPH reagent solution, the mixtures were sufficient to 5 mL with methanol p.a. Homogenize the mixture and incubated for 30 minutes, absorption at a wavelength of 515 nm was measured with UV-VIS spectrophotometer [13].

Antioxidant activity was calculated using the formula:

$$\text{Antioxidant activity} = \frac{\text{Abs Blanko} - \text{Abs sample}}{\text{Abs blanko}} \times 100\%$$

Cytotoxicity Test on Cancer Cells by MTT assay:

H460 cells in RPMI-1640 media with 10% FBS, were put 90µL into 96-well plates with a density of 1.0×10^4 cells per well, incubated 24 hours at 37°C with 5% CO_2. Pipette 10µL of sample solution containing different concentrations of 10, 20, 30, 40, and 50µg/mL and Cisplatin as a positive control, incubated for 72 hours

under the same conditions. Discard the media and replace with 100µL MTT in RPMI-1640 with 10% FBS (0.5mg/mL), and the cells are incubated for 3 hours at 37°C with 5% CO_2. The MTT solution was discarded, and formazan crystals were dissolved in DMSO. After 15 minutes of incubation, absorbance was measured with Elisa reader at 570nm[14].

RESULTS AND DISCUSSION

This research is a follow-up study from previous research, where in this study the results were obtained that the ethyl acetate extract of *T. decurrens* Bory has antioxidant activity with an IC_{50} value of 180.54 µg/mL. Ethyl acetate extract in our study also determined its total flavonoid by colorimetric method and the results of total flavonoid was obtained of 4.8 mgEQ/g (mg Equivalent quercetin/g) extract. The existence of this information, so that this research continues the process of isolation and purification in order to obtain compounds that have bioactivity. The isolation process several fractions were obtained and then purification was carried out. This process obtained a pure isolate named T-8 with a weight of 15 mg. The T-8 isolates obtained were then tested for bioactivity as antioxidants and anticancer using H460 cells.

Determination of antioxidant activity in our study was carried out using the DPPH method (2,2-diphenyl-1-picrylhydrazyl). Free radical reduction activity that was approved with an IC_{50} value[15]. The smaller IC_{50} value mean higher antioxidant activity where the T-8 isolate from *T.decurrens* Bory has antioxidant activity obtained IC_{50} value of 4.23 µg/ml with a very strong activity category[16]. This study was also complemented with an in vitro anticancer against H460 cell and compared with cisplatin as positive control. Table 1 shows that the anticancer activity of T-8 isolates. Isolate which had an IC_{50} value of 10.95 µg/ml and cisplatin as a positive control of 5.64 µg/ml.

Table 1. Anticancer activity of isolate T-8 against H460 by MTT Assay

Sample	Concentration µg/ml	Cytotoxicity (%)	IC_{50} µg/ml
T-8	10	33.67±3.01	10.95
	20	78.38±1.13	
	30	85.05±0.09	
	40	96.4±0.21	
	50	100.0±0.09	
Cisplatin	5	41.82±0.21	5.64
	10	66.45±0.04	
	15	75.40±0.14	
	20	83.19±0.05	
	25	91.21±0.39	

The data were express as mean±SD with triplicate.

Identification of isolate is substance form yellow solids with FT IR spectroscopy shows the wave number at 3412.06 cm^{-1} is -OH, 3288.63 cm^{-1} is -CH unsaturated, 2850.79 cm^{-1} characteristic for aliphatic, 1688.43 cm^{-1} is carbonyl (C = O), 1517.98 cm^{-1} is C=C aromatic, and 1166.93 cm^{-1} is C-O (ether). ESI-TOFMS analysis was obtained $[M + H]^+$ $C_{15}H_{10}O_7$ m/z 303.0495. This isolate structure was determined by ^1H and ^{13}C NMR spectroscopy by comparing the data in Table 1. The existence of ABX proton aromatic system at δH 7,729 (1H, d, J = 2.0 Hz, H-2'), δH 7,621 (1H, dd, J = 7.0 Hz, H-6′), and δH 6,878 (1H, d, J = 7.0 Hz, H-5′) because 3′, 4′ is mated to ring B and the typical meta-coupled pattern for H-6 and H-8 protons (δH 6,181 and 6,385, d, J = 2.0 Hz). The NMR ^{13}C spectrum shows the presence of 15 aromatic carbon, based on NMR data and compared with previous data[17].

Table 2. ^1H-NMR and ^{13}C-NMR (500 MHz), compound 1[a] ($CDOD_3$, in ppm) and Quercetin[b] (in $CDOD_3$)

Position C	^1H NMR[a]	^{13}C NMR[a]	^1H NMR[b]	^{13}C NMR[b]
2		148.92		147.7
3		137.36		135.7
4		177.46		176.8
5		162.64		160.7
6	6.181 1H d, J= 2.0 Hz	99.42	6.20 1 H d, J= 2.0 Hz	98.2
7		165.71		163.9
8	6.385 1H d, J=2.0 Hz	94.59	6.40 1H d, J= 2.0 Hz	94.5
9		158.41		156.1
10		104.69		103
1'		121.86		121.9
2'	7.729 1 H, d, J= 2.0 Hz	116.19	7.65 1H, d, J=2.1 Hz	115
3'		146.37		145
4'		148.2		145.8
5'	6.878 1H d, J= 7 Hz	116.4	6.85 1H, d, J=8.4 Hz	115.6
6'	7.621 1H dd, J= 7.0, 2.0 Hz	124.32	7.50 1H dd, J=8.4, 2.1 Hz	124.5

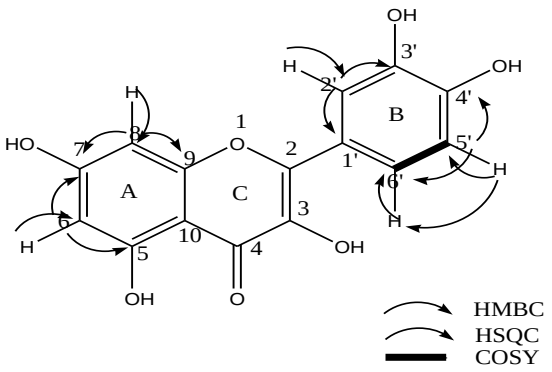

Figure 1 Correlation of HMBC, HSQC and COSY from isolate

To confirm the position of the functional group in isolate, the COSY, HSQC, and HMBC experiments were carried out and the results can be seen in Figure 1. Two aromatic protons at δH 6.878 and 7.621 are interlocked with each other and correlated with C-4' (δC 148.2) and C1' (δC 121.86), while the aromatic protons at δH 7.729 correlate with C-4' (δC 148.2) and C-1' (δC 121.86), from the HMBC spectrum. The aromatic proton δH 6.181 correlates with C-5 (δC 162.64) and C-7 (δC 165.71), while the aromatic proton at δH 6.385 correlates with C-7 (δC 165.71) and C-9 (δC 158.41). The HSQC spectrum shows the aromatic proton δH 6.181 correlates with C-6 (δC 99.42), δH 6.385 with C-8 (δC 94.59) shows that the hydroxyl group is located at C-5 and C-7 on ring A. The aromatic proton δH 7.729 correlates with C-2' (δC 116.19), δH 6.878 correlates with C-5' (δC 116.4), suggesting that the two hydroxyl groups at C-3' and C-4' on ring B of the flavonols skeleton. COSY data shows that δH 6,878 correlates with δH 7.621 shows a very high conformity, so the isolate is identified as quercetin[18]. Myricetin a flavonoid group has also been found in *T. ornata* from Mandapan coast [18], quercetin for the first time reported from the *T. decurrens* Bory species. Base on bioactivity data indicate Quercetin has potential as an anticancer, also got quercetin inhibits HepG2 liver cancer cell[19].

CONCLUSION:

In this study, the total flavonoids produced from ethyl acetate extract were 4.8 mgEQ/g. One flavonoid compound, quercetin was isolated from the ethyl acetate extract of *T. decurrens* Bory, this compound is the first report of *T. decurrens* Bory. Quercetin showed very strong antioxidant activity with a value of IC_{50} 4.23 µg/ml. This compound also has the potential to inhibit H460 lung cancer cells with IC_{50} values < 20 µg/ml, but when compared with positive controls (cisplatin), quercetin activity was still lower.

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CONFLICT OF INTEREST:

The authors declare no conflict of interest.

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RESEARCH ARTICLE

Sustained ophthalmic delivery of pH triggered Cromolyn sodium *in situ* gel

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ABSTRACT:

The research study intends to formulate pH triggered *in situ* gel of Cromolyn sodium composed of Polyacrylic acid (carbopol 934) polymer in combination with Hydroxypropyl Methylcellulose (HPMC K4M) polymer at 1:1, 1.5:1, 2:1 molar ratio by utilizing pH trigger method. Formulations were evaluated for pH, viscosity, gelling capacity, drug content and *in vitro* drug release. Results of Carbopol 934 and HPMC K4M based *in situ* gelling systems at 1:1, 1.5:1, 2:1 shown that the formulations were fluid state at room temperature in a formulated pH (pH 4.5) and went through fast progress into the viscous gel phase at the pH of the tear fluid 7.4. The viscosity of formulated pH triggered *in situ* gel at 2:1 molar ratio shown excellent result compares to 1:1, 1.5:1 molar ratio. The *in vitro* drug release of the developed *in situ* gelling formulations at 1:1, 1.5:1, 2:1 molar ratios increases the contact time and showed a non – fickian diffusion type of release behavior with 94.45%, 83.26%, 70.48% respectively over 8 hours periods compared with that of marketed formulation that shows 99.4% over 4 hours. Thus, the developed system at 2:1 molar ratio acts as a viable alternative to conventional eye drops and also prevent the rapid drainage.

KEYWORDS: *In situ* gel, pH trigger method, Cromolyn sodium, Carbopol 934, HPMC K4M.

1. INTRODUCTION:

Eye is important sensory organs, convert light to recognize brain[1]. Also, restrict the entry of all exogenous substance due to defense mechanisms[2]. Eye may be infected by different diseases such as conjunctivitis, dry eye syndrome, glaucoma and keratitis[3]. Targeting drug to the ocular site with therapeutic dose is task[4]. Factors of nasolacrimal drainage, drugs binding to lachrymal protein, increased lachrymation, minimum availability of corneal area are reducing absorption of drug in ocular routes[5]. The conventional eye drops are having problem with blinking and results in 10 folds decrease in drug concentration within 4-20 minutes necessities regular dosing and bring about pulse kinetics of the drugs in the eye[6].

Ophthalmic inserts, ointment, aqueous gel and nanosuspension are some of the traditional approaches exploited to make longer residence time and enhance bioavailability of instilled ophthalmic dose.

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However, despite these advantages, the ocular delivery systems show shortcomings such as blurred vision (ointment) or low patient compliance (inserts) [7, 8].

In recent times, *in situ* gel forming systems have been used as vehicles for controlled drug delivery as it has advantages like improved patient compliance, reduced frequency of administration, and ease of administration[9]. Moreover, polymeric solutions in this system on exposure to the physiological temperature, pH or ionic composition of the lachrymal fluid changes its physical state from solution to a gel thereby it improves the bioavailability and pre-corneal residence time of drugs. Based on the approach deployed to initiate sol-to-gel phase transition, it is put on as three types of system: pH triggered systems, temperature-dependent system and ion activated systems[10, 11].

Generally, pH sensitive polymers are polyelectrolytes having acid or basic groups that responses with pH changes in the local surrounding environment[12]. One such polymer is Carbopol 934, a polyacrylic acid polymer with mucoadhesive property usually employed in the preparation of ophthalmic pH triggered gel systems[13-15]. In solid-state the Carbopol 934 molecule is in strongly coiled spiral form, on hydration of this spiral

form ends in gel formation thereby enhances the viscosity[16]. Additionally, Carbopol 934 shows excellent organoleptic characteristics, compatibility and stability with many API's, and sol-to-gel phase transition at low concentration[17]. Acidic nature of this polymer may cause eye irritation. Therefore, another polymer such as HPMC K4M was added as a viscosity enhancer to reduce the concentration of Carbopol 934[18].

Cromolyn sodium (2% W/V) eye drop is used as mast cell stabilizer in the treatment of seasonal allergic conjunctivitis. The objective of the present investigation work was to formulate a pH triggered *in situ* gelling system of Cromolyn sodium for sustained ophthalmic delivery. The polymeric combination of Carbopol 934 and HPMC K4M was explored as a vehicle for the Cromolyn sodium (0.2% W/V) eye drops formulation and was characterized for gelling capacity, viscosity, *in vitro* release and its kinetic.

2. MATERIALS AND METHODS:

Cromolyn sodium was gifted sample from Aurolab private limited (Madurai, Tamilnadu), Carbopol 934 and HPMC K4M polymers were purchased from Hi media laboratories Pvt. Ltd (Mumbai). Benzalkonium chloride were obtained from Loba Chemie Pvt. Ltd (Mumbai).

2.1. Identification of Cromolyn sodium:

Before the development of pharmaceutical formulation, the drug's intrinsic chemical and physical properties have been taken into account to provide the framework for drug's combination with ingredients in the fabrication of dosage form. Preformulation studies serve as an important establishment tool every in the development of both API and drug products.

2.1.1. Description (appearance of drug):

Cromolyn sodium was observed visually to check the colour, odour and nature of the powder.

2.1.2. Melting point:

Melting point of cromolyn sodium was determined by the capillary tube method. The sample was filled into a capillary tube which was sealed at one side. Then, the tube was placed inside the melting point apparatus and the temperature at which drug was changed over to fluid was recorded as melting point[19,20].

2.1.3. Saturation solubility studies of Cromolyn sodium:

2.1.3.1. Preparation of simulated tear fluid:

Simulated tear fluid (STF) of pH 7.4: 0.68 g of sodium chloride, 0.20 g of sodium bicarbonate, 0.008 g calcium chloride dehydrate was weighed accurately, dissolved and made up to 100 ml with distilled water[21].

2.1.3.2. Procedure:

The solubility of Cromolyn sodium was determined in different solvents like distilled water and simulated tear fluid, pH 7.4. The samples were added to each test tube containing 2 ml of different solvents with continuous shaking for 30 minutes to prepare a saturated solution. Then the saturated solution was transferred to 2 ml centrifuge tube and kept in centrifugation for 15 minutes at 3000 rpm. The samples were filtered through a Whatman filter and aliquots were suitably diluted. It was measured by UV spectrophotometer at 239 nm[22].

2.1.4. Determination of lambda max:

From the stock solution of Cromolyn sodium (1000 µg/ml), an appropriate dilution was made to 10 µg / ml by using distilled water and it was scanned between 200-400 nm by using UV spectrophotometer. The peak showing maximum absorbance was noted as the lambda max of drug[21].

2.1.5. Preparation of standard curve for Cromolyn sodium:

The standard stock solution of Cromolyn sodium was prepared by dissolving 50 mg of drug in 50 ml of simulated tear fluid (pH 7.4) having a concentration of 1000 µg/ml. Then secondary stock solution (100 µg/ml) was prepared from the primary stock solution by pipetting out 1ml and transferred into a 99ml of STF in 100 ml volumetric flask. From the secondary stock solution, the aliquots of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 ml were pipetted out in a 10ml volumetric flasks and the volume was made up to 10 ml with STF to get concentrations of 1 - 10 µg /ml. The absorbance of the sample solutions was measured spectrophotometrically at 239 nm by using distilled water as a blank. The calibration curve was plotted by using concentrations versus absorbance[23].

2.1.6. Drug-Excipient compatibility study:

Fourier transform infrared studies was performed to identify the interaction between drug and polymer. The FT-IR spectrum of Cromolyn sodium, polymer, physical mixture of Cromolyn sodium and polymer (1:1) were obtained by KBr pellet method which involves grinding samples with KBr powder and compressed into pellets[24, 25]. The FT-IR spectra were estimated over the range of 4000-400cm^{-1}.

2.2. Preparation of *in situ* gel:

Preparation of *in situ* gel involves 2 phase, the first phase involves dissolving the required quantity of HPMC K4M in distilled water kept on magnetic stirrer to get a clear solution followed by addition of the desired amount of Carbopol 934 with continues stirring for overnight. The second phase involves dissolving the desired amount of drug in distilled water to get 2% w/v concentration followed by addition of disodium EDTA in 0.1% w/v and Benzalkonium chloride in 0.001% w/v concentration was added to the previously prepared first phase (polymeric solution) under constant stirring at

room temperature to get a clear solution. Then volume was made up to 100 ml by adding distilled water[26].

Table 1: Composition of Cromolyn sodium *in situ* gel

INGREDIENTS	F-1 (W/V)	F-2 (W/V)	F-3 (W/V)
Cromoyln sodium	2%	2%	2%
Carbopol 934	0.1%	0.15%	0.2%
HPMC K4M	0.5%	0.5%	0.5%
Disodium EDTA	0.1%	0.1%	0.1%
Benzalkonium chloride	0.01%	0.01%	0.01%
water	100ml	100ml	100ml

2.3. Characterization of formulation:

2.3.1 Clarity:

Clarity test for all formulations was visually inspected against a white and black background to inspect the turbidity[27].

2.3.2. pH:

The pH of the prepared formulations was measured by a digital pH meter. This was previously calibrated by standard pH 4 and pH 7[28, 29].

2.3.3. Gelling capacity:

The gelling capacity study was performed by introduction of a drop of formulation in 2ml of simulated tear fluid (STF) that was equilibrated at 37ºC n a clear glass vial[30, 31]. Then, it was visually assessed based on account of the time taken for gelation and the time taken for the gel formed to dissolve.

2.3.4. Drug content:

The drug content determination involves the sample preparation by diluting 1ml of the formulation to 100 ml with STF solution pH 7.4 from this 1 ml was withdrawn and further diluted to 10 ml with STF[32]. Then, it was spectroscopically determined at 239 nm by using UV-visible spectrometer against balnk of STF solution.

2.3.5. Viscosity study:

The developed *in situ* gel formulations were poured into the small adapter of Brook field viscometer and the angular velocity was increased gradually from 10 to 50 rpm. The optimum viscosity of the formulation should be in the range of 5 to 1000 m Pas before gelling and, 50 to 50,000 m Pas after the formation of gel [33, 34].

2.4. In vitro drug release:

By dialysis bag diffusion method *in vitro* drug release study of Cromolyn sodium *in situ* gel formulations was performed. The formulated i*n situ* gel (2 ml) dispersed in a dialysis bag was studied in a 200 ml of simulated tear fluid (pH 7.4) medium contained beaker placed over a magnetic stirrer at 100 rpm that was maintained at 37ºC±1º C. At predetermined time intervals, 2 ml of samples were withdrawn and replaced it with equal amounts of fresh pH 7.4 simulated tear fluid and the collected samples were immediately analyzed by using UV-visible spectrometer at 239 nm. *In vitro* drug release profile of prepared formulation was compared with marketed eye drop[35, 36].

2.4.1. In vitro drug release kinetic studies:

The release kinetics of optimized Cromolyn sodium *in situ* gel formulation was assessed by considering four unique models including zero order, first order, Higuchi model, korsmeyer's-peppas model [37, 38].

3. RESULT AND DISCUSSION:

3.1. Identification of Cromolyn sodium:

3.1.1. Description or appearance of drug:

The visually assessed appearance of the drug was found to be white colored, odorless, crystalline powder.

3.1.2. Melting point:

Melting point of drug was measured by capillary tube method. The result was found to be 241ºC-242ºC respectively, which confirms the identification of the drug.

3.1.3. Saturation solubility study:

The saturation solubility of Cromolyn sodium in distilled water and simulated tear fluid of pH 7.4 was determined and found that Cromolyn sodium was more soluble in distilled water and STF (pH 7.4).

3.1.4. Determination of lambda max:

The lambda max of the Cromolyn sodium was found to be 239 nm and their absorbance 0.265[39].

3.1.5. Standard curve:

Calibration curve of Cromolyn sodium was done in simulated tear fluid pH 7.4. The regression coefficient was discovered to be 0.998 which was shown in Figure 1.

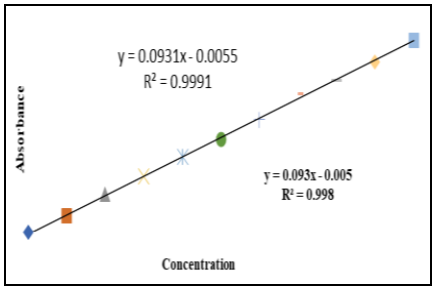

Fig. 1: Standard graph of Cromolyn sodium in STF

3.1.6. Drug - Excipient compatibility studies:

The FT-IR spectra of pure drug, polymer, its physical mixture were obtained. Cromolyn sodium shows the broad characteristic peaks at 3393.86 cm^{-1} (OH stretching), 1702.24 cm^{-1} (-C=O stretching), 1475.59 cm^{-1} (-C=C- stretching), 1051.24 cm^{-1} (-C-O stretching), 951.90 cm^{-1} (-C-H aromatic bending). All these peaks were present both in the physical mixture (drug and polymer). There was no interference of any functional groups in all the spectra. Thus, it showed that there is no specific physicochemical interaction between the drug and polymer used in the formulation.

3.2. Characterization of formulation:

3.2.1. Clarity:

The prepared *in situ* gel formulations was found to be clear without any turbidity and suspended particles.

3.2.2. pH:

The pH of all formulations from F-1 to F-3 was found to be in the range of 4.3 to 4.5 (non physiological condition).

3.2.3. Gelling capacity:

Gelling capacity of all *in situ* gel formulations was given in table 2. All the formulation showed better gelling capacity at physiological condition.

Table 2: Gelling capacity of *in situ* gel

FORMULATION	GELLING CAPACITY
F-1	+++
F-2	+++
F-3	+++

'+++' Gelation immediate, and for extended period

3.2.4. Drug content:

Drug content of all formulations was discovered to be in the range of 73-81%. Least % of drug content was observed in F-1, where the amount of Carbopol 934: HPMC K4M (1:5). The loading of drug was found to be increased, when the Carbopol 934 concentration in the formulation was enhanced, which may be attributed to the charge-based interaction between Cromolyn sodium and Carbopol 934[40].

3.2.5. Viscosity study:

The viscosity of *in situ* gel formulations was measured by Brookfield viscometer and the results were shown in table 5. F-1 formulation shows least viscosity and F-3 was more viscous. This says an increase in viscosity of the formulation when increasing the polymer concentration[41].

The rheological studies of the optimum formulation were studied by plotting graph of shear rate versus viscosity which was shown in Figure 2. This showed that the viscosity of the formulation decreased with an increase in shear rate, which indicates the character of pseudoplastic fluids.

3.3. *In vitro* drug release studies:

In vitro drug release profile of Cromolyn sodium from the gels containing different concentration of Carbopol 934 along with HPMC K4M in the same ratio was tabulated in table 6. Formulation F-3 had shown the least drug release 70.48% in 8 hours compared to formulation F-1 that is 94.45% and formulation F-2 had shown drug release 83.26%.

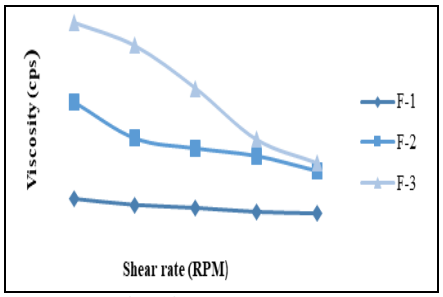

Fig. 2: Rheological profile of the *in situ* gelling system

In vitro drug release study of marketed eye drop (conventional dosage form) demonstrated that around 99.4% medication discharges inside 4 hours.

Table 3: *in vitro* drug profile of Cromolyn sodium

Time (hours)	cumulative % drug release		
	F-1	F-2	F-3
1	46.21	33.20	17.80
2	50.65	39.58	22.67
3	55.44	43.19	31.77
4	56.10	60.89	48.09
5	66.23	66.22	60.18
6	77.34	75.18	61.39
7	85.12	78.46	65.90
8	94.45	83.26	70.48

In vitro drug release study of marketed eye drop and *in situ* gel formulation as a function of time is plotted (figure 3). The result showed that when the polymer concentration decreases drug discharge increases when polymer concentration increases *in vitro* drug discharge from the formulation decreases. *In situ* gel formulations slowly release the drug compared with a marketed eye drop.

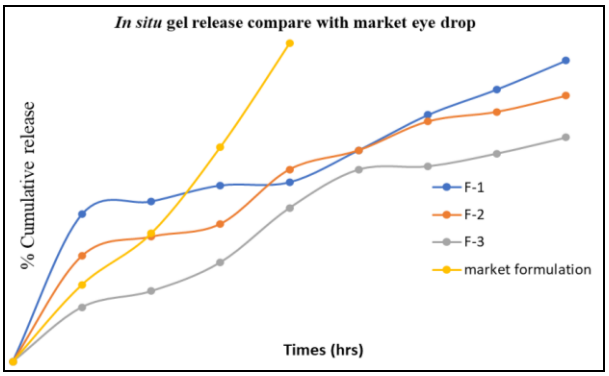

Fig.3: Market formulation compare with *in situ* gel

3.3.1. *In vitro* drug release kinetic studies:

The result of *in vitro* drug release of optimized *in situ* gel formulation was fitted to different kinetics models to discover the drug release mechanism, as shown in table 4.

Table 4: Kinetic modeling of in vitro drug release form optimized ocular in situ gel F-3

Formulation code	Mathematical model kinetics					Best fit model
	Zero order	First order	Higuchi model	Korsmeyer- peppas model		
	R^2	R^2	R^2	R^2	n	
F-3	0.93	0.96	0.85	0.95	0.84	First order

The korsemeyer-peppas release exponent (n) was found to be 0.84 which was followed non-fickian diffusion (n value was found between 0.43-0.85) mechanism (combination of diffusion and erosion) and first order release pattern [42, 43].

CONCLUSION:

Cromolyn sodium *in situ* gel was successfully prepared by using Carbopol 934 and HPMC K4M by pH triggered *in situ* gel technique. F-3 formulation evaluated here has potential in ophthalmic use for reason that it is easily administered and hence the pH triggered *in situ* gelling considered to be promising for improving the ocular residence time without irritating eyes and a viable alternative to marketed eye drops.

CONFLICTS OF INTEREST:

The author declares no conflict of interest with the data contained in the paper.

ACKNOWLEDGEMENTS:

I am thankful to my guide Dr. S. Subramanian, and teaching and non-teaching staffs, Department of Pharmaceutics from PSG College of Pharmacy for providing their innovative ideas and necessary research facilities for performing my work without any fail. I heartily thank my friend vignesh for support every stage.

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RESEARCH ARTICLE

Phytochemical analysis and investigation of Antimicrobial and Antioxidant potential of the Leaf Extracts of *Putranjiva ruxburghi*

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ABSTRACT:

Objectives: Herbs are in use as medicine worldwide from the time immemorial. Many drugs derived from plant, animal or minerals are in use as medicine till date. This is a study on leaf extract of *Putranjiva roxburghii Wall*. (Euphorbiaceae) involving pharmacognosy, phytochemistry, activity against microbes and oxidation to substantiate its use. The leaf extracts were collected by successive soxhlation using solvents like petroleum ether, chloroform, benzene, acetone & ethanol. Chemicals present in the crude leaf extracts and isolated constituents were analyzed for both quality and quantity followed by TLC, UV–Visible Spectrophotometric analysis, HPTLC, Phytochemical tests and TLC examination confirmed to contain flavonoids in acetone, chloroform, aqueous and ethanol extract. The activity of Petroleum-ether, chloroform, acetone, alcoholic and aqueous extracts against the microbes was assessed by cup plate method. Okada & Okada method was followed to evaluate the DPPH free radical for aging property. The reducing, total antioxidant and peroxide radical for aging action of the extracts were assessed. The study confirmed significant antimicrobial and antioxidant property of the *Putranjiva roxburghii* Wall leaves may be for the presence of constituents like flavonoids, saponins, Phytosterols, favoring its traditional usage as medicine.

KEYWORDS: *Putranjiva roxburghii* Wall; antimicrobial; antioxidant; reducing power; peroxide radical scavenging.

INTRODUCTION:

The world is rich in medicinal plants. From ancient times herbs are in use as medicine throughout the world. Many of the drugs currently in use have been derived from herb, faunae or mineral deposits. Around the globe, 80% of inhabitants apparently use herbs for remedies[1]. The remedial property of the plants is endorsed to the chemicals as secondary metabolites available in the plant.

The leaves of *Putranjiva roxburghii* Wall plant was used for the study, which belongs to family Euphorbiaceae**.** They are refrigerant and used in catarrh and to improve fertility. Seeds are sweet, anti-inflammatory and are used to treat vata and pitta, hyperdipsia, opthalmopathy, azoospermia, habitual abortion and sterility[2]. While the fruits are anodyne, anti dysentric, stimulant and tonic. Leaf decoction sometimes prescribed for washing infected eyes[3]. The fruit stones are believed to protect from evil eye and the lives of children. The leaves, the fruits and the fruit stones are used in fever and cold[4]. The nuts of *Putranjiva roxburghii*, wall; in Sanskrit putra-jiva or putram-jiva, "that which makes the child live", are hung round the necks of children to keep them in good health. It is mentioned in the nighantus as being also Garbha-kara, "productive of impregnation" and medicinal properties are attributed to them. The band wrinkled nuts are generally worn only as a charm, but are sometimes given internally in colds on account of

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their supposed heating properties[5]. The beads prepared from the roots of Putranjva tree is used to prevent miscarriage by the locals of Gandaiarea of Chhattisgarh, India[6]. Phytochemical analysis, antimicrobial and antioxidant study on different leaf extracts of Putranjiva was undertaken to substantiate the above facts.

MATERIALS AND METHODS:

Materials:

Streptomycin (CSC Pharmaceuticals, Mumbai), Nutrient agar, mycological peptone (Mitushibiopharma, Ahmedabad), Laboratory grade solvents was procured locally. Strains of organisms (ATCC), *Bacillus subtilis* (6633), *Staphylococcus aureus* (29737), *Salmonella typhi* (19430), *Klebsiella pnemoniae* (10031), *Escherichia coli* (10536) and *Pseudomonas aeurginosa* (9027) were provided by microbiology laboratory, Bharat Institute of Technology, Hyderabad, India.

Collection of plant materials:

Putranjiva roxburghii leaves and fruits of were collected from the forests of Phulbani in Kandhamala district of Odisha, India. Certification of the plant specimen was done by Department of Botany, Bharat Institute of Technology, Hyderabad. Leaves were shade dried and in room temperature.

Pharmacognostical evaluation:

The leaf powder's organoleptic properties (taste, colour and odor) were documented. Leaf powder was observed under microscope for the presence of cell fragments, fibers, epidermal cells, vessels, crystals of calcium oxalate and starch grains. The ash and extractive values, moisture level, other parameters were found using standard methods.

Extraction:

The leaf extract of *Putranjiva* were obtained by successive Soxhlet extraction using different solvents. The leaf powder was packed in a pouch of filter paper and put in the Soxhlet extractor. The extraction was done successively starting with petroleum ether (60-80°C) followed by chloroform, benzene, acetone, ethanol. Aqueous extract was obtained by 24h maceration of dried marc in chloroform-water. The solvents in the extracts were removed by evaporation on the water-bath. The consistency, color and produce of the extracts were charted.

Screening of the phytochemicals:

Chemical analysis (Qualitative and Quantitative) was done on crude extracts and the isolated constituents. Chemical components of the extracts were detected by standard phytochemical tests.

Chromatography (TLC):

Sample extracts were prepared by dissolving in their solvent. Samples were and applied to the silica gel G and GF TLC plates followed by development in suitable solvent system to detect chemical components[7,8]. Developed plates were dried, the spots on the chromatogram were located to observe the separation of the components of extracts. The locations of steroidal glycosides were observed visually and under UV lamp. Carbohydrates were located by using Anisaldehyde and Sulphuric acid. Saponins by vanillin and sulphuric acid, flavonoids by UV lamp and natural product reagent.

Isolation of chemical components:

The method of Harborne along some changes was followed for the separation of chemical compounds. The leaf powder was mixed with 2M HCl and boiled for one hour in a beaker, cooled and filtered followed by successive extraction with petroleum ether and ethyl acetate. Phytochemical test was done on the crystalline compounds collected in the parts of ethyl acetate and petroleum ether[9]. TLC of the isolated compounds were done on silica gel GF and solvent system benzene: ethyl acetate: glacial acetic acid (9:12:0.5). Spots on the TLC were located using UV lamp.

High performance thin layer chromatography studies:

HPTLC (Camag2016) was used to analyze the ethanol extract and isolated compound.

Visible Spectrophotometric estimation of total Polyphenolic constituent:

The total polyphenolic constituent in the ethanol, water extracts and the separated compound were determined with reference to gallic acid. One ml of 5, 10, 15, 20, 25 µg/ml concentration of the extract was added separately to 1ml of 95% ethanol, 5ml distilled water and 50% folin-ciocalteu reagent (0.5ml). The mixtures were kept away from light for 1 hr followed by immediate recording of absorbance at λmax i.e., 725nm.

Assay of antimicrobial activity:

Petroleum-ether, chloroform, alcoholic and water extracts of *Putranjiva* was taken to determine their activity against gram +ve and –ve test bacteria using streptomycin as standard and DMSO as control. Cup plate method was followed for the study. All bacteria used were American Type Culture collection.

Preparation of test solutions:

Extracts having 500, 1000 and 1500mg/0.1ml concentration in DMSO, Streptomycin (100mg/ml) was prepared as standard.

Procedure:
The antibacterial activity of the extracts were done following the procedure reported by Kothari and Shrivasthava[10,11], 3-4mm thickness layer of nutrient agar media in 9cm diameter petri dish containing 25ml of media was used. Using sterile borer, cups of 8mm diameter (5 nos) were made after solidification of the medium. Using micropipette 0.1ml solution of extracts and standard were put in the cups. Diffusion of the extracts and standard was facilitated by keeping the plates in room temperature, followed by incubation for one day at 37±1°C. The above steps were followed simultaneously for control and DMSO vehicle for the extract to assess the activity. The study was done in triplicate. Zone of inhibition (diameter) was measured and recorded.

Free radical scavenging activity using DPPH:
Okada & Okada method[12] technique was followed to assess the DPPH free radical for aging action. Fresh ethanolic solution of DPPH (0.05mM) was prepared by adding 96% ethanol with vigorous shaking then kept in dark at 4^0C. 300µl of the prepared DPPH was mixed with 40µl each from 0.025- 2mg/ml concentration of extract solution. The absorbance (Abs) of the mixture was checked at 517nm using spectrophotometer after 5 minutes. Ethanolic solution of DPPH was used as blank. The determinations were repeated three times. Following equation was used to determine percentage of inhibition, which is the radical scavenging activity[13-17].

Percent of DPPH inhibition= [(Abs blank – Abs test)/Abs blank] × 100

Where Abs blank and Abs test are the absorbance values of the blank and test sample respectively.

Assay of reducing power:
2.5ml phosphate buffer (2 M, pH 6.6), 2.5ml potassium ferricyanide (10g/l) and1 ml of 20-100µg/ml plant extract solution was mixed and kept for 20min at 50°C, added 2.5ml 10% Tri-chloroacetic acid and centrifuged at 1500rpm for 10 min. 2.5ml of the supernatant solution was diluted with equal volume of distilled water and to this 0.5ml $FeCl_3$ (0.1%) was added. The absorbance was measured at 700nm in UV-Visible Spectrophotometer (Elico UV visible Spectrophotometer 196, INDIA) taking phosphate buffer as reference and ascorbic acid as standard. The absorbance was presented as mean ± standard deviation of the end mixture of two corresponding experiments. Higher absorbance of the mixture indicates higher reducing power[18].

Assessment of peroxide radical scavenging activity:
20-100µg/ml of extracts were prepared in phosphate buffer saline (PBS), mixed with 0.6ml of 4mM H_2O_2 solution prepared in PBS and incubated for 10min. The absorbance at 230nm using ascorbic acid as standard was measured[19].

Assessment of total antioxidant activity:
0.2ml of the extracts (100-500µg/ml) was diluted with 1.8ml of distilled water, to this 2ml of phosphomolybdium reagent solution containing 0.6M Sulphuric acid, 28mM sodium phosphate and 4mM ammonium molybdate was added, stored at 95°C for 90min, allowed for cooling to room temperature and absorbance was measured at 695nm[20-25]. Ascorbic acid equivalent was used to represent the antioxidant activity.

Table 1: Rf values for different components by thin layer chromatography of different extracts of *p.roxburghii*

Compound	Rf value					
	Pet Eth Ext	**Benz Ext**	**Ace Ext**	**Chlor. Ext**	**Eth. Ext**	**Wat. Ext**
Steroidal glycosides	0.44	0.35	-	-	-	-
Carbohydrate	-	-	-	-	0.22 0.53 0.71	0.80 0.71 -
Saponins	-	-	-	0.53 0.94	0.93 -	0.82 -
Flavonoids	-	-	-	0.26 0.44 0.86	0.44 0.62 0.86	0.26 0.82 0.93

Pet Eth E: Petroleum ether extract; AE: Acetone extract; Eth Ext: Ethanol extract; Benz Ext: benzene extract; Ace Ext: Aqueous extract; Chlorof Ext: Chloroform extract.

Table 2: Assessment of total phenolic compound in *p.roxburghii* leaf extract.

Concentration	Absorbance at 725nm			
	Ethanolic extract	Water extract	Isolated compound	Gallic acid (std)
10	0.003±0.001	0.004±0.001	0.004±0.007	0.17±0.014
20	0.005±0.003	0.071±0.007	0.062±0.002	0.28±0.002
30	0.22±0.002	0.26±0.001	0.26±0.015	0.37±0.006
40	0.26±0.002	0.44±0.001	0.35±0.02	0.53±0.002
50	0.51±0.002	0.69±0.003	0.62±0.15	0.87±0.003

n=5; mean±SE

Table 3: Zone of inhibition for extracts of *putranjivaroxburghii* leaf

Extract	Bacillus subtilis	Staphylococcus aureus	Klebsiellaphuemoniae	Escherichia coli	Pseudomonas aeurginosa	Salmonella typhi
streptomycin	5.4±1.9	6.2±1.2	6.0±1.9	6.0±0.2	9.0±0.1	6.0±1.5
petroleum ether	1.4±1.3	1.1±1.6	2.0±1.4	1.8.0±0.8	2.0±0.13	1.6.0±1.7
chloroform	3.3±1.7	4.5±1.8	5.0±1.2	5.2±0.2	7.7±0.6	5.1±1.3
ethanolic	6.3±1.5	6.7±1.4	5.6±1.8	5.7±0.8	8.0±0.5	7.0±1.6
aqueous extracts	2.2±1.4	3.2±1.8	4.0±1.2	3.0±0.8	5.4±0.4	3.0±1.7

n=3; mean±SE

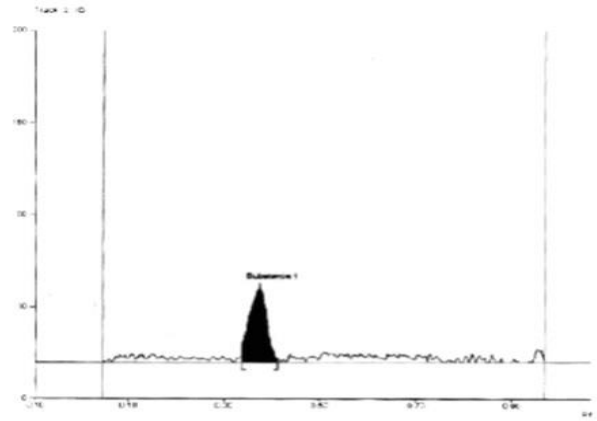


Fig. 1: HPTLC Chromatogram of isolated compound (?).

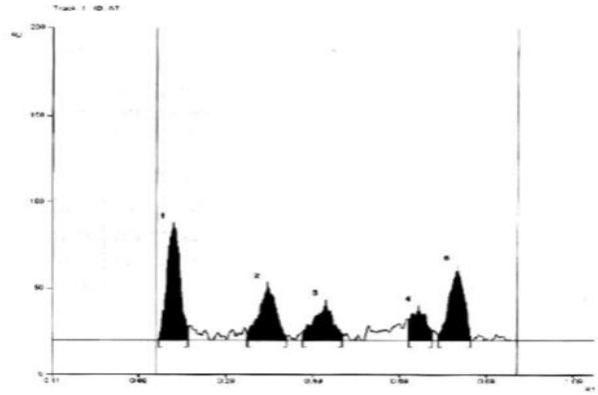


Fig. 2: HPTLC Chromatograms of Ethanol extract.

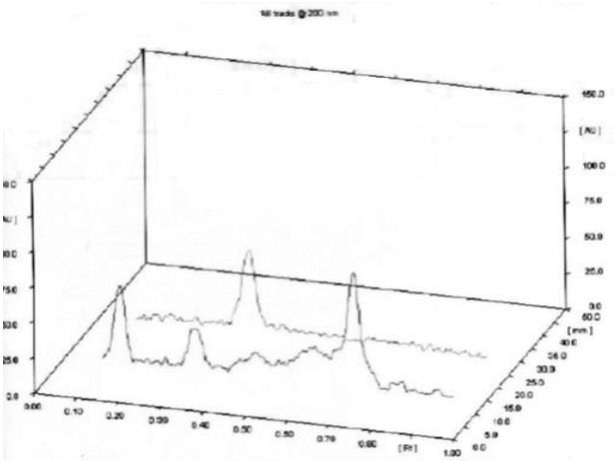
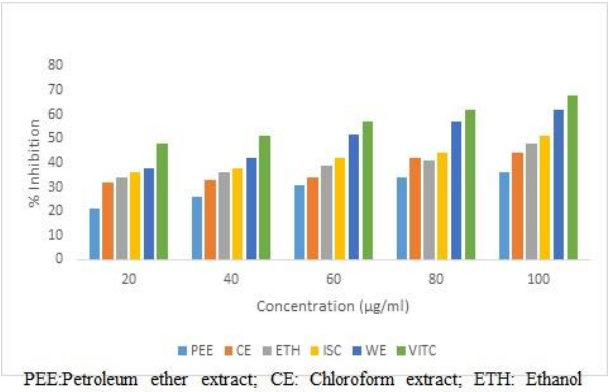
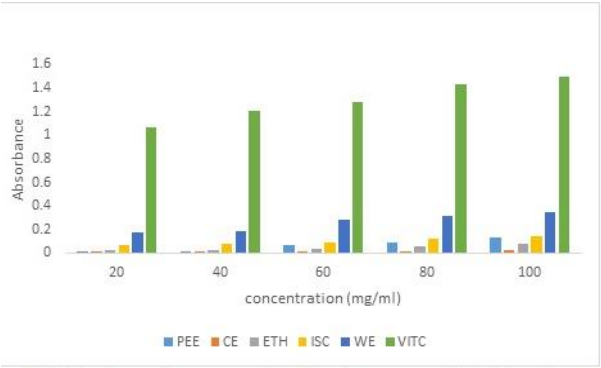


Fig. 3: 3D photographs of the isolated compound and ethanol extract.



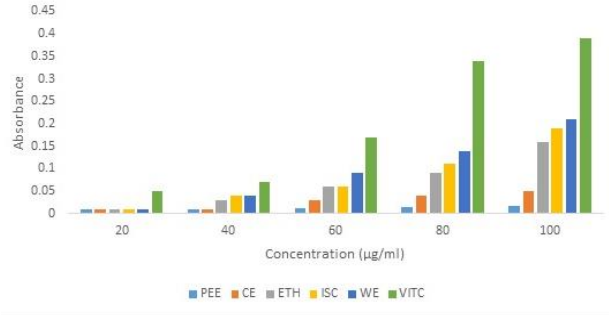
PEE:Petroleum ether extract; CE: Chloroform extract; ETH: Ethanol extract; ISC: Isolated compound; WE: Aqueous extract; VITC: Vitamin C.

Fig. 4: Radical Scavenging activity DPPH, of leaves extracts of *P. roxburghii* and Vitamin- C



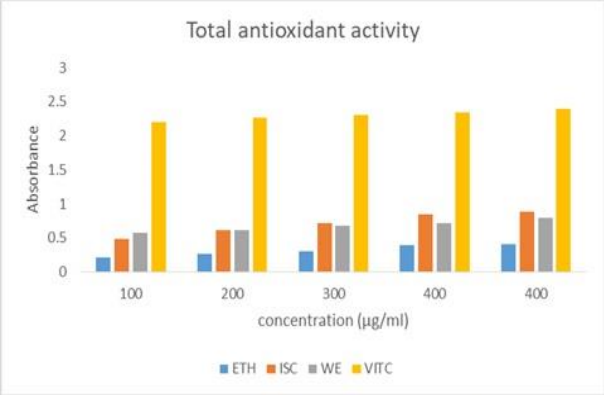
PEE:Petroleum ether extract; CE: Chloroform extract; ETH: Ethanol extract; ISC: Isolated compound; WE: Aqueous extract; VITC: Vitamin C.

Fig. 5: Reduction power of leaves extracts of *P. roxburghii* and Vitamin-C



PEE:Petroleum ether extract; CE: Chloroform extract; ETH: Ethanol extract; ISC: Isolated compound; WE: Aqueous extract; VITC: Vitamin C.

Fig. 6: H_2O_2 Scavenging activity, of leaves extracts of *P.roxburghii* and Vitamin-C



ETH: Ethanol extract; ISC: Isolated compound; VITC: Vitamin C

Fig.7: Sum of Antioxidant activity of leaves extracts of *P.roxburghii* and Vitamin-C

RESULTS AND DISCUSSION:

Study on Pharmacognosy:

Shade dried *Putranjiva*leaves were coarsely powdered. The organoleptic, microscopic and physical characteristics of the powdered drug were evaluated. The color of the powders was greenish red with distinct odor without taste. The leaves were coriaceous dark green, shining, 6.3–10cm by 2.2–3.8cm, elliptic oblong, obtuse or acuminate, distantly serrulate, main nerves slender, with reticulate venation between; petioles 6mm long; stipules small triangular acute. Microscopic evaluation of the leaf powder was done. The result showed presence of rectangular to square shaped thick walled epidermal cells with and prominent cuticle, wide angular thick walled short radial multiples of xylem elements, stomata is anomocytic and presence of sporadic Calcium oxalate (Spherocrystals).The powdered leaves found to have 8%, 3.6%, 5.8% of total, acid insoluble and water soluble ash with water soluble extractive 6.2%, 3.9% of alcohol soluble extractive and 8.6% total moisture content. These differences in values are due to the variation in the chemicals, specifically the polar plant constituents.

Phytochemical analysis:

Powdered drug was extracted by successive soxhlation with different solvents like petroleum ether, chloroform, benzene, acetone, ethanol and water. Finally, maceration of the marc was done with chloroform water I.P. Primary phytochemical analysis of the extracts was done. The *P.roxburghii* leaf extracts contain carbohydrates, flavonoids, saponins and phytosterols. Carbohydrate was found the successive ethanol and aqueous extract. Saponins are present in ethanol, acetone and aqueous extract. Flavonoids were found in all extraction solvent except petroleum ether and benzene extracts, whereas phytosterols were found in Petroleum ether extracts.

Thin layer chromatography findings:

R_f values were calculated for all the spots presented in Table 1. Benzene and petroleum ether extracts do not contain Carbohydrates, saponins or flavonoids. Saponins and flavonoids were found in acetone, chloroform and ethanol extract. Carbohydrates were found in acetone and ethanolic extract. Extracts of petroleum ether and benzene found to have steroidal glycosides. The difference in R_f value may be resulting due to impurity in the solvent, composition of solvent system, used adsorbent, solvent polarity, absorbent substance etc.

Isolation of chemical compounds:

The ethyl acetate, petroleum ether fraction and isolated crystalline compounds were tested for presence of phyto chemicals.The results revealed the presence of carbohydrates and tannins, ethyl acetate fraction contained flavonoids. Petroleum ether fraction contained steroids and steroidal glycosides. Isolated compounds were only positive to phenolic compounds test. TLC of ethanolic extract was developed. The R_f value for the spot of the isolated compound was 0.29matching to a spot found in ethanolic extract. Five spots were seen in the HPTLC profile of ethanolic extract. The R_f values for the 5 spots were 0.11, 0.29, 0.41, 0.56, 0.71 and the % area under curve of the were 30.7, 19.13, 16.81, 11.31 and 23.03, respectively. The isolated compound has R_f value 0.27 and % area under curve 33.11, matching with a spot of Rf value 0.29 seen in the ethanolic extract. The concordance spot found in both TLC and HPTLC reveals the co-presence of isolated compound with others. Presence of phenolic content in the isolated compound from the ethanol extract was confirmed by the chemical analysis. Figures 1 and 2 represents the HPTLC chromatograms of isolated compound and ethanol extract and figure 3 shows the 3D photographs of the isolated compound and ethanol extract.

Estimation of total polyphenolic content:

Sum of the polyphenolic content for the aqueous, ethanolic extract and separated compound was assessed by UV-Visible Spectrophotometric method.The findings are given in Table 2. The water extract showed presence of significant amounts of phenolic compounds in comparison to ethanolic extract and the standard.

Antimicrobial activity:

The antimicrobial activity was assessed by cup plate method against various strains of bacteria. Chloroform, petroleum ether, ethanolic and aqueous extracts of the leaves of *P. roxburghii* was used for the study. The zone of inhibition in mm (Mean ± standard deviation, n = 3) for the standard streptomycin was found to be. 6.0±1.9, 5.4±1.9, 6.2±1.2, 6.0±0.2, 9.0±0.1 and 6.0±1.5 for *Klebsiella pneumoniae*, *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas*

aeurginosa and *Salmonella typhi*, respectively. In this study, the extracts did shown activity against mentioned microorganism (table 3), which was in concordance with the report[26,27] that stated that *P. roxburghii* methanolic extract showed a significant antimicrobial activity[28] and this justifies its use to wash infected eyes.

In vitro antioxidant activity:

Leaf extracts (Petroleum ether, Chloroform, aqueous and alcoholic) and isolated compound of *P. roxburghii* were evaluated for antioxidant action using Vitamin-C as standard. Radical scavenging activity DPPH, reduction power, H_2O_2 scavenging activity and sum of antioxidant activity of leaves extracts of *P. roxburghii* and Vitamin-C are expressed in Figures 4, 5, 6 and 7. There are reports about the antioxidant or free radical scavenging activity of flavonoids, tannins or phenolic compounds of plants[29]. The DPPH test gives insight about the scavenging activity of the compounds against steady free radicals. DPPH shows high absorption in the visible range at 517nm. DPPH upon reaction with open radical, the single electrons gets paired, which causes change in color from deep violet to light yellow results in reduced absorption. The reduction in absorbance is the indicator of the radical scavenging potential. The aqueous extract of *P. roxburghii* leaves seems to be equipotent as Vitamin-C with a highest inhibition of 59.16% at 100µg/ml whereas for Vitamin-C it was 66.4% for the same concentration. Extracts by petroleum ether had less inhibition in comparison to isolated compound, other extracts and Vitamin-C. Reductants are responsible for the antioxidant activity, and they cause breaking of the chain of free radicals by giving a hydrogen atom. The reductants in *P.roxburghii* leaves extracts reduces Fe^{3+} ferricyanide complex to the ferrous (Fe^{2+}) which can be measured spectrophotometrically at 700nm due to formation of Perl's Prussian blue. Reductive potential of the extracts and ascorbic acid has been represented in Figure 5. *P.roxburghii* leaves extracts have strong reducing power, which is dose dependent. The aqueous leaf extract (100µg/ml) of *P. roxburghii* has maximum absorbance of 0.35 where as Vitamin-C has 1.5 at the same concentration. Water extracts found to have better hydrogen peroxide scavenging activity than Vitamin-C in terms of the absorbance value. Petroleum ether and chloroform extracts has poor antioxidant activity, so other extracts and isolated compounds have been considered for estimating antioxidant in total and compared with Vitamin-C taking five concentrations ranging from 100-500µg/ml with a difference of 100µg/ml. The water extract was found to have better total antioxidant activity in terms of absorbance than Vitamin-C. This investigation revealed the antioxidant potential of the plant against the oxidative damage. In the recent past there several reports about the usage of plant having antioxidant activity for the green synthesis of nanoparticles[27,30,31].

CONCLUSION:

The plant *P. roxburghii* has phenolic compounds and other compounds contributing significantly to its antimicrobial and antioxidant property favoring its use as traditional medicine. However structural elucidation of the isolated compound using IR, NMR and mass spectroscopy is highly recommended as a part of future study.

ABBREVIATION USED:

TLC-Thin Layer Chromatography, HPTLC- High Performance Thin Layer Chromatography, DPPH-α, α-diphenyl-β-picryl hydrazyl, DMSO-Dimethyl Sulfoxide\

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RESEARCH ARTICLE

Synthesis, characterization and biological activity of pyrazoline derivatives

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ABSTRACT:
Pyrazoline is dihydropyrazole having only one endocyclic double bond. Synthesis of pyrazoline derivatives from chalcones is an active arena of investigation due to established pharmacological result. In present study, chalcones were prepared by reacting of acetophenone with benzaldehyde. Then prepared chalcones react with isoniazid in the presence of glacial acetic acid results pyrazoline derivative. Physical parameters such as color, crystal structure, solubility, melting point, retardation factor (R_f) and % yield was evaluated. Confirmations of structures of all synthesized derivatives, were done by spectroscopic data of IR, ^{1}HNMR and Mass spectroscopy. Also, all synthesized compounds were evaluated for antibacterial activity against *B. subtilis*, *S. aureus*, *P.aeruginosa* and *E.coli*, and antifungal activity was accomplished against *C. albicans* and *A. niger at* the different conc. and concluded that some compounds have excellent antimicrobial activity and most of the synthesized compounds exhibits moderate antimicrobial activity.

KEYWORDS: Chalcone, Pyrazoline, IR spectroscopy, NMR spectroscopy, Mass spectroscopy, antibacterial activity, antifungal activity.

INTRODUCTION:
Pyrazoline are the members of nitrogen holding heterocyclic compounds.[1] Structurally, Pyrazoline have two nitrogen atoms in five-membered ring systems. Pyrazoline nucleus covers a C=N endocyclic double bond. [2] Among the two nitrogen atoms; one is basic one the other is neutral in nature.[3]

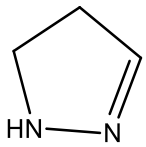
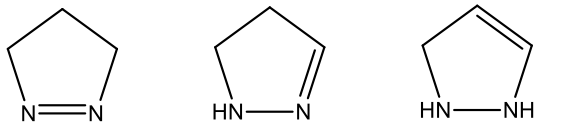


Fig 1: Pyrazoline ring

Pyrazoline is dihydropyrazole holding only one endocyclic double bond. Depending on the position of the double bond three forms of pyrazoline are potential. These are 1-pyrazoline, 2-pyrazoline and 3-pyrazoline. Among all the pyrazolines, 2-pyrazoline has increased fascination and is regularly studied one.[4]



1- Pyrazoline 2- Pyrazoline 3- Pyrazoline

Fig 2: Types of pyrazoline

Pyrazoline derivatives have been establish in natural products in the form of vitamins, alkaloids and pigments.[6] Pyrazoline display a number of biological actions like antimicrobial[7,8,9,10,11,12,13], anti-inflammatory[14,15], antitumor[16], anti-tubercular[17], anticonvulsant activity[18], antioxidant[19], hepatoprotective activity, antimalarial, analgesic and COX-2 inhibitor[20], MAO inhibitors, antidiabetic[21]etc.

MATERIAL AND METHODS:
Material:
All the chemicals were procured from reputed firms like Qualigens, CDH, Hi-Media, Sigma-Aldrich etc. were procured through local dealers of reputed companies, All the glass wares used in research work were procured from Borosil company, Mumbai through local dealer. All the instrument used in research work were available in college and scientific laboratories.

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Methods:

In the first step, synthesis of chalcone, take 0.01 mole of substituted acetophenone and 0.01 mole of substituted benzaldehyde were dissolve in 25 ml of methanol and then gradually mix the 6ml of 40% NaOH solution.[22] This mixture solution stirred at room temperature for 24 Hrs. then diluted with 100ml of water and acidified with diluted HCL. Precipitate obtained was filtered, washed and recrystallized.

In the second step, synthesis of 1, 3, 5-trisubstituted pyrazoline derivatives from substituted chalcones, for this take 0.01 mole of substituted chalcone and 0.01 mole of isoniazid with 25 mL of glacial acetic acid refluxed for 6 hrs. Excess of solvent was removed below reduced pressure and the reaction mixture was transferred into ice cold water.[23] Precipitated product was achieved by filtration, washing and recrystallization.

Physical parameters e.g. color, crystal structure, solubility, melting point, retardation factor (R_f) (Chloroform: Methanol (99.5:0.5)) and % yield was estimated. Confirmations of synthesized compound's structures were done by spectroscopic data of IR, ^{1}H NMR, and Mass spectra.

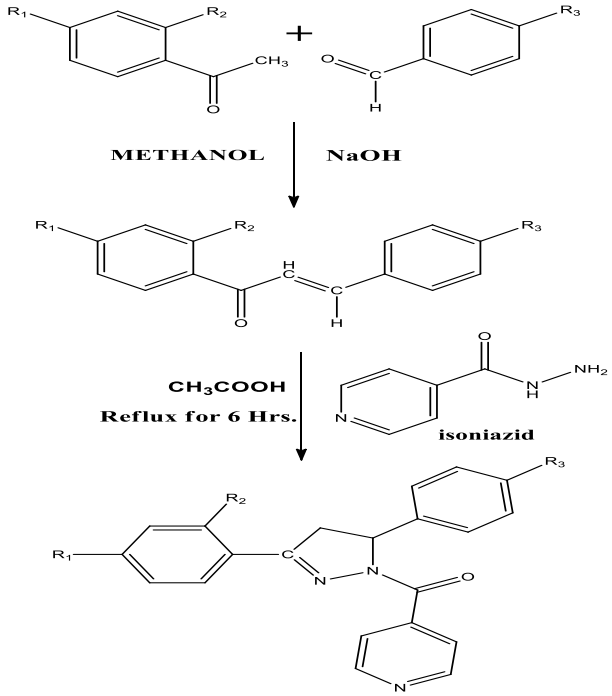

Fig. 3: Scheme to synthesized pyrazoline derivatives

Table 1: List of substitutions on Pyrazoline nucleus

S.No.	Compound Name	R_1	R_2	R_3
1	PD-1	OCH_3	H	CH_3
2	PD-2	OCH_3	H	Cl
3	PD-3	OCH_3	H	NO_2
4	PD-4	OCH_3	H	OCH_3
5	PD-5	OCH_3	H	NH_2
6	PD-6	OCH_3	H	4-OH
7	PD-7	OCH_3	H	$N(CH_3)_2$
8	PD-8	CH_3	H	CH_3
9	PD-9	CH_3	H	Cl
10	PD-10	CH_3	H	NO_2
11	PD-11	CH_3	H	OCH_3
12	PD-12	CH_3	H	NH_2
13	PD-13	CH_3	H	4-OH
14	PD-14	CH_3	H	$N(CH_3)_2$

Antimicrobial Screening: Preparation of the nutrient media:

The broths were prepared by dissolving the specified quantities of the dehydrated broth (Hi media) in purified water and were distributed 4 ml quantities in to each test tube. The tubes were closed with cotton plugs and sterilized by autoclaving at 121ºC for 15 minutes.

Cultivation of microorganisms:

The bacterial cultures were aseptically inoculated into nutrient broth and incubated under aerobic conditions at 37ºC for 24 h. Fungal cultures were inoculated into Sabouraud's broth and incubated under aerobic conditions at 25 ºC for 48 h.

Determination of antimicrobial activity:

Disc-diffusion methods was used for the evaluation of antimicrobial activity of the synthesized compounds. Circular paper disks of 6 mm diameter was impregnated with the specific amount of the test sample and were placed on a suitable nutrient/sabouraud's agar medium in a petri plate which was inoculated on its surface with one of the test organisms. After incubation, the plates were observed for the growth inhibition zones around the disks. The diameter of the zone of inhibition is proportional to the antimicrobial activity of the substance. The diameters of the zone of inhibition were compared with that produced by the standard antibiotics.

Preparation of the disks and samples:

Paper disks of 6 mm diameter and 2 mm thickness were used for the test. These disks were sterilized by autoclaving at 121 ºC (15 lb PSIG) for 15 minutes. Almost all samples were tested at 50 µg level. To obtain this, sample solutions containing 10 mg/ml were prepared in sterile dimethyl formamide (DMF) and 5µl each of the solutions was added on each disk using a micropipette. All the solutions were added on each disk using a micropipette. All the solutions were prepared using aseptic precautions. Ciprofloxacin (10 µg/disk) was taken as standard antibiotics for the comparison of the antibacterial activity of the synthesized compounds. Griseofulvin (10µg/disk) was used as standard drugs for antifungal activity studies.

General procedure:

Each petri plate containing nutrient/ sabouraud's agar

medium was inoculated with one bacterial/ fungal culture by spreading the suspension of the organism with a sterile cotton swap. Each plate was divided into six equal portions along the diameter. Each portion was used to place one disk. Four disks of each sample were placed on four portions, one disk with standard drug and a disk impregnated with the solvent (DMF). All the plates were kept in the refrigerator for 30 minutes to allow the diffusion of the sample in-to the refrigerator for 30 minutes to allow the diffusion of the sample into the surrounding agar medium. Then the plates inoculated with bacterial cultures were incubated at 37ºC for 18 h and those with incubated at 25 ºC for 48 h. Diameter of the zones of inhibition wherever produced were measured and the average diameter for each sample was calculated. The diameters obtained for the test samples were compared with that produced by the standard antibiotics, ciprofloxacin for antibacterial activity and griseofulvin and clotrimazole for antifungal activity.

Table 2: Antibacterial activity of pyrazoline derivatives

S. No.	Compound Code	Diameter of zone of inhibition (mm)			
		B. subtilis	***S. aureus***	***P. aeruginosa***	***E. coli***
1	PD-1	21(6.25)	16(6.25)	14(6.25)	20(6.25)
2	PD-2	22(6.25)	15(6.25)	13(6.25)	14(6.25)
3	PD-3	13(6.25)	13(25)	22(6.25)	15(6.25)
4	PD-4	12(6.25)	22(25)	16(6.25)	13(6.25)
5	PD-5	12(6.25)	14(6.25)	13(6.25)	11(6.25)
6	PD-6	13(6.25)	19(6.25)	14(6.25)	10(6.25)
7	PD-7	10(6.25)	17(6.25)	12(6.25)	14(6.25)
8	PD-8	18(25)	8(12.5)	8(6.25)	18(6.25)
9	PD-9	16(6.25)	22(6.25)	10(6.25)	20(6.25)
10	PD-10	19(6.25)	13(6.25)	10(6.25)	10(6.25)
11	PD-11	13(6.25)	16(6.25)	16(6.25)	20(12.5)
12	PD-12	12(6.25)	10(6.25)	14(6.25)	22(12.5)
13	PD-13	12(6.25)	11(6.25)	15(6.25)	26(6.25)
14	PD-14	13(6.25)	18(6.25)	13(6.25)	19(6.25)
15	Control	-	-	-	-
16	Ciproflaxacin	22(6.25)	24(12.5)	25(6.25)	23(6.25)

Table 3: Antifungal activity of pyrazoline derivatives

S. No.	Compound Code	Diameter of zone of inhibition (mm)	
		C. albicans	***A. niger***
1	PD-1	15(12.5)	17(12.5)
2	PD-2	17(12.5)	26(12.5)
3	PD-3	13(6.25)	14(12.5)
4	PD-4	12(6.25)	24(12.5)
5	PD-5	12(6.25)	15(12.5)
6	PD-6	13(6.25)	18(12.5)
7	PD-7	11(6.25)	26(12.5)
8	PD-8	18(12.5)	25(12.5)
9	PD-9	17(6.25)	12(12.5)
10	PD-10	19(6.25)	10(12.5)
11	PD-11	20(6.25)[a]	15(12.5)
12	PD-12	20(6.25)	20(12.5)
13	PD-13	21(6.25)	14(12.5)
14	PD-14	22(6.25)	25(12.5)
15	Control	-	-
16	Griseofulvin	20(6.25)	26(12.5)

PD-1:(3-(4-methoxyphenyl)-5-(p-tolyl)-4,5-dihydro-1H-pyrazol-1-yl)(pyridin-4-yl) methanone, **m.p.:** 332^{O}C**, Yield(%):**65, **R$_f$:**0.52, **IR:**3479, 1597, 1434, 1343, 1248,**^{1}H NMR (CDCl$_3$-400Mz) δ:** 2.34(s, 3H, C**H$_3$**), 3.72(t, 1H, C**H$_2$(**Pyz)), 3.83(s, 3H, Ar-O-C**H$_3$**), 3.96(t, 1H, C**H$_2$**(Pyz)), 4.93(t, 1H, C**H(**Pyz)), 7.09(d, 2H, *J*=7.56, C**H**(Ar)), 7.18(d, 4H, *J*=6.96, C**H**(Ar)), 7.83(d, 2H, *J*=9.92, C**H**(Ar)), 7.93(d, 2H, *J*=9.2, C**H**(Ar)), 8.90(d, 2H, *J*=10.6, C**H**(Ar)) **ESI-MS (m/z):**372(M+1)

PD-2:(5-(4-chlorophenyl)-3-(4-methoxyphenyl)-4,5-dihydro-1H-pyrazol-1-yl)(pyridin-4-yl) methanone, **m.p.:**276 ^{O}C, **Yield(%):**83, **R$_f$:**0.71, **IR:**3280, 1552, 1439, 1307, 1230,**^{1}H NMR (CDCl$_3$-400Mz) δ:** 3.74(t, 1H, C**H$_2$(**Pyz)), 3.84(s, 3H, Ar-O-C**H$_3$**), 3.96(t, 1H, C**H$_2$**(Pyz)), 4.93(t, 1H, C**H(**Pyz)), 7.07(d, 2H, *J*=6.8, C**H**(Ar)), 7.42(d, 2H, *J*=8.72, C**H**(Ar)), 7.48(d, 2H, *J*=8.24, C**H**(Ar)), 7.83(d, 2H, *J*=6.56, C**H**(Ar)), 7.94(d, 2H, *J*=7.2, C**H**(Ar)), 8.91(d, 2H, *J*=7.96, C**H**(Ar)) **ESI-MS (m/z):**392(M+1)

PD-3:(3-(4-methoxyphenyl)-5-(4-nitrophenyl)-4,5-dihydro-1H-pyrazol-1-yl)(pyridin-4-yl) methanone, **m.p.:**329 ^{O}C, **Yield(%):**62, **R$_f$:**0.62, **IR:**3289, 1557, 1489, 1339, 1235,**^{1}H NMR (CDCl$_3$-400Mz) δ:** 3.73(t, 1H, C**H$_2$(**Pyz)), 3.83(s, 3H, Ar-O-C**H$_3$**), 3.96(t, 1H, C**H$_2$**(Pyz)), 4.93(t, 1H, C**H(**Pyz)), 7.06(d, 2H, *J*=6.76, C**H**(Ar)), 7.57 (d, 2H, *J*=9.6, C**H**(Ar)), 7.82(d, 2H, *J*=7.48, C**H**(Ar)), 7.92(d, 2H, *J*=6.00, C**H**(Ar)), 8.24(d, 2H, *J*=9.52, C**H**(Ar)), 8.90(d, 2H, *J*=7.24, C**H**(Ar)) **ESI-MS (m/z):**403(M+1)

PD-4:(3,5-bis(4-methoxyphenyl)-4,5-dihydro-1H-pyrazol-1-yl)(pyridin-4-yl)methanone **m.p.:**256 ^{O}C, **Yield(%):**64, **R$_f$:**0.67, **IR:**3307, 1556, 1448, 1312, 1230,**^{1}H NMR (CDCl$_3$-400Mz) δ:** 3.73(t, 1H, C**H$_2$(**Pyz)), 3.83(s, 6H, Ar-O-C**H$_3$**), 3.97(t, 1H, C**H$_2$**(Pyz)), 4.93(t, 1H, C**H(**Pyz)), 6.94(d, 2H, *J*=8.24,

C**H**(Ar)), 7.08(d, 2H, *J*=8.96, C**H**(Ar)), 7.20(d, 2H, *J*=8.96, C**H**(Ar)), 7.83(d, 2H, *J*=8.08, C**H**(Ar)), 7.92(d, 2H, *J*=7.76, C**H**(Ar)), 8.90(d, 2H, *J*=9.24, C**H**(Ar)) **ESI-MS (m/z):**388(M+1)

PD-5:(5-(4-aminophenyl)-3-(4-methoxyphenyl)-4,5-dihydro-1H-pyrazol-1-yl)(pyridin-4-yl) methanone,**m.p.:**287^{O}C, **Yield(%):**64, **R_f:**0.53, **IR:**3294, 1566, 1448, 1312, 1244,**^{1}H NMR ($CDCl_3$-400Mz) δ:** 3.72(t, 1H, C**H_2(**Pyz)), 3.83(s, 3H, Ar-O-C**H_3**), 3.96(t, 1H, C**H_2**(Pyz)), 4.93(t, 1H, C**H(**Pyz)), 6.27(s, 2H, Ar-N**H_2**), 6.58(d, 2H, *J*=6.52, C**H**(Ar)), 7.04(d, 2H, *J*=10.4, C**H**(Ar)), 7.08(d, 2H, *J*=4.48, C**H**(Ar)), 7.83(d, 2H, *J*=10.84, C**H**(Ar)), 7.94(d, 2H, *J*=11.04, C**H**(Ar)), 8.90(d, 2H, *J*=8.92, C**H**(Ar)) **ESI-MS (m/z):**373(M+1)

PD-6:(5-(4-hydroxyphenyl)-3-(4-methoxyphenyl)-4,5-dihydro-1H-pyrazol-1-yl)(pyridin-4-yl)methanone,**m.p.:**354 ^{O}C,**Yield(%):** 63, **R_f:**0.61, **IR:**3520, 3303, 1547, 1443, 1303, 1225,**^{1}H NMR ($CDCl_3$-400Mz) δ:** 3.72(t, 1H, C**H_2(**Pyz)), 3.83(s, 3H, Ar-O-C**H_3**), 3.96(t, 1H, C**H_2**(Pyz)), 4.93(t, 1H, C**H(**Pyz)), 5.35(s, 1H, O**H**), 6.72(d, 2H, *J*=8.48, C**H**(Ar)), 7.08(d, 2H, *J*=10.04, C**H**(Ar)), 7.14(d, 2H, *J*=6.8, C**H**(Ar)), 7.83(d, 2H, *J*=7.76, C**H**(Ar)), 7.93(d, 2H, *J*=8.88, C**H**(Ar)), 8.92(d, 2H, *J*=6.24, C**H**(Ar)) **ESI-MS (m/z):** 374(M+1)

PD-7:(5-(4-(dimethylamino)phenyl)-3-(4-methoxyphenyl)-4,5-dihydro-1H-pyrazol-1-yl) (pyridin-4-yl)methanone, **m.p.:**321^{O}C, **Yield(%):**69, **R_f:**0.62, **IR:**3314, 1548, 1439, 1312, 1235,**^{1}H NMR ($CDCl_3$-400Mz) δ:**3.06(s, 6H, N-C**H_3**), 3.72(t, 1H, C**H_2(**Pyz)), 3.83(s, 3H, Ar-O-C**H_3**), 3.96(t, 1H, C**H_2**(Pyz)), 4.92(t, 1H, C**H(**Pyz)), 6.73(d, 2H, *J*=9.24, C**H**(Ar)), 7.07(d, 2H, *J*=6.72, C**H**(Ar)), 7.13(d, 2H, *J*=7.56, C**H**(Ar)), 7.83(d, 2H, *J*=7.6, C**H**(Ar)), 7.93(d, 2H, *J*=8.96, C**H**(Ar)), 8.90(d, 2H, *J*=9.44, C**H**(Ar)) **ESI-MS (m/z):**401(M+1)

PD-8:(3,5-di-p-tolyl-4,5-dihydro-1H-pyrazol-1-yl)(pyridin-4-yl)methanone, **m.p.:**342^{O}C, **Yield(%):**62, **R_f:** 0.72, **IR:**3293, 1548, 1450, 1439, 1312,**^{1}H NMR ($CDCl_3$-400Mz) δ:**2.34(s, 3H, C**H_3**), 3.74(t, 1H, C**H_2(**Pyz)), 3.96(t, 1H, C**H_2**(Pyz)), 4.93(t, 1H, C**H(**Pyz)), 7.18(d, 4H, *J*=7.00, C**H**(Ar)), 7.29(d, 2H, *J*=8.52, C**H**(Ar)), 7.73(d, 2H, *J*=8.28, C**H**(Ar)), 7.83(d, 2H, *J*=8.96, C**H**(Ar)), 8.90(d, 2H, *J*=9.12, C**H**(Ar)) **ESI-MS (m/z):**356(M+1)

PD-9:(5-(4-chlorophenyl)-3-(p-tolyl)-4,5-dihydro-1H-pyrazol-1-yl)(pyridin-4-yl)methanone, **m.p.:**287^{O}C, **Yield(%):**68, **R_f:**0.71, **IR:**3396, 1359, 1450, 1425, 1325,**^{1}H NMR ($CDCl_3$-400Mz) δ:** 2.34(s, 3H, C**H_3**), 3.72(t, 1H, C**H_2(**Pyz)), 3.96(t, 1H, C**H_2**(Pyz)), 4.93(t, 1H, C**H(**Pyz)), 7.29(d, 2H, *J*=5.52, C**H**(Ar)), 7.42(d, 2H, *J*=5.16, C**H**(Ar)), 7.48(d, 2H, *J*=5.88, C**H**(Ar)), 7.74(d, 2H, *J*=8.72, C**H**(Ar)), 7.83(d, 2H, *J*=6.8, C**H**(Ar)), 8.89(d, 2H, *J*=8.08, C**H**(Ar)) **ESI-MS (m/z):**376(M+1)

PD-10:(5-(4-nitrophenyl)-3-(p-tolyl)-4,5-dihydro-1H-pyrazol-1-yl)(pyridin-4-yl)methanone, **m.p.:**325^{O}C, **Yield(%):**75, **R_f:** 0.52, **IR:**3280, 1547, 1450, 1448, 1316,**^{1}H NMR ($CDCl_3$-400Mz) δ:**2.35(s, 3H, C**H_3**), 3.73(t, 1H, C**H_2(**Pyz)), 3.96(t, 1H, C**H_2**(Pyz)), 4.94(t, 1H, C**H(**Pyz)), 7.29(d, 2H, *J*=5.36, C**H**(Ar)), 7.55(d, 2H, *J*=8.72, C**H**(Ar)), 7.72(d, 2H, *J*=6.72, C**H**(Ar)), 7.84(d, 2H, *J*=7.28, C**H**(Ar)), 8.22(d, 2H, *J*=6.52, C**H**(Ar)), 8.92(d, 2H, *J*=8.56, C**H**(Ar)) **ESI-MS (m/z):**387(M+1)

PD-11:(5-(4-methoxyphenyl)-3-(p-tolyl)-4,5-dihydro-1H-pyrazol-1-yl)(pyridin-4-yl) methanone, **m.p.:**289 ^{O}C, **Yield(%):**73, **R_f:** 0.63, **IR:** 3284, 1547, 1450, 1439, 1307, 1234**, ^{1}H NMR ($CDCl_3$-400Mz) δ:** 2.33(s, 3H, C**H_3**), 3.72(t, 1H, C**H_2(**Pyz)), 3.83(s, 3H, Ar-O-C**H_3**), 3.95(t, 1H, C**H_2**(Pyz)), 4.94(t, 1H, C**H(**Pyz)), 6.93(d, 2H, *J*=7.2, C**H**(Ar)), 7.22(d, 2H, *J*=8.56, C**H**(Ar)), 7.28(d, 2H, *J*=5.52, C**H**(Ar)), 7.72(d, 2H, *J*=8.8, C**H**(Ar)), 7.82(d, 2H, *J*=5.16, C**H**(Ar)), 8.90(d, 2H, *J*=8.92, C**H**(Ar)) **ESI-MS (m/z):**372(M+1)

PD-12:(5-(4-aminophenyl)-3-(p-tolyl)-4,5-dihydro-1H-pyrazol-1-yl)(pyridin-4-yl)methanone, **m.p.:**313^{O}C, **Yield(%):**73, **R_f:**0.62, **IR:** 3396, 1567, 1450, 1487, 1387, **^{1}H NMR ($CDCl_3$-400Mz) δ:** 2.34(s, 3H, C**H_3**), 3.72(t, 1H, C**H_2(**Pyz)), 3.96(t, 1H, C**H_2**(Pyz)), 4.92(t, 1H, C**H(**Pyz)), 6.27(s, 2H, Ar-N**H_2**), 6.58(d, 2H, *J*=8.04, C**H**(Ar)), 7.04(d, 2H, *J*=10.8, C**H**(Ar)), 7.29(d, 2H, *J*=5.36, C**H**(Ar)), 7.73(d, 2H, *J*=9.08, C**H**(Ar)), 7.82(d, 2H, *J*=5.88, C**H**(Ar)), 8.92(d, 2H, *J*=6.84, C**H**(Ar)) **ESI-MS (m/z):**357(M+1)

PD-13:(5-(4-hydroxyphenyl)-3-(p-tolyl)-4,5-dihydro-1H-pyrazol-1-yl)(pyridin-4-yl) methanone, **m.p.:**295^{O}C, **Yield(%):**79, **R_f:**0.58, **IR:**3551, 3391, 1551, 1450, 1441, 1351,**^{1}H NMR ($CDCl_3$-400Mz) δ:** 2.34(s, 3H, C**H_3**), 3.72(t, 1H, C**H_2(**Pyz)), 3.96(t, 1H, C**H_2**(Pyz)), 4.92(t, 1H, C**H(**Pyz)), 5.35(s, 1H, Ar-O**H**), 6.72 (d, 2H, *J*=10.6, C**H**(Ar)), 7.14(d, 2H, *J*=10.4, C**H**(Ar)), 7.29(d, 2H, *J*=7.12, C**H**(Ar)), 7.73(d, 2H, *J*=11.04, C**H**(Ar)), 7.83(d, 2H, *J*=7.04, C**H**(Ar)), 8.90(d, 2H, *J*=10.44, C**H**(Ar)) **ESI-MS (m/z):**358(M+1)

PD-14:(5-(4-(dimethylamino)phenyl)-3-(p-tolyl)-4,5-dihydro-1H-pyrazol-1-yl)(pyridin-4-yl) methanone, **m.p.:**341 ^{O}C, **Yield(%):**71, **R_f:**0.59**, IR:**3351, 1551, 1450, 1432, 1341,**^{1}H NMR ($CDCl_3$-400Mz) δ:** 2.34(s, 3H, C**H_3**), 3.05(s, 6H, Ar-N(C**H_3)$_2$**), 3.72(t, 1H, C**H_2(**Pyz)), 3.96(t, 1H, C**H_2**(Pyz)), 4.92(t, 1H, C**H(**Pyz)), 6.74 (d, 2H, *J*=7.6, C**H**(Ar)), 7.13(d, 2H, *J*=7.72, C**H**(Ar)), 7.29(d, 2H, *J*=9.88, C**H**(Ar)), 7.74(d, 2H, *J*=4.48, C**H**(Ar)), 7.83(d, 2H, *J*=8.68, C**H**(Ar)),

8.90(d, 2H, *J*=5.68, C**H**(Ar)) **ESI-MS (m/z):**385(M+1).

RESULT and DISCUSSION

All the pyrazoline derivatives were synthesized and confirmed by the physical parameters such as Solubility, Melting Point, Retardation factor (Rf) value and spectral data such as IR, NMR and Mass spectra. All the pyrazoline derivatives were evaluated for antimicrobial activity against *B. subtilis, S. aureus, P. aeruginosa, E.coli, C. albicans* and *A. niger at the different conc. Most* of the compounds showed moderate activity with low MIC. Specially, PD-2 against *B. subtilis,* PD-4 and PD-9 against *S. aureus,* PD-13 against *P. aeruginosa PD-12 and PD-13 against E. coli, PD-13 and PD-14 against C. albicans and, PD-2, PD-7, PD-8 and PD-14 against A. niger exhibits excellent activity.*

CONCLUSION:

From this study, we determined that molecular modification of pyrazoline derivative has led to potential antimicrobial agents**.** PD-2, PD-3, PD-4, PD-7, PD-8, PD-9, PDC-12, PD-13 and PD-14 can be main molecules in this respect.

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CONFLICT OF INTEREST:

There is no conflict of interest.

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RESEARCH ARTICLE

Development of a combined solution of pyrimidine nucleotides with vitamin B_6

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ABSTRACT:
Pyrimidine nucleotides, namely uridine monophosphate and cytidine monophosphate, play an important role in the cellular metabolism of nerve fibers. The combination of these nucleotides with pyridoxine hydrochloride (vitamin B_6) in one dosage form will allow us to fully implement the strategy of the complex neurotropic pharmacotherapy in neuropathies of various origins. To develop a stable solution, an important step at the stage of the composition development is to study the compatibility of active substances (active pharmaceutical ingredients - APIs) in solution. Samples of binary solutions and solutions containing all active substances were prepared and examined. The API interaction was determined by various parameters – changes in color, transparency, pH, the total impurity content, etc. Based on a comprehensive study of the processes of the API dissolution the optimal pH limits of the solution required for the stable existence of a combination of substances with different pH values of the medium have been substantiated and experimentally confirmed. As a result of the research, the optimal pH value of the solution recommended is 4.0-4.8. In the composition of substances the amount of water can be in an adsorbed or crystallized, or combined form. This fact should be taken into account in order to correctly calculate the actual amounts of initial ingredients when preparing the solution. The nature of the water component of APIs was clarified when studying the phase composition of samples on a powder diffractometer. The studies conducted have allowed us to determine the directions of further research for developing the composition of an injection drug. This research is in choosing the optimal buffer system and excipients-antioxidants.

KEYWORDS: Pyrimidine nucleotides, Uridine monophosphate, Cytidine monophosphate, Pyridoxine hydrochloride, Parenteral dosage form.

INTRODUCTION:
Progress in the clinical practice of pathologies of the peripheral nervous system (PNS) is impossible without solving the problem of effective and safe pharmacotherapy of diabetic and alcoholic neuropathies. The share of these neurological complications of diabetes and alcoholism is increasing and is accompanied by high indicators of temporary disability, disability and significant socio-economic losses[1,2].

Pharmacotherapy of nerve lesions in many cases should be comprehensive, affect different links of pathogenesis. To treat alcoholic polyneuropathy (APN) and diabetic polyneuropathy (DPN) nonsteroidal anti-inflammatory drugs, tricyclic antidepressants, anticonvulsants, analgesics, and others, which act on the symptoms of diseases, are used[3,4].

The range of medicines containing active components that act at the level of peripheral nerves and promote their therapeutic regeneration is currently not rather wide[5]. Among them drugs based on pyrimidine nucleotides – uridine and cytidine are known[6,7].

Nucleotides are endogenous substances and are generally considered harmless. Therefore, therapy with these drugs is very well tolerated; practically, it is not

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accompanied by undesirable side effects. Pyrimidine nucleotides – uridine monophosphate and cytidine monophosphate are neurotrophic medicinal substances that play a key role in molecular metabolic processes. Exogenous replenishment of these substances contributes to the restoration of impaired neuronal functions, normalization of metabolic shifts and acceleration of regeneration, and it clearly correlates with the regression of clinical symptoms[6].

The treatment of diabetic and alcoholic complications of PNS is very often accompanied with administration of vitamin preparations, among them B vitamins or neurotropic vitamins take the main place[8,9]. B vitamins are involved in the processes of axonal transport of neurons, transmission of nerve impulses along motor and sensory fibres, regulate the balance of the nociceptive and antinociceptive systems[10].

Currently, there are some drugs based on pyrimidine nucleotides and vitamin B_6 preparations that are used as part of the complex therapy of DPN and APN. Among them, there are drugs for parenteral use. They are "Nucleo C.M.P. forte"[11,12], lyophilized powder for the preparation of a solution for injection produced by "Ferrer International", Spain, and "Keltican", lyophilized powder for the preparation of a solution for injection produced by Nicomed. Pyridoxine hydrochloride (PHC) solutions for injection are also well known[13,14,15,16].

Thus, the combination of such active pharmaceutical ingredients (APIs) as two pyrimidine nucleotides – uridine monophosphate and cytidine monophosphate with B vitamin – PHC in one drug will allow us to fully implement the strategy of the complex neurotropic pharmacotherapy in neuropathies of various origins, i.e. the effective prevention of the nervous tissue destruction and promotion in preserving the functional life. This is an urgent and socially significant task.

The aim of this work was to determine the compatibility and conditions of a stable existence of uridine monophosphate, cytidine monophosphate and PHC in solution in the necessary therapeutic concentrations in order to create a new combined drug for parenteral use based on pyrimidine nucleotides and vitamin B_6.

MATERIAL AND METHODS:

The study objects were such substances as uridine monophosphate of disodium salt (UMP), cytidine monophosphate of disodium salt (CMP) and PHC, solutions based on these pyrimidine nucleotides and vitamin B_6.

The substances of uridine monophosphate, disodium salt, batch 111020, cytidine monophosphate, disodium salt, batch 111018, manufactured by "Shanghai Oripharm Co. Ltd.", China were used as active substances for the research. The quality of substances met the requirements regulated by the manufacturer in the Drug Master Files (DMF) and the manufacturer certificates. The quality of PHC substance, batch UQ 11113337, manufactured by "DSM Nutritional Products GmbH", Germany met the requirements of the European Pharmacopoeia (Ph. Eur.) and the State Pharmacopoeia of Ukraine (SPhU)[17,18].

To achieve the goal, we developed a methodological approach that included the following stages:

- Determination of critical characteristics of APIs, which were the raw material and main drug substances;
- Analysis of their quality specifications, determination of the indicators that could affect the compatibility of APIs in solution;
- Study of the compatibility of active substances in solution under the action of influencing factors, determination of factors that could affect the critical quality characteristics of APIs in the product;
- Determination of the optimal pH range of the solution for the injectable dosage form.

During the research work the qualitative and quantitative control of experimental samples of solutions was carried out by the indicators characterizing stability: pH, the impurity content, transparency, color, particulate matter according to the methods given in the Ph. Eur., SPhU[17,18] and the product specification file.

The ranges of critical parameters and characteristics were determined based on the experimental data and the previous work experience.

RESEARCH RESULTS:

The qualitative and quantitative composition of the new combined drug was developed on the basis of studying the literature data and the experimental work. Therapeutic concentrations of active substances were proposed by pharmacologists based on the results of exploratory pharmacological studies of the neuropathic activity of mixtures containing different ratios of these ingredients in injection administration. The maximum neuropathic effect was exhibited by the composition containing all these ingredients in the following amounts:

- Uridine monophosphate, disodium salt, 2.0 mg/mL
- Cytidine monophosphate, disodium salt, 5.0 mg/mL
- Pyridoxine hydrochloride, 25.0 mg/mL

When creating a new drug, in addition to the rationality from a medical point of view, including the pharmacological compatibility of the active components of the drug combination, their physico-chemical compatibility in solution should be considered. This is especially important for parenteral drugs, which are mainly solutions[19].

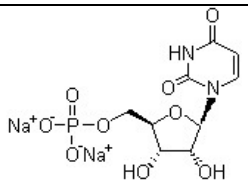
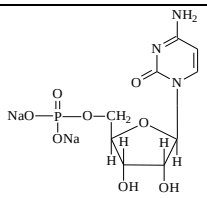
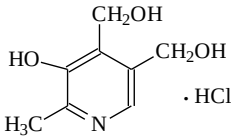
UMP and CMP are salts of weak organic acids and a strong base; in more detail – these are salts of nucleotides, which, in turn, consist of a nucleoside and a phosphoric acid residue. Nucleotides have strong acidic properties with a high-density negative charge. Nucleotides are also monoesterified derivatives of phosphoric acid. Due to the presence of phosphoric acid residues they are strong dibasic acids, which ionization is added to the ionization determined by the acidic properties of heterocyclic bases.

PHC is a pyridine derivative, and it also belongs to the category of salts. It is a salt of an organic base and an inorganic acid.

All main active ingredients of the combined drug developed are complex organic compounds that have reactive groups and can interact in solution.

We analyzed the quality characteristics of UMP, CMP and PHC, determined indicators and characteristics affecting the stability of these compounds in solution with their simultaneous presence. The information concerning the API properties and quality requirements is given in Table 1.

Table 1: Characteristics and quality indicators of substances of UMP, CMP and PHC

Name	UMP	CMP	PHC
Structural formula			
Solubility	Readily soluble in water	Readily soluble in water	Readily soluble in water
Transparency	Transparent or does not exceed the standard I by turbidity (1 % solution)	Transparent or does not exceed the standard I by turbidity (1 % solution)	Transparent (5 % solution)
Color	Colorless or does not exceed the standard BY_5 by the degree of color (1 % solution)	Colorless or does not exceed the standard BY_5 by the degree of color (1 % solution)	The color of 5.0 % solution should not be more intense than the standard Y_7
pH	7.0-8.5 (1 % solution)	8.0-9.5 (1 % solution)	2.4-3.5 (5 % solution)
Related impurities	Impurity A – not more than 0.15%; impurity B – not more than 0.15%; any other impurity – not more than 0.10%; total impurities – not more than 1.5%	Impurity A – not more than 0.15 %; impurity B – not more than 0.15 %; impurity C – not more than 0.15 %; impurity D – not more than 0.15 %, impurity E – not more than 0.15 %, impurity F – not more than 0.30 %, impurity G – not more than 0.30 %, impurity a H – not more than 0.15 %; any unidentified impurity – not more than; total impurities – not more than 1.5%	Impurity B – not more than 0.15%; unspecified impurities – not more than 0.10%; total impurities – not more than 0.2%. Peaks with the area less than 0.5 of the area of the main peak on the chromatogram of the reference solution (a) (0.05 %) are not considered
Water	Not more than 26.0 %	Not more than 25.0 %	Not more than 0.5 %
Assay	Not less than 98.0% and not more than 102.0% calculated with reference to the anhydrous substance	Not less than 98.0% and not more than 102.0% calculated with reference to the anhydrous substance	Not less than 99.0% and not more than 101.0% calculated with reference to the anhydrous substance

UMP and CMP have a similar chemical structure, similar physical and chemical properties, and a close pH range in the alkaline region; therefore, they are predictably compatible in the same solution.

PHC has a different structure, and its solutions have a pH in the acidic region; thus, there may be incompatibilities in the solution between this compound and nucleotides.

At different pH ranges different ionic forms of pyridoxine are present in the PHC solution (Fig. 1).

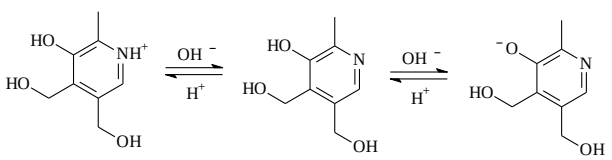


Fig. 1: Ionic forms of pyridoxine

The presence of different ionic forms can lead to differences in the physical and chemical properties of PHC at different pH ranges. In the acidic region, up to pH 4.31, there is mainly a protonated form, solutions are colorless. At pH from 4.31 to 8.37 a neutral form forms solutions of a slightly yellow color; in the region of alkaline pH values (above 8.37) there is a negatively charged form. The second and third forms are more labile and subject to oxidation processes.

The stability studies for PHC were performed in buffer solutions with a pH from 1.2 to 9.18. The analysis of pyridoxine solutions at pH levels corresponding to the existence of each of the three forms showed the following. In acidic solutions PHC was more stable. At pH 1.2 (Form 1) impurities were detected at the level of the unidentified limit and impurity B due to pyridoxine oxidation was insignificant (less than 0.03%). At pH 5.0 an increase in impurities was observed, but their amount did not exceed the level of the unidentified limit, impurity B was at the level of the unidentified limit (0.1%). At pH 9.18 impurities could be compared with impurities at pH 5.0, and the amount of impurity B at the level of the qualified limit was 0.4%. Solutions of pyridoxal as a product of PHC decomposition in solution had a yellow color at alkaline pH values.

Therefore, it is most acceptable to create a pH level for a new combined drug not higher than 4.8 in order to avoid the appearance of pyridoxine impurities, and not lower than 3.0 for pharmacological reasons.

Since the pH of UMP and CMP solutions of disodium salts were in the alkaline region (Table 1), their properties and resistance to destruction at neutral and acidic pH values were studied. Uridine monophosphate did not form various ionic forms, unlike cytidine monophosphate, which second ionic form was due to protonation of nitrogen. In the experiment, this form did not show significant differences in stability. Some slight difference in the composition of UMP and CMP impurities was found at pH 1.2: there was an insignificant formation of an impurity of uridine in the UMP solution (0.15%) and cytidine in the CMP solution (0.12 %), both impurities were at the level of the identified limit. Other impurities were up to 0.2 and 0.4% for UMP and up to 0.2% for CMP.

At pH 5.0 and 9.18 other impurities for UMP and CMP were below the identified limit. An impurity of cytidine with a slight increase (up to 0.15%) at pH 5.0 was detected in the CMP solution. Uridine was not formed in the UMP solution at this pH. At pH 9.18 the main impurity in the solutions of UMP and CMP was not formed.

The analysis of UMP and CMP solutions in buffer solutions from 3.5 to 5.3 under the temperature exposure (at 120°C for 15 minutes and at 40°C for 7 days) showed an increase in the impurity of uridine and cytidine, and it was due to the hydrolysis reaction by the ester bond. At pH of 4.2 or more, this process was somewhat slowed down and stabilized.

Based on these data the conclusion about the predicted compatibility of APIs in solution with a slightly acidic pH level was made.

To experimentally confirm the compatibility of APIs, the samples of binary solutions and solutions containing all APIs were prepared. All solutions were prepared in compliance with therapeutic concentrations of APIs.

When calculating the actual amounts of substances for the preparation of solutions in therapeutic concentrations it is necessary to take into account the amount of water, which can be adsorbed or crystallized, or of combined composition, in the substances. The nature of the water component of API substances was clarified in a powder X-ray diffraction study of the phase composition of samples using "Siemens D500" a powder diffractometer[20] (Bragg-Brentano geometry /2, radiation CuKα, = 1.54184Å, a monochromator or a filter on the secondary beam, step-scanning 2 = 0.02 °).

Each sample was ground in a mortar and compacted in a glass cell with a 20x10x0.5 mm cavity for the sample, the cell was then placed in a sample holder for imaging. The phase identification was performed using a PDF-1 file included in the diffractometer software and data from the Cambridge structural data bank. The quantitative calculation of X-ray diffraction patterns was performed using the Rietveld method[21,22] (FullProf program). The instrumental profile of the lines required for calculating the average crystallite size using this method was determined by a LaB_6 X-ray diffraction pattern obtained under similar conditions. The calculation results are given in Fig. 2.

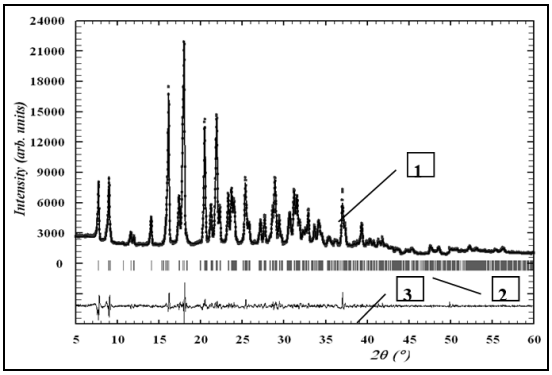


Fig. 2: The results of refinement of the X-ray diffraction pattern for sample 1 (UMP) using the Rietveld method

Fig. 2 shows the experimental and calculated X-ray diffraction patterns that practically coincide (Mark 1), a number of vertical lines (Mark 2) indicates the Bragg position of the lines; the lower curve (Mark 3) demonstrates the difference between the experimental and calculated intensity at each point.

It has been found that sample 1 (UMP) is UMP in the form of a heptahydrate, and the Cambridge bank has data only for this form; moreover, these data are not entirely accurate – there is a disordering of sodium atoms in the structure, which makes it impossible to reliably separate sodium atoms and water molecules that are part of their coordination sphere.

The results of refinement of the X-ray powder diffraction pattern show a satisfactory correspondence between the diffraction pattern observed and the bank model of the structure, but in the X-ray pattern there are several weak lines at the background level, and they do not correspond to this model. It can be assumed that these lines appear as a result of the partial loss of crystallization water and the formation of one or more hydrates with the different water content, and there are no literature data for them[23].

To confirm the assumption of the water loss, three additional X-ray diffraction patterns were taken: the initial freshly ground sample (sample 2a); the same sample after exposure in a cell for 3 days in air (sample 2b); the same sample after exposure in a cell for 4 days in a sulfuric acid desiccator (sample 2c).

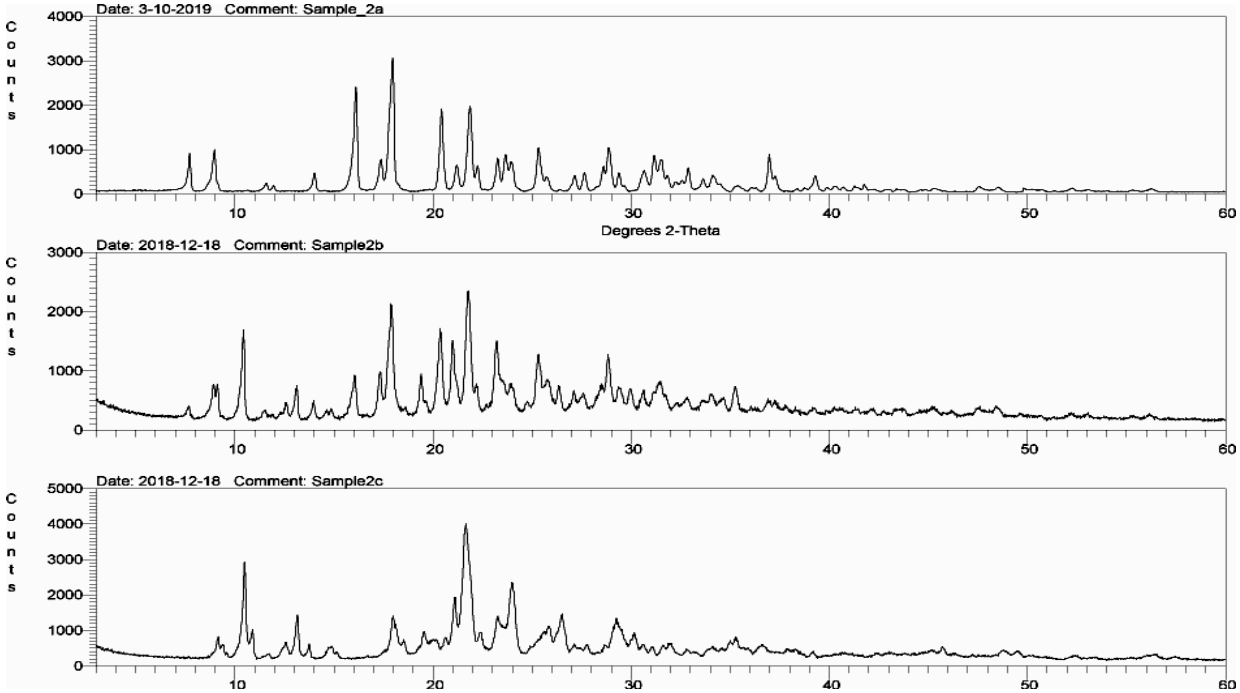


Fig. 3: The X-ray diffraction patterns of sample 1 (UMP) during the drying process

It can be seen from X-ray diffraction patterns given in Fig. 3 that all three samples differ significantly in their composition and, most likely, samples 2b and 2c are not single-phased. Thus, it can be concluded that sample 1 is a heptahydrate of UMP, which, being an unstable substance, slowly loses crystallization water during trituration and maturation.

Calculation of the X-ray diffraction pattern obtained for sample 2 (CMP) has allowed us to find out that it is CMP in the form of a thirteen-water crystal hydrate with a small admixture of another hydrate (Fig. 3). Both crystallohydrates are in a nanocrystalline state; thus, it has been assumed that efflorescence processes can also occur in this salt as in sample 1. It should be noted that the bank data from the structure of 13-hydrogen crystallohydrate is similar to that of sample 1 (disordered sodium and unreliable separation of sodium and oxygen peaks), while the structure of the second crystallohydrate contains a lot of crystallization water, which is not part of the coordination sphere of sodium atoms. Therefore, the phase content of both substances in the sample may differ markedly from that obtained as a result of the X-ray diffraction pattern calculation. The results of the X-ray diffraction pattern refinement of sample 2 (CMP) are presented in Fig. 4 (marks 1-3 indicate lines and curves as in Fig. 1).

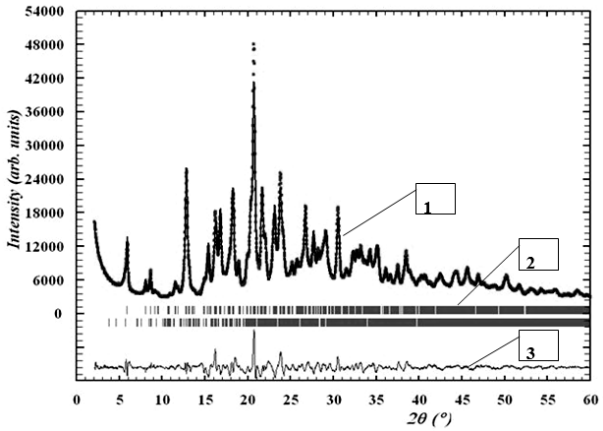


Fig. 4: The results of refinement of the X-ray diffraction pattern for sample 2 (CMP) using the Rietveld method

The phase composition of the PHC substance sample was also studied. It was found that sample 3 (PHC) was in the form of pyridoxinium chloride, which corresponded to the literature data and confirmed by calculation. No impurity lines were observed on the X-ray diffraction pattern.

The results of the study of the phase composition of APIs substances determined the identity of substances, the presence of impurities and the type of water present in the substance (adsorbed or crystallized). These data were taken into account when choosing a method for determining the actual water content in substances (for UMP and CMP – according to Fischer, for PHC – by the loss on drying) and for factorizing their quantities when preparing binary solutions and solutions of all APIs in compliance with therapeutic concentrations to study compatibility.

The API interaction was determined by various parameters – changes in color, transparency, pH, the total impurity content, etc. In these studies, if binary mixtures showed an increase in impurities associated only with the influencing factors and the absence of impurities that would characterize the API interaction, it was considered that the ingredients were compatible[24].

The solutions were exposed to various factors (heating, pH, the action of an oxidizer, a reducing agent).

No impurities characterizing the API interaction were detected in binary solutions of UMP and CMP. In binary solutions of UMP with PHC and CMP with PHC there was an increase in the formation of impurity A of pyridoxine when heating on a boiling bath for one hour compared to the fresh solution. At room temperature no increase of impurities in the solutions was observed. Thus, an increase in impurities associated only with the influencing factor was detected. There were no impurities that would characterize the API interaction.

The study of binary model samples and solutions containing all APIs at pH 4.0-4.8, which is acceptable for PHC, has shown that the stable existence of disodium salts of UMP and CMP is also possible in this range. Therefore, it can be assumed that a new combined drug may include these APIs provided that the pH is maintained at approximately 4.0-4.8.

The next potential factor of instability of the APIs studied is the effect of oxidizing agents. The behavior of APIs in binary model samples and in solutions containing all APIs under the action of an oxidizer was also studied. The contribution of this influencing factor to the instability of model solutions was determined by the parameter "total impurity content". Based on the analysis of the results the absolute value of the increase of impurities relative to the freshly prepared solution (in %) by the factor "action of oxidizing agents (hydrogen peroxide)" was calculated. This value was 11 %.

Therefore, based on the analysis of the effect of factors affecting the stability of API solutions of the drugs developed the ability to hydrolytic and oxidative processes was determined due to the pH level, the action of the elevated temperature and oxidizing agents.

CONCLUSIONS:

Thus, as a result of the research, the compatibility of APIs that have an alkaline and acidic nature has been studied, and the optimal pH level for a combined solution based on them has been determined. The studies conducted have allowed us to determine the directions of further research for developing the composition of a parenteral dosage form. This research is in choosing the optimal buffer system, which is very important for maintaining the stability[25,26] of active substances with different pH levels and their simultaneous presence. To achieve the chemical stability of the solution, it is necessary to choose a system of excipients that prevent possible oxidation processes.

CONFLICT OF INTEREST:

The authors declare no conflict of interest.

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RESEARCH ARTICLE

Fabrication and characterization of nanostructured lipid carrier system for effective delivery of poorly water-soluble drug quetiapine fumarate

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ABSTRACT:
The aim of present study is to investigate the potential of nanostructured lipid carriers (NLCs) in improving the oral bioavailability of quetiapine fumarate, a second-generation antipsychotic drug. Quetiapine Fumarate (QF) loaded NLC were prepared by hot homogenization followed by an ultrasonication method. Response surface methodology - central composite design (CCD) was used to systemically examine the influence of concentration of capmul MCM EP, concentration of poloxamer 188 and concentration of egg lecithin on particle size (PS) and % entrapment efficiency (% EE) and to optimize the NLC formulation. The CCD consists of three factored design with five levels, plus and minus alpha (axial points), plus and minus 1 (factorial points) and the centre point. A mathematical relationship between variables was created by using Design Expert software Version 12. The statistical evaluations revealed that three independent variables were the important factors that affected the PS and % EE of QF loaded NLC. The best fitted mathematical model was linear and quadratic for PS and % EE respectively. The optimized formulations found with 218.1±0.14nm of PS and 93±0.16% of % EE. Results illustrated the superiority of developed QF loaded NLC formulation as a stable drug delivery system, providing better bioavailability with the possibility of better treatment for psychological disorders.

KEYWORDS: Central composite design; Nanostructured lipid carriers; Poloxamer 188; Quetiapine Fumarate; Response surface methodology.

INTRODUCTION:
Since last decades a tremendous rise has been observed in the psychological disorders, particularly bipolar disorder and Schizophrenia[1]. Quetiapine is one of the extensively prescribed drugs as monotherapy in treatment bipolar disorder and schizophrenia [2, 3., 4, 5] QF belongs to Biopharmaceutics Classification System class II [6, 7] drug and exhibits 9% oral bioavailability. QF being weak acid get rapidly absorbs in the stomach but suffers from high first-pass effect. QF possesses half-life of 6 hours and requires frequent administration which in turn enhances chance of dose skipping [8, 9].

Low oral bioavailability and high first pass effect lead to reduction in absorption which is a major obstacle in QF therapy. QF exhibits low solubility and low absorption at high pH. QF undergoes P-glycoprotein (P-gp) efflux leading to less concentration in the brain even after absorption.

Colloidal drug carriers have been used to address solubility issues of a drug. But these systems suffer from certain disadvantages like drug leakage, stability problems, high production cost and sometimes cytotoxicity. Similarly, lipid containing delivery systems such as lipid drug conjugate (LDC), solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC) can be effectively used to solve customary drug delivery systems issues such as low bioavailability and low drug loading.

However, LDC suffered with few demerits such as particle size growth, uncertain gelation tendency, sudden

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polymeric transitions and low drug loading [10, 11]. In order to address LDC disadvantages, SLN system was developed [8]. SLN can be characterized as small lipid containing preparations which possess large surface area and have been considered as biocompatible and biodegradable systems [12, 13, 14, 15].

NLC (Lipid based systems) - The second-generation lipid carrier is usually composed of solid lipids and liquid lipids together in a system with surfactant[16, 17]. This mixing causes depression in melting point of substrates and converts the mixture into solid form at body temperature. NLC shows a high drug loading with minimum drug expulsion [18, 19, 20] as compared to SLN. NLC bypass P-gp efflux due to other way for uptake (non receptor mediated endocytosis). In view of these facts, NLC system can be considered as effective alternate to overcome issues associated with QF therapy.

MATERIALS AND METHODS:

Materials:

QF was obtained as a gift sample from Aurobindo Pharma Limited, Hyderabad, India. For the production of NLC, Monegyl - T18 and Monegyl - D207 (Mohini organics Pvt. Ltd. Mumbai), Glyceryl monostearate, GMS (Micro Labs Limited, Bangalore), Capmul MCM EP (Intas pharmaceuticals Ltd., Ahmedabad), Poloxamer 188 (BASF India Limited, Navi Mumbai), Egg lecithin (Naproid life sciences Pvt. Ltd. Mumbai) were used. All the other reagents used were of analytical grade.

Methods:

Optimization of ratios of solid lipid to liquid lipid:

Solid lipid (Stearic acid, Monegyl- T18, Monegyl-D207, GMS) was screened by method given in literature survey [21, 22]. Liquid lipid (oils) was selected depending on the solubility of the QF in liquid lipid [23, 24, 25, 26, 27].

The mixture comprising of both solid and liquid lipids were prepared homogenous. To optimize the ratio of lipids, miscibility test between the selected lipids namely GMS and capmul MCM EP was performed. The selected solid lipids and oil were weighed in the different % ratios (60:40, 70:30, 63.636: 36.363) in glass vials. This blend was heated to a temperature 10°C above the M.P. of the solid lipid. Thereafter, the liquid blend vortexed and smeared on glass slide. Upon solidification, a dry filter paper was pressed on this lipid blend and observed for sign of oil drops, if any. The mixture that does not showed any oil drop on filter paper was considered as miscible and was selected for the development of QF loaded NLCs [22].

Preparation of QF loaded NLCs:

QF loaded NLCs were fabricated by a slight modification of hot homogenization method followed by the ultrasonication method. Preliminary batches were prepared, examined and optimized for various process parameters such as water volume, sonication time, amplitude, PS, Zeta potential (ZP), Polydispersity index (PDI) of the NLC dispersion. This method resulted in consistent production of smaller size NLCs (<250 nm) with good PDI and good % EE.

Egg lecithin was dissolved in 3ml mixture of methanol and chloroforms (1:1) and vaporized for 15 min to completely evaporate the solvents to achieve a film of egg lecithin. The GMS and capmul MCM EP were melted (10ºC above the M.P. of the lipids used) and was added to above mixture. QF was added to attain a drug-lipid mixture. The hot surfactant solution (poloxamer 188) in water preheated to 10ºC above the lipid's M.P. (70ºC) was added to the clear lipid melt containing egg lecithin. Resulting mixture was exposed to high-shear homogenization at 2000 rpm for 10 min and heated to obtain a coarse emulsion. This coarse emulsion was then subjected to probe sonicator (PCi *analytics* PKS -750FL, Mumbai) for 15 minutes at 50°C by maintaining output amplitude at 50% and gap of 5 second to form NLC dispersion. Then hot NLCs obtained was cooled to room temperature to precipitate the lipid into the solid form to yield QF loaded NLCs [28, 29, 30, 31]. This technique is simple to prepared NLCs without any sophisticated instruments and can also be used for large scale production [32, 33].

Preliminary batches of NLC and optimization of process variables:

Based on literature survey and trial batches, critical process variables were identified and subjected to optimization. QF suspension was formulated using 0.025gm of QF, 0.125gm of sodium carboxy methyl cellulose as a suspending agent and distilled water up to 25ml as vehicle for further studies. Selected process variable used were temperature (70ºC), magnetic stirrer rpm (2000 rpm) for 10 min. The sonicator process used variable were amplitude at 50% and gap of 5 sec. for 15 min. (Table 1)

Table 1: Preparation of trial NLC batches

Sr. No.	Components (gm)	Trial 1	Trial 2	Trial 3	Trial 4	Trial 5
	Ratio of Solid lipid % to liquid lipid %	63.636: 36.363	**63.636: 36.363**	60:40	60:40	70:30
1	QF	0.025	**0.025**	0.025	0.025	0.025
2	Monegyl T 18	0.175	-	0.0875	-	-
3	GMS	-	**0.175**	0.0875	0.165	0.1923
4	Capmul MCM EP	0.1	**0.1**	0.1	0.110	0.0827
5	Poloxamer 188	0.375	**0.375**	0.375	0.375	0.375
6	Egg lecithin	0.250	**0.250**	0.250	0.250	0.250
7	Distilled water (ml)	25	**25**	25	25	25

Experimental design for QF loaded NLC:

Preliminary batches indicated that the variables *viz.* concentration of capmul MCM EP (X_1), concentration of poloxamer 188 (X_2), and concentration of egg lecithin (X_3) were the important factors that affected the PS and % EE of NLC. Thus, response surface methodology-(CCD) was used to systemically examine the influence of these three independent variables on PS (nm) and % EE of the prepared NLCs. The CCD consisting of three factored design with five levels, plus and minus alpha (axial points), plus and minus 1 (factorial points) and the center point, hence 15 runs was used in this study. On the basis of the results obtained from preliminary batches, the optimize concentrations of different components was selected (Table 1). The details of the formulation design are listed in Table 2.

Table 2: Independent variables, dependent variable and levels for design of NLCs

Variables	Levels				
1. Independent variables in gms	-1.68179	Low (-1)	Center	High (+1)	+1.68179
Concentration of capmul MCM EP (X_1)	0.0829552	0.1	0.125	0.150	0.167045
Concentration of poloxamer 188 (X_2)	0.140343	0.2	0.2875	0.375	0.434657
Concentration egg lecithin (X_3)	0.11591	0.150	0.2	0.250	0.28409
2. Dependent variable	Constraint				
Y1: PS (nm)	Minimize				
Y2: EE (%)	Maximize				

For all batches the quantity of QF (0.025gm), GMS (0.175gm), water up to (25 ml) was kept constant. The formulations were prepared in triplicate[34]. Results of the study were validated through design expert software version 12 (State Ease, Inc., USA) for further analysis [35].

Optimization of QF loaded NLC formulation:

The solution that has 'Desirability Factor' close to 1 was selected as given by Design Expert software [31].

Freeze-Drying (FD) Study:

Several ratios of total lipids: cryoprotectant (Trehalose) at 1:2, 1:4, 1:6 and 1:8 were tried. The selected cryoprotectant was added after homogenization process. The blend was then subjected to freeze-drying (Labconco, 25 Plus) at −40 °C for 24 hrs. The ratio that produced the smallest PS and PDI was selected[34, 35, 36].

***Evaluation and characterization* of QF loaded NLCs**

Particle size and polydispersity index (PDI):

The FD samples of QF loaded NLCs were used after reconstitution with distilled water for analysis of PS and PDI. PS was calculated by using photon correlation spectrometer (Malvern Zeta sizer nano ZS 90) with a 4.0 mW internal laser. Dynamic light scattering was based on the particle diffusion associated with Brownian motion, which gives an idea about PS. PS was than estimated by the translational diffusion coefficient using the Stokes-Einstein equation by software[37]. PDI was determined to confirm distribution of PS in the formulation[38].

Zeta potential:

The FD samples were used after dilution with distilled water to determine ZP of prepared formulation was evaluated by using the Malvern Zetasizer Nano ZS 90. The electrophoretic mobility of particles was transformed to the zeta potential [39, 40, 41]. Zeta potential was determined using the 'Dip cell' with application of field strength 20 V/cm with 30 runs.

Entrapment efficiency and drug loading:

The drug loaded NLC was subjected to centrifuge at 45,000 rpm for 35 min. Then 1.0 ml of the supernatant was diluted with methanol. The samples were analyzed by using UV spectroscopy at 244 nm (UV 1650, Shimadzu, Japan) to calculate % EE[42]. For drug loading, QF from freeze dried powder was mixed with methanol. The QF quantity was analyzed by using UV spectroscopy at 244, against methanol as blank [41, 43].

Drug excipients compatibility studies, (FTIR) study:

The FTIR spectrum of QF and final formulation were recorded by FTIR (FTIR-4800, Shimadzu, Japan). Briefly, solid sample (1 mg) along with 100 mg dried potassium bromide was compressed into a disc. For liquid sample, sample drops were dripped onto NaCl or KBr aperture plate and sandwiched it under other aperture plate by forming thin liquid membrane. Then, sample was scanned for absorbance in between 4000 ~

400 cm^{-1}. The obtained spectrum was then matched with standard group frequencies of QF.The obtained FTIR (4800, Shimadzu, Japan) spectrum was then compared with standard frequencies of QF [44, 45, 46, 47].

Morphological study (SEM):
The surface morphology of FD sample (batch F3) was studied using SEM (TESCAN MIRA3, Czech Republic). The sample was dusted on double-sided tape onto aluminum stub, coated with gold by using cold sputter coater in SEM chamber of thickness 400 Å. The graphs were recorded with voltage of 15 kV electron beam [43].

Differential scanning calorimetry (DSC):
Thermal analysis data was recorded using a DSC (DSC 204 F1 Phoenix, NETZSCH). The freeze-dried sample (2 mg) was sealed in pin-holed standard 40 µl aluminum pan. The heating rate was10˚C/min from 30˚C to 400˚C and nitrogen purged rate of flow was 10 ml/min. The data was also recorded for QF (drug), GMS (solid lipid) and physical mixture [48, 49].

X-ray diffraction (XRD):
The physical state of QF loaded NLCs was evaluated by XRD (Bruker AXS D8).The freeze dried NLC was smeared on low background sample holder and fixed onto stage in goniometer. Device was set with B-B geometry. The voltage and current were set to 35 mA and 40 mV respectively and XRD was recorded. The data was also recorded for QF (drug), GMS (solid lipid) and physical mixture [48].

***In vitro* drug release:**
The *in vitro* release of QF from optimized formulation F3 and prepared QF suspension was recorded using the dialysis bag diffusion method [50]. The optimized NLC formulation equivalent to 5 mg of drug was transferred to a dialysis bag, sealed and suspended for 2 hrs in USP (type II) apparatus containing 900 ml 0.1 N HCl (pH 1.2), and then in phosphate buffer (pH 6.8) for 24 hrs with 50 rpm at 37°C ± 0.5°C. The 5 ml of the samples were taken out at intervals *viz.* 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 7, 8, 12 and 24 hrs filtered through 0.45 µm membrane filter and the same was replaced with fresh media to enables sink condition throughout release period. The QF release was analyzed after dilution at 207 nm using a UV–Visible spectrophotometer by taking the respective medium as a blank. Same procedure was repeated for QF suspension. All measurements were run in triplicates [51]. Data was fitted to different kinetic models to obtain the release kinetics of NLCs [52]. The highest r^2 value showed the actual mode of drug release [53, 54].

Stability studies:
The stability study of FD batch F3 of NLCs was carried out in accordance with ICH guidelines. Formulation F3 were stored at 25ºC±2ºC /60% RH, ambient temperature and 40ºC±2ºC /75% RH) for 6 months. Effects of temperature and RH on PS and % EE were studied at the end of 0, 3 and 6 months respectively [29, 31, 55].

Determination of pharmacokinetic parameters in rats Administration of optimized formulation of NLC and QF suspension in rats:
Present protocol was approval by Institutional Animal Ethical Committee (IAEC) of Pinnacle Biomedical Research Institute (PBRI), Bhopal (Reg. No. 1824/PO/ERe/S/15/CPCSEA) with reference no. PBRI/IAEC/PN-19025 dated 23/01/2019. In house breed wistar rats acquired from Pinnacle Biomedical Research Institute, Bhopal, (M.P.) were used for the present study. The male wistar albino rats (approx. 150-220g) were used for the present study under fasting conditions. Rats were divided in two groups (n-6), first group was treated with optimized formulation of NLC (equivalent to 25 mg of QF) via oral route whereas rats of second group were treated with pure drug suspension (equivalent to 25 mg of QF) orally.

Blood sample collection and processing:
Blood samples (250 µl) were serially taken out into heparinized tubes from retro-orbital plexus at intervals of 0.5, 1, 2, 4, 6, 8, 12 and 24 hours. The saline solution equivalent to blood samples withdrawn was injected to rats to compensate blood loss. Plasma was isolated by using centrifuge at 5000 rpm for 10 min at 4°C.The plasma samples were stored at -20ºC.

Extraction procedure for drug from blood plasma:
The QF in rat plasma samples were analyzed from the calibration curve. Drug in plasma was analyzed by HPLC (LC-2010CHT) equipped with C_{18} column, UV-Visible detector, an auto injector and a system controller. The mobile phase consists of a mixture with ratio 40:60 v/v of water and acetonitrile respectively at 25 ± 2°C. Mobile phase was passed through membrane filter (0.45 µm) followed by deaeration for 5 minutes using sonicator. The flow rate was adjusted to 1 ml/min. Sample injection volume was 20 µl and analyzed at 207 nm.

Estimation of various pharmacokinetics parameters:
Various pharmacokinetics parameters such as maximum concentration (C_{max} in ng/ml), time taken to reach C_{max} (T_{max} in hrs.), $AUC_{(0-24)}$ in ng hr/ml, $AUMC_{(0-24)}$ in ng hr^2/ml, mean residence time (MRT in hrs), relative bioavailability (F in %) were determined using rat plasma samples and compared with drug (QF) suspension [21].

RESULTS:

Optimization of ratios of solid lipid to liquid lipid:

After solidification of selected solid lipid and liquid lipid in different % ratios (60:40, 70:30, 63.636: 36.363), they were applied to dry piece of filter paper and the sample which did not show any oil droplets on the surface of filter paper was considered miscible was selected for use in the development of trial batches of QF loaded NLCs.

Results of preliminary batches and optimization of process variables of NLCs:

Preliminary optimization of stirring time, rpm and temperature was decided by literature survey and by conduction of the different experiments [56]. NLC Trial 2 batch demonstrated good result with PS as 240.6nm, ZP of -25.3 mv, PDI of 0.492 good result.

Experimental design with results for NLC formulation

The experimental results were showed in Table 3.

Table 3: Experimental design for formulation of QF loaded NLC

Batch	Independent variables			Dependent variables			
				Observed value		Predicted value	
	X_1	X_2	X_3	Y_1	Y_2	Y_1	Y_2
F1	0.1	0.2	0.15	240.5±1.08	92.2±0.16	240.13	91.86
F2	0.15	0.2	0.15	242±0.81	83.5±1.22	241.94	83.84
F3	**0.1**	**0.375**	**0.15**	**218.1±0.14**	**93±0.16**	**220.46**	**92.59**
F4	0.15	0.375	0.15	220.8±0.16	84.2±0.29	222.26	84.16
F5	0.1	0.2	0.25	243.2±0.61	92.5±0.08	243.3	92.14
F6	0.15	0.2	0.25	245.1±0.08	83.5±0.4	245.1	83.51
F7	0.1	0.375	0.25	225±0.32	93.2±0.16	223.62	92.46
F8	0.15	0.375	0.25	225±0.81	83.5±0.08	225.43	83.43
F9	0.0829552	0.2875	0.2	231.3±0.08	95±0.81	231.26	95.91
F10	0.167045	0.2875	0.2	235±1.41	81.9±0.48	234.3	81.56
F11	0.125	0.140343	0.2	249.5±1.22	87±1.08	249.33	87.01
F12	0.125	0.434657	0.2	218.3±0.35	87±1.55	216.23	87.56
F13	0.125	0.2875	0.11591	232±0.73	88±0.81	230.12	88.08
F14	0.125	0.2875	0.28409	234.8±0.97	87.2±0.16	235.44	87.7
F15	0.125	0.2875	0.2	232.5±1.00	87.5±0.08	232.78	87.48

mean ± SD , *n*=3, X1: concentration of capmul MCM EP (gm); X2: concentration of poloxamer 188 (gm), X3: concentration of egg lecithin (gm), Y1: PS (nm), Y2:% EE (%)

Polynomial equation:

Polynomial equations for Y_1 and Y_2 were as follows:

$Y_1 = +254.26800+36.09216X_1 -112.44751X_2+31.64568 X_3$ ----------(1)

$Y_2 = +115.34921-310.43642 X_1+17.39099X_2-3.41783X_3-45.71429 X_1X_2-120X_1X_3-22.85714X_2 X_3+708.09282X_1^2-9.15519 X_2^2+ 56.81505X_3^2$ ----------(2)

Response 1 (Y_1): Effect on PS

Results of all batches from ANOVA analysis, showed that independent factors were significantly affect the PS (p-value : <0.0001). The R^2 value (0.9862) found near to 1 showed linearity. The linear model F value of 381.13 showed that the model was significant. PS of all 15 batches were in the range of 218.1±0.14nm to 249.5±1.22nm (Table 3). Equation (1) shows factors X_1 (concentration of capmul MCM EP), X_3(concentration of egg lecithin) have positive effect on PS of NLCs and X_2 (concentration of poloxamer 188) have a negative effect on PS of NLCs. This is also confirmed by equation of response Y_1.

The PS of NLCs increased with an increment in liquid lipid concentration, which was found similar to reports published by Hu et al., Tamjidi et al. and Dai et al [57, 58, 59]. This may be due to disruption of lipid wall followed by aggregation and ultimately particle size growth with increase in liquid lipid content [57]. Elevation in interfacial tension results in swelling of NLC and higher liquid lipid concentration causes increase in PS [58, 59].

Poloxamer in 1.5% concentration was sufficient to cover the surface of QF loaded NLC effectively during the formulation process due to reduction in interfacial tension between the water and oil phase which lead to separation of particle and surface area enlargement. But beyond the appropriate surfactant concentration, saturation occurs where the affinity between lipid and water phase gets decrease which would not permit the PS to decrease further [60].

Response 2 (Y_2): Effect on % entrapment efficiency:

Results from the ANOVA analysis indicates that all independent factors were greatly affect the % EE of NLCs (p-value: <0.0001). The R^2 value (0.9899) was found near to 1 and showed linearity. The quadratic model with F value of 108.73 showed the model was significant. The % EE of all batches was in the range of 81.9±0.48 to 95±0.81% (Table 3).

The equation (2) showed linear and quadratic

interactions for response Y_2. Factors X_1 (concentration of capmul MCM EP) and X_3(concentration egg lecithin) had negative impact on % EE of NLCs and X_2 (concentration of poloxamer 188) had a positive impact on % EE of NLCs.

The 3D plots showed the negative impact of X_1X_2, X_1X_3, X_2X_3 interactions on % EE of NLCs. The negative impact of X_1X_2 interaction (increase in concentration capmul MCM EP and concentration of poloxamer 188) or X_1X_3 interaction (increase in concentration capmul MCM EP and concentration of egg lecithin) or X_2X_3 interaction (increase in concentration of poloxamer 188 and concentration of egg lecithin) would decrease the % EE of NLCs.

The drug QF is lipid soluble which gets entrapped in the lipid matrix and showed good % EE. Its solubility decreases during cooling of lipid melt. Further, capmul MCM EP in the lipid mixture increases drug dissolution and also help in preventing separation and crystallization of QF from lipid blend on cooling, which is in good accordance with the findings of the earlier studies of Lohan et al and Muller et al [28, 61].

The % EE of NLCs reduced greatly with an increment in liquid lipid concentration, which was found in agreement to earlier reports of Zhu et al., Ali et al., Soleimanian et al., Kudarha et al [62, 63, 64, 65]. This may be due to lipid precipitation during NLCs production. During cooling, re-crystallization of lipids results low drug entrapment. Thus, increase in the lipid concentration beyond certain limit leads to poor % EE.

Optimization of QF loaded NLC:
Optimization of QF loaded NLC was carried out to determine the appropriate concentration of independent variable. Results derived from design expert software 12, indicated that F3 has all charactistics to be considered as optimized batch (0.4% of capmul MCM EP, 1.5% poloxamer 188 and 0.6% egg lecithin).

Freeze-Drying Study:
Various total lipid: trehalose ratio (1:1, 1:2, 1:4, 1:6) were tried. But 1:4 ratio was used in the formulations for further investigation as this ratio showed superior result with PS of 220nm, ZP of -29.5mv and PDI of 0.380 than other ratios.

***Evaluation and characterization* of QF loaded NLCs**
Particle size and polydispersity index of QF loaded NLCs:
The particle size and PDI of optimized batch F3 was found 218.1±0.14nm and 0.382 respectively. (Fig. 1)

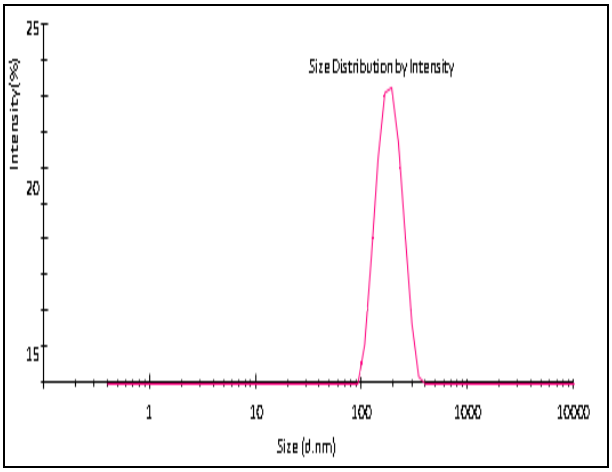

Fig. 1: Particle size and polydispersity index of QF loaded NLCs

Zeta potential::
The ZP of F3 batch was found to be -29.5 mV, which imparts good stability of NLCs dispersion. (Fig. 2)

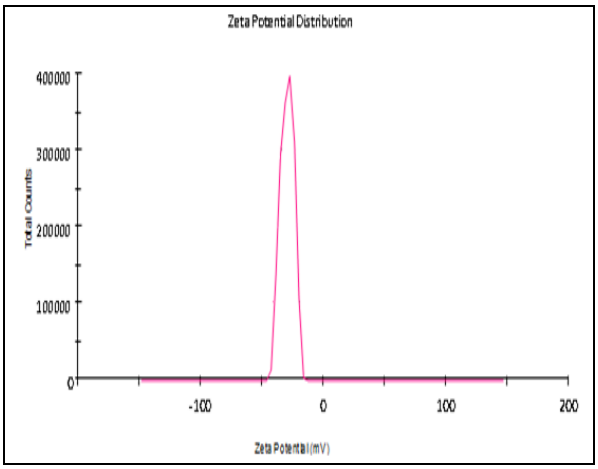

Fig. 2: Zeta potential of QF loaded NLCs

Percentage entrapment efficiency and drug loading:
All batches showed good % EE ranging from 81.9% to 95% (Table 3). The % EE for the batch F3 was found to be 93.00 ± 0.163 %. The DL was found to be 8.65 ± 0.067 %.

Fourier transforms infrared spectroscopy (FTIR) study:
As there is no change in the nature and position of the peak in the formulation, it can be concluded that the drug maintains its identification without any chemical interaction with excipients used.

Morphological study (SEM):
The micrograph of the optimized NLC (F3) demonstrated spherical droplets with average diameter of 50.47nm [66, 67].

Differential Scanning Calorimetry (DSC):

The absence of peak in the endotherm of optimized formulation was due to Kelvin effect (nanoparticles melt at a temperature lower than the M.P. of solid lipid) [68]. This finding suggests the conversion of the crystalline QF to its amorphous state. Thus indicate no incompatibility [53].

X-ray diffraction (XRD):

The XRD spectra of QF in Fig. 3 showed intense peaks at 2θ scale, indicating the high crystalline structure of QF (i.e., two sharp peaks at 2θ = 22.617°and 25.2341°). The peaks of QF were absent in the XRD pattern of physical mixture and QF loaded NLC formulation, confirmed that the QF molecule was converted to an amorphous complex with the lipid matrix.

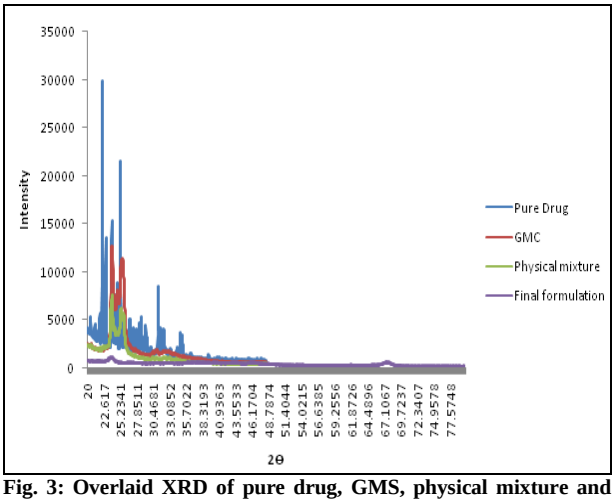

Fig. 3: Overlaid XRD of pure drug, GMS, physical mixture and final formulation

***In vitro* drug release:**

The optimized formulations of NLC showed significant enhancement in QF release profile as compared with QF suspension (Fig. 4). Higuchi model (r^2 =0.9964) was found to be best fitted model for optimized formulation of NLC F3 [69].

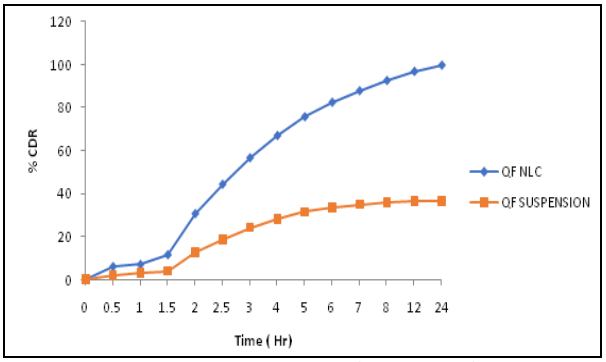

Fig. 4: *In vitro* QF release behavior **of optimized formulations of NLC and QF suspension**

Stability study:

From stability results it can concluded that the formulation had satisfactory stability over 6 months period [60]. (Table 4)

Table 4: Stability study of optimized formulation of NLC

Time (months)	0	3	6
Condition	25ºC±2ºC /60%±5 RH		
PS (nm)	218.1	220	220
% EE	93	93	93
Condition	Ambient temperature		
PS (nm)	218.1	218.1	218.1
% EE	93	93	93
Condition	40ºC±2ºC /75% ±5 RH		
PS (nm)	218.1	218.1	218.1
% EE	93	93	92

Pharmacokinetic study:

Enhanced bioavailability of newly developed formulation of QF was validated using pharmacokinetic study in wistar albino rats**.** The drug in the plasma samples was analyzed by using HPLC method. Plasma concentration-time profile and various pharmacokinetic parameters *viz.* C_{max}, T_{max}, and $(AUC)_{0\text{-}24h}$, $AUMC_{(0\text{-}24)}$, MRT and F were estimated using optimized formulation and QF suspension after an oral administration of dose equivalent to 25mg/kg has been presented in Fig. 5 and Table 5.

Table 5: Comparative pharmacokinetic parameters of optimized formulation of NLC and QF suspension in wistar rats

Parameters	Optimized Formulation	QF suspension
T_{max} (hr)*	2.00 ± 0.19	1.00 ± 0.36
C_{max} (ng/ml) *	2200.17 ± 200.36	364.83 ± 85.63
$AUC_{(0\text{-}24)}$ (ng hr/ml)*	23966.41 ±1172.01	5110.83 ± 421.47
$AUMC_{(0\text{-}24)}$ (ng hr^2/ml)*	54249.89 ± 2119.12	11487.23 ± 967.24
MRT (hr)	2.26 ± 0.41	2.24 ± 0.35
Relative bioavailability (% F)	4.69	-

mean ± SD, *n*=3, *p<0.05 level of significant difference

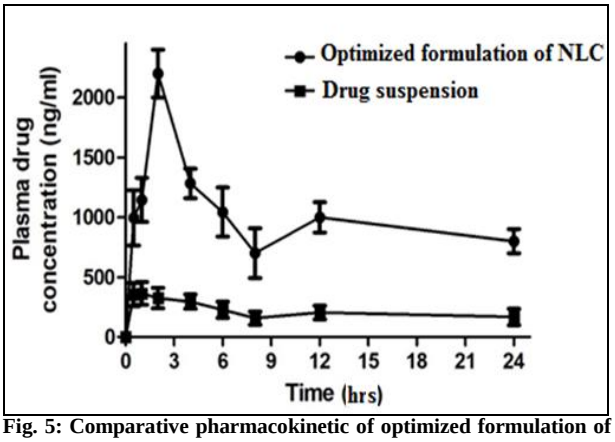

Fig. 5: Comparative pharmacokinetic of optimized formulation of NLC and QF suspension in wistar rats

The C_{max} of optimized NLC (2200.17 ± 200.36ng/ml) was significantly higher than that of QF suspension (364.83 ± 85.63ng/ml). Peak plasma concentration and T_{max} of pure drug and formulation had significant difference. The mean observed plasma $(AUC)_{0\text{-}24h}$ of formulation was also significantly improved to 4.69-fold compared to pure drug suspension confirming improved bioavailability of drug from formulation. The optimized NLC formulation profile is superior to that of QF suspension. The oral bioavailability of QF from optimized batch F3 was higher than that of suspension.

Notably, at all time points, the NLC were remarkably higher than those administered with QF suspension defining performance superiority of NLC over QF suspensions. This increase in bioavailability of QF may be due to lymphatic transport from NLC formulation and avoiding first-pass metabolism of QF.

DISCUSSION:

The particle size of NLCs increased with an increment in liquid lipid conc., same results as per earlier reports [57, 58, 59]. This may be due to disruption of lipid wall then aggregation and ultimately particle size growth with increase in liquid lipid content [57]. Interfacial tension increased and core of NLC swelled with greater liquid lipid concentration and thus observed increase in PS [58, 59].

But beyond the appropriate surfactant conc., there exists saturation where the affinity between lipid and water phase gets explored entirely which would not permits the PS to decrease further [60].

The drug QF is lipid soluble, so it mostly entrapped in the lipid matrix and showed good % EE. The solubility decreases during cooling of lipid melt. Further, capmul MCM EP in the lipid mixture increases drug dissolution and also help in preventing separation and crystallization of QF from lipid blend on cooling [61].

The % EE of NLCs reduced greatly with an increment in liquid lipid conc., which was same that found in earlier reports [59, 60, 61, 62]. This may be due to lipid precipitation during NLCs production. During cooling, re-crystallization of lipids results into a core with reduced drug content. So, increase in the lipid conc. beyond certain limit leads to poor % EE. These results were in good agreement with earlier reports [70, 71, 72].

QF loaded showed excellent stability specified by ZP, high % EE value, drug loading capacity with sustained action. Thus it can be concluded that NLC is a smarter drug delivery system with unique advantages such as higher drug loading; higher entrapment of drug, sustained drug release behavior and eventually enhanced drug absorption as compared with other lipid-based drug delivery system. The feasibility of large-scale production makes NLC as versatile delivery system. Thus, NLCs seems to be reasonable delivery systems for oral administration of QF and may be used as alternate strategy to achieve ameliorated release and prolonged action of QF. In future, QF loaded NLCs may be used in clinical subjects for achieving better outcomes.

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CONFLICT OF INTEREST::

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this paper.

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RESEARCH ARTICLE

In Silico Study of Secondary Structure of Hemoglobin Protein

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ABSTRACT:
Protein structure prediction is one of the important goals in the area of bioinformatics and biotechnology. Prediction methods include structure prediction of both secondary and tertiary structures of protein. Protein secondary structure prediction infers knowledge related to presence of helixes, sheets and coils in a polypeptide chain whereas protein tertiary structure prediction infers knowledge related to three dimensional structures of proteins. Protein secondary structures represent the possible motifs or regular expressions represented as patterns that are predicted from primary protein sequence in the form of alpha helix, betastr and and coils. The secondary structure prediction is useful as it infers information related to the structure and function of unknown protein sequence. There are various secondary structure prediction methods used to predict about helixes, sheets and coils. Based on these methods there are various prediction tools under study. This study includes prediction of hemoglobin using various tools. The results produced inferred knowledge with reference to percentage of amino acids participating to produce helices, sheets and coils. PHD and DSC produced the best of the results out of all the tools used.

KEYWORDS: Protein, hemoglobin, secondary structure prediction.

INTRODUCTION:
Protein is a biomolecule which is an important dietary source which we as humans consume. Protein is a polypeptide made up of amino acids and is present in its three-dimensional arrangement in nature. Protein is studied in four different levels as primary, secondary, tertiary and quaternary which are mentioned as following:

Primary structure:
It is the first level of protein structure which contains amino acid sequence in the form of polypeptide chain. During protein biosynthesis amino acids are bound together with peptide bonds. Based on the nature of free groups at the extremities of the sequence the protein has two ends: carboxyl terminal (C-terminus) end and the amino terminal (N- terminus) end. Primary structure of any protein is determined from the gene from which it is translated.

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As we know as per central dogma DNA transcribes to produce mRNA which further translates to produce protein. Primary structure of any protein can be studied from protein databases. The annotated information for any protein sequence can be retrieved from various protein databases like UNIPROT, PDB, etc. Primary sequences of proteins can be extracted from these databases in fasta format and can be used for secondary as well as tertiary structure prediction.

Secondary structure:
It is the second level of protein structure that is represented in the form of alpha helixes, beta sheets and turns or coils. Basically the backbone of any protein consists of structures that are in the form of helixes or sheets which are connected by the help of turns or coils. Thus, connections producing structures such as helix-helix, sheet-sheet and helix-sheet are seen in the backbone of protein structure. Secondary structure prediction of proteins provides information regarding presence of helix, sheet and coil that is which amino acid participates in specific type of secondary structure represented as H, S, C (helix, sheet and coil). Secondary structure prediction of any protein can be done using any secondary structure prediction methods like Chou

Fasman, GOR, Artificial Neural Network, etc.

Tertiary structure :
Itis the third level of protein structure which represents the three-dimensional conformation of protein that includes arrangement of amino acids into helixes, sheets and turns with backbone structure arrangement based on psi, phi and omega angles. Ramachandran plot explains the possible allowed and disallowed regions for the amino acids that further participates to form the three-dimensional structure of protein. The polypeptide chain is a folded structure produced due to interactions between the R groups of participating amino acids. The possible interactions seen in tertiary structure includes hydrogen bonds, hydrophobic interactions, Wander wall interaction, disulphide bonds etc. Tertiary structure databases such as PDB provides annotated information regarding three dimensional structures of proteins. X-ray crystallography and NMR spectroscopy are the techniques used to predict the three dimensional of proteins. There are in silico methods that can also predict the three-dimensional protein structures. The methods includes Ab-initio method, Homology modeling and Threading.

Quaternary structure:
It is the fourth level of protein structure which represents multiple polypeptide chains connected to produce a single protein structure. Basicaly quaternary level represents number of polypeptide chains that are connected to each other like for example hemoglobin is a protein that is made up of four subunits with two alpha and two beta types.

Protein Secondary Structure Prediction
Secondary structure prediction[1] is an important method used in the field of bioinformatics. Its main motive is to predict secondary structures of proteins based on their amino acid sequences. It provides the complete information of the amino acid sequence like alpha helices, beta strands or turns along with their parameters. The prediction process to search for helices, sheets and coils includes the following six methods:-

Chou Fasman Method:
Chou Fasman algorithm is extensively used to predict secondary structure of proteins. This method is based on an algorithm that calculates prediction values of each participant amino acid. Conformational parameter for each amino acid is calculated on the basis of specific position frequency of every amino acid present in given polypeptide chain. The conformational parameters are calculated for the 20 amino acids based on information collected from standard proteins and are represented as P(α) P (β) and P(turn) for helixes, sheets and coils. The algorithm includes various steps initialized by assigning relevant parameters to all the amino acid residues of the protein for which prediction needs to be done.In further steps combination of six residues is identified for helixes, five residues are sheets and four residues for turns. This method shows 50- 60% accuracy for secondary structure prediction.[2]

Nearest neighbor method:
Nearestneighbor method is also known as homologous method, memory-based method and exemplar-based method as it is based on a hypothesis that small length homologous sequences of polypeptide chain will represent similar secondary structures. This method uses structural databases for standard protein information. In this method small fragments are collected to prepare a sliding window. For every window the central amino acid residue is predicted for its secondary structure based on the rest of the residues from the training dataset. The same process is followed for prediction of other residues in the protein to be predicted.[3]

HMM (Hidden Markov model):
Hidden markov model is another method used for prediction of protein sequences based on markov model. The output producing probabilities to produce helix, sheet and coil are used while predicting the secondary structure of protein needed.[4, 5]

GOR (Garnier-Osguthorpe-Robson):
GOR is another secondary structure prediction method that is based on information theory. It can also predict the helix, β sheets, turn or random coils. The method is better for helix as compared to sheets because sheet depends on interactions with long range between two non-adjacent amino acid residues. In this method sliding window of 17 amino acid residues is used to predict the secondary structure of central residue for the polypeptide chain classifying amino acids into helixes, sheets and coils. The method shows 64% accuracy as being sheet, helix or coil.[6, 7]

Artificial Neural Network:
ANN is based on biological neural network and is used to predict secondary structure of proteins based on standard protein training datasets. ANN uses classification method to categorize amino acid residues into helixes sheets and coils. Information is given as primary protein sequence to the ANN tool which is predicted for the presence of helix, sheet and coil based on weight training and updation of output produced to predict the secondary structure of proteins. The method shows 63% accuracy as being sheet, helix or coil.[8, 9]

Self-optimized prediction method (SOPMA):
SOPMA is a secondary structure prediction method based on predicting helixes, sheets and coils on multiple alignments using self optimization method. The method shows 63% accuracy as being sheet, helix or coil.[10]

Protein Secondary Structure Prediction Tools:

There are various tools based on secondary structure prediction method that includes the following:

- *AGADIR (**http://agadir.crg.es/**)*
- *APSSP (**http://crdd.osdd.net/raghava/apssp/**)*
- CFSSP*(**http://www.biogem.org/tool/chou-fasman/**)*
- GOR *(**https://npsa-prabiR.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_gor4.html**)*
- HHPRED*(**http://toolkit.tuebingen.mpg.de/hhpred**)*
- JPRED *(**http://www.compbio.dundee.ac.uk/www-jpred/**)*
- PROF *(**https://www.aber.ac.uk/~phiwww/prof/**)*
- PSIPRED *(**http://bioinf.cs.ucl.ac.uk/psipred/**)*
- SOPMA *(**https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_sopma.html**)*
- STRAP *(**http://www.bioinformatics.org/strap/Scripting.html**)*
- TOPMATCH *(**https://bio.tools/topmatch**)*
- SPIDER2*(**http://sparks-lab.org/yueyang/server/SPIDER2/**)*
- SYMPRED *(**http://www.ibi.vu.nl/programs/sympredwww/**)*
- YASSPP*(**http://glaros.dtc.umn.edu/yasspp/**)*
- PSSPRED *(**http://bioinf.cs.ucl.ac.uk/psipred/**)*
- FRAG1D*(**http://frag1d.bioshu.se/**)*
- SPIDER2*(**http://sparks-lab.org/yueyang/server/SPIDER2/**)*
- RAPTORX-SS8*(**http://raptorx.uchicago.edu/**)*

MATERIALS AND METHODOLOGY:

The main aim of this research work is the comparative analysis of various secondary structure prediction tools. Primary sequence for hemoglobin used to study the secondary structure results. For this prediction analysis Hemoglobin subunit gamma-2 protein sequence was retrieved from UNIPROT database in FASTA file format ***(https://www.uniprot.org/uniprot/P69892.fasta)*. The** tools used for comparative analysis includes

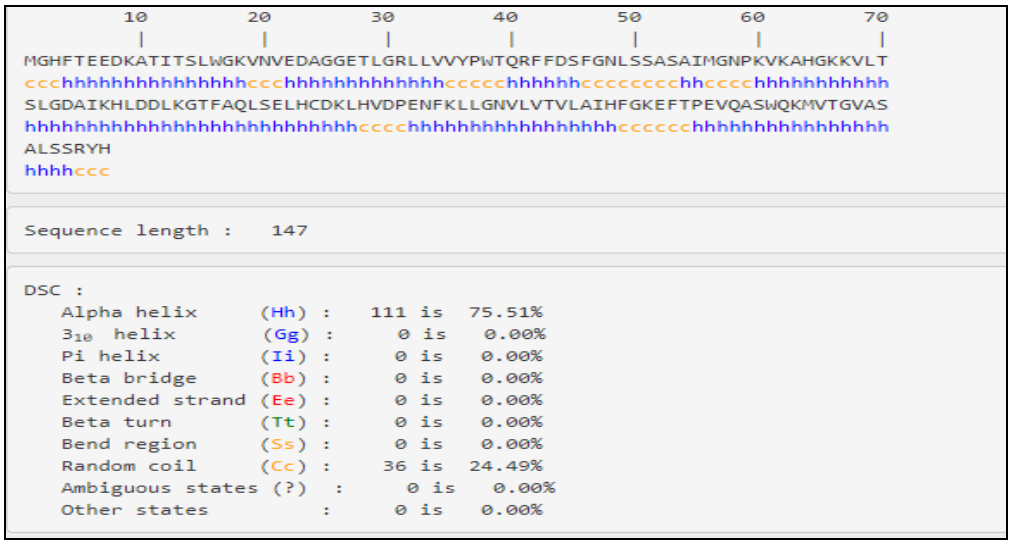
- CFSSP
- PHD
- DSC
- GOR
- SOPMA
- MLRC

RESULTS:

Secondary structure was predicted using hemoglobin sequence taken from the UNIPROT database. Prediction tools produced results that are represented in percentage form for the percentage of amino acids converted into helices, sheets and coils. Comparative analysis of all the following tools revealed that amino acids participating in hemoglobin tend to produce helices greater then sheets or coils. Comparative analysis of the results also reveals that these tools tend to produce variant results out of which PHD and DSC tools were close enough to the actual protein secondary structure studied from protein data bank.

The results of the six tools under study are mentioned from Fig. 1.1 to Fig 1.6.as following:

Figure 1.1 : Result of CFSSP

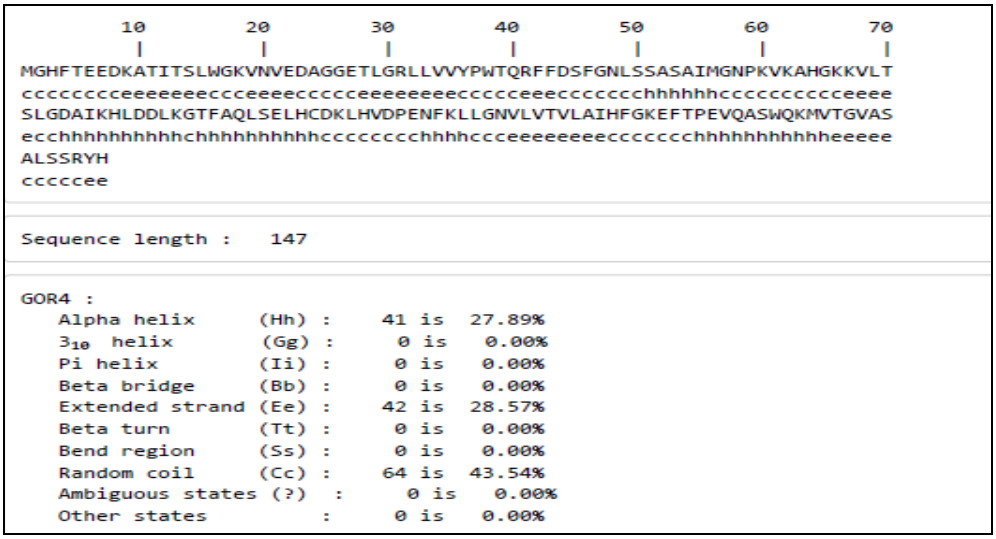


```
         10         20         30         40         50         60         70
          |          |          |          |          |          |          |
MGHFTEEDKATITSLWGKVNVEDAGGETLGRLLVVYPWTQRFFDSFGNLSSASAIMGNPKVKAHGKKVLT
cccHHHHHHHHHHHHHcccHHHHHHHHHHHHcccccHHHHHHcccccccchhcccchhhhhhhhhhh
SLGDAIKHLDDLKGTFAQLSELHCDKLHVDPENFKLLGNVLVTVLAIHFGKEFTPEVQASWQKMVTGVAS
hhhhhhhhhhhhhhhhhhhhhhhhhhhcccchhhhhhhhhhhhhhhhhhcccccchhhhhhhhhhhhhhhh
ALSSRYH
hhhhccc

Sequence length :   147

DSC :
   Alpha helix      (Hh) :   111 is  75.51%
   3₁₀  helix       (Gg) :     0 is   0.00%
   Pi helix         (Ii) :     0 is   0.00%
   Beta bridge      (Bb) :     0 is   0.00%
   Extended strand  (Ee) :     0 is   0.00%
   Beta turn        (Tt) :     0 is   0.00%
   Bend region      (Ss) :     0 is   0.00%
   Random coil      (Cc) :    36 is  24.49%
   Ambiguous states (?)  :     0 is   0.00%
   Other states          :     0 is   0.00%
```

Figure 1.2 : Result of DSC

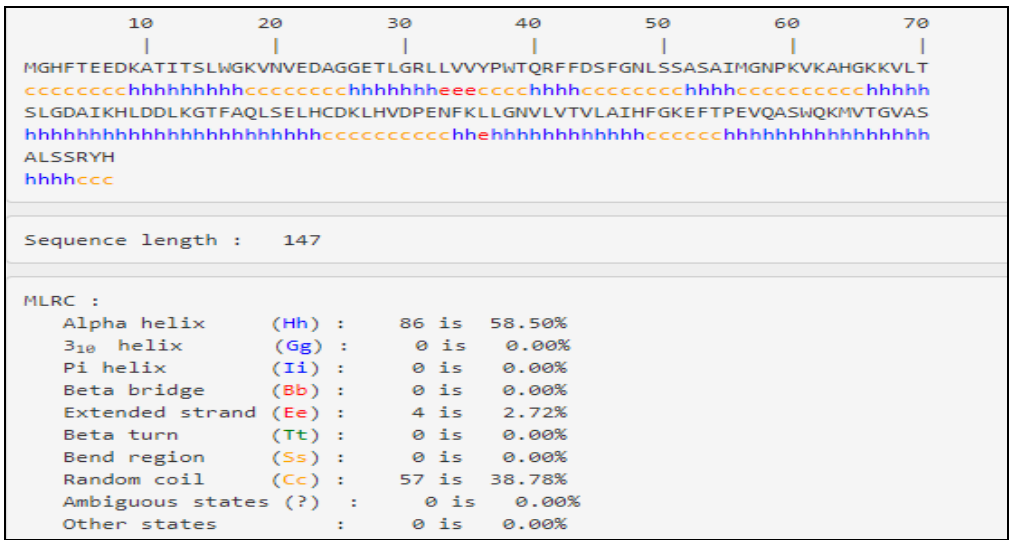


```
         10         20         30         40         50         60         70
          |          |          |          |          |          |          |
MGHFTEEDKATITSLWGKVNVEDAGGETLGRLLVVYPWTQRFFDSFGNLSSASAIMGNPKVKAHGKKVLT
ccccccceeeeeeeccceeeeccccceeeeeeeeeccccceeeccccccchhhhhhccccccccceeeee
SLGDAIKHLDDLKGTFAQLSELHCDKLHVDPENFKLLGNVLVTVLAIHFGKEFTPEVQASWQKMVTGVAS
ecchhhhhhhhhhchhhhhhhhhhcccccccchhhhccceeeeeeeeccccccchhhhhhhhhheeeee
ALSSRYH
ccccee

Sequence length :   147

GOR4 :
   Alpha helix      (Hh) :    41 is  27.89%
   3₁₀  helix       (Gg) :     0 is   0.00%
   Pi helix         (Ii) :     0 is   0.00%
   Beta bridge      (Bb) :     0 is   0.00%
   Extended strand  (Ee) :    42 is  28.57%
   Beta turn        (Tt) :     0 is   0.00%
   Bend region      (Ss) :     0 is   0.00%
   Random coil      (Cc) :    64 is  43.54%
   Ambiguous states (?)  :     0 is   0.00%
   Other states          :     0 is   0.00%
```

Figure 1.3 : Result of GOR4



```
         10         20         30         40         50         60         70
          |          |          |          |          |          |          |
MGHFTEEDKATITSLWGKVNVEDAGGETLGRLLVVYPWTQRFFDSFGNLSSASAIMGNPKVKAHGKKVLT
cccccccchhhhhhhhhcccccccchhhhhhheeecccchhhhcccccccchhhhcccccccccchhhhh
SLGDAIKHLDDLKGTFAQLSELHCDKLHVDPENFKLLGNVLVTVLAIHFGKEFTPEVQASWQKMVTGVAS
hhhhhhhhhhhhhhhhhhhhhhhhcccccccccchhehhhhhhhhhhhhcccccchhhhhhhhhhhhhhhh
ALSSRYH
hhhhccc

Sequence length :   147

MLRC :
   Alpha helix      (Hh) :    86 is  58.50%
   3₁₀  helix       (Gg) :     0 is   0.00%
   Pi helix         (Ii) :     0 is   0.00%
   Beta bridge      (Bb) :     0 is   0.00%
   Extended strand  (Ee) :     4 is   2.72%
   Beta turn        (Tt) :     0 is   0.00%
   Bend region      (Ss) :     0 is   0.00%
   Random coil      (Cc) :    57 is  38.78%
   Ambiguous states (?)  :     0 is   0.00%
   Other states          :     0 is   0.00%
```

Figure 1.4 : Result of MLRC

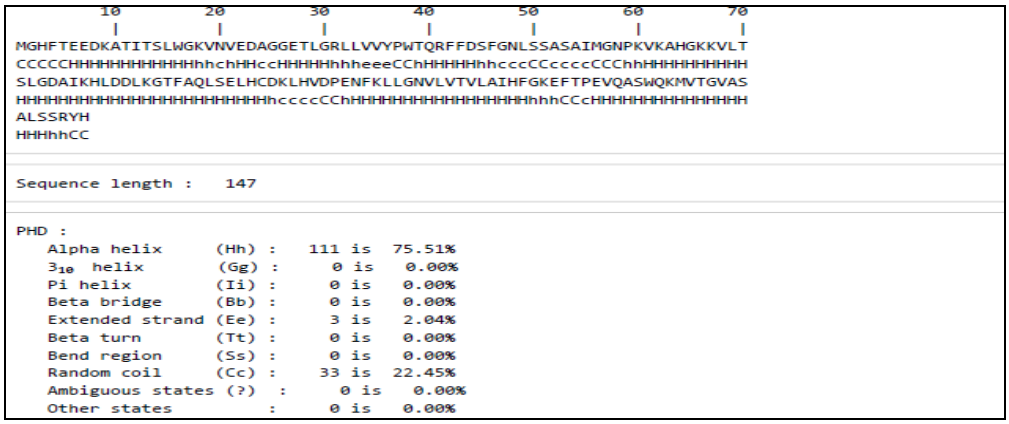


Figure 1.5 : Result of PHD

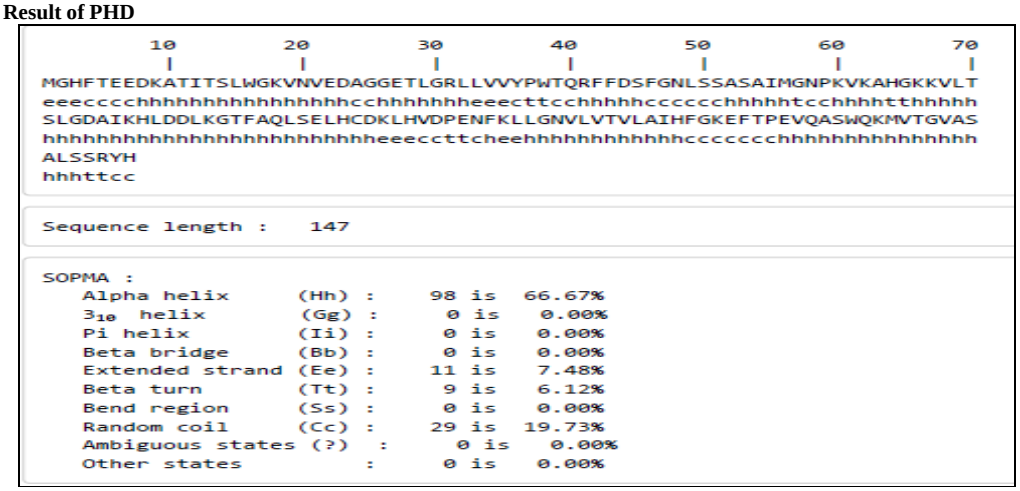


Figure 1.6 : Result of SOPMA

Table 1: Comparative analysis of prediction tools used

Name	GOR4	PHD	SOPMA	DSC	CFSSP	MLRC
Helix	27.89%	75.51%	66.67%	75.51%	83.7%	58.50%
Sheets	28.57%	2.04%	7.48%	0%	59.2%	2.72%
Coils	43.54%	22.45%	19.73%	24.49%	12.2%	38.78%

CONCLUSION:

The results produced using CFSSP, GOR, PHD, SOPMA, DSC, MLRC tools were different and variation in results was seen with respect to helices, strands and coils. Out of all these tools PHD and DSC had predicted helixes to be approximately 75%. Literature studies reveal that almost 75% of all the amino acids are participating in the formation of helical structures in hemoglobin. Thus, we conclude had predicted the best of the results. The same will be verified after predicting the three-dimensional structure of hemoglobin and comparing the same from tertiary databases. Future work will include study of three-dimensional structures of hemoglobin and possible predicted helices, sheets and coils from the secondary structure.

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RESEARCH ARTICLE

Stability Indicating HPTLC Method for Simultaneous Determination of Amlodipine Besylate and Lisinopril in Combined Dose Tablet Formulation

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ABSTRACT:
A combined dose tablet formulation containing Amlodipine besylate and Lisinopril is used for the treatment of essential hypertension. The present study reports development and validation of stability indicating high performance thin layer chromatographic method for simultaneous estimation of these drugs in combined dose tablet formulation. The two drugs were satisfactorily resolved on aluminum plates precoated with silica gel $60F_{254}$ using n-butanol : methanol: ammonia (4:4:1 v/v/v) as mobile phase. The Rf value for lisinopril and amlodipine besylate were 0.27±0.02 and 0.62±0.02, respectively. Densitometric evaluation of the separated bands was performed at 215nm. The calibration curves for lisinopril and amlodipine besylate were found to be linear in the concentration range of 1000-6000ng/band. The method was validated as per ICH guidelines for accuracy, precision, robustness, specificity, limit of detection and limit of quantitation. Statistical analysis proves that the method is suitable for simultaneous analysis of Lisinopril and Amlodipine besylate in pharmaceutical formulation without any interference from the excipients/degradant. The developed method offers several advantages such as sensitive, rapid, cost effective and less time consuming as compared to the reported methods. As the method could effectively separate the drugs from its degradation products, it can be employed as a stability indicating method.

KEYWORDS: Amlodipine besylate, Lisinopril, HPTLC, Stability Indicating Method.

INTRODUCTION:
Antihypertensive monotherapy, although commonly used, does not address the multifactorial nature of hypertension as a disease with many pathways. Using more than one drug makes more therapeutic sense because combination agents cover more than one pathway.

The use of low-dose combination antihypertensive agents is a good contemporary strategy. The combination of Lisinopril (5mg) a long acting angiotensin converting enzyme inhibitor and Amlodipine (5mg) a long acting 1,4-dihydropyridine calcium channel blocker, is well established and is widely used in patients suffering from essential hypertension.

Lisinopril (LSN) is chemically (2S)-1-[(2S)-6-amino-2-{[(1S)-1-carboxy-3- phenyl propyl] amino} hexanoyl] pyrrolidine- 2-carboxylic acid1,2[1]. Amlodipine (AML) chemically is 2-[(2-Aminoethoxy)-methyl]-4-(2-chlorophenyl)-1,4-dihydro-6-methyl-3,5-pyridinedicarboxylic acid 3-ethyl 5-methyl ester benzene sulfonate[2]. Both the drugs are official in IP, BP and USP. The combination tablet is not yet official in any of the pharmacopoeia. Hence, there is no pharmacopoeial method for simultaneous estimation of

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these drugs in tablet dosage form. However number of spectrophotometric[3-5], HPLC[6-16], LC-MS/MS[17], and HPTLC[18], methods are reported in the literature for estimation of AML, both individually as well as in combination with other drugs. LSN is also reported to be estimated, alone or in combination with other drugs, using Spectrohpotomtery[19], Spectrofluorimetry[20], HPLC[21-24] and HPTLC[25-26]. Few HPLC[27-29] and HPTLC[30] methods have been reported for simultaneous determination of AML and LSN.

The objective of the present work was to develop a economical, sensitive and less time consuming HPTLC method for simultaneous determination of AML and LSN in pharmaceutical formulation as an alternative to the reported HPLC/HPTLC methods. The proposed method is optimized and validated as per the international conference on harmonization (ICH) guidelines[31].

MATERIAL AND METHODS:

Chemicals and reagents:

The standard of Lisinopril and amlodipine was supplied by Genpharma International Pvt. Ltd. Pune. Fixed-dose combination tablet AMLOVAS™-L contains 5mg Lisinopril and 5mg of Amlodipine was purchased from local market. All chemical and reagents were of analytical grade and were purchased from Merck Chemicals, Mumbai, India.

Instrumentation:

The sample were spotted in the form of bands of 6mm width with a 100µL sample syringe (Hamilton,Bonaduz, Switzerland) on silica gel precoated aluminum 60F_{254} plates, (10×10cm with 250mm thickness; E Merk,Darmstadt, Germany) using a CAMAG Linomat 5 (Muttenz,Switzerland) sample applicator. The plates were prewashed with methanol. A constant application rate of 0.1mL/s was used, and the space between two bands was 5.6mm. The slit dimension was 5 ×0.45mm, and the scanning speed was 10mm/s. Linear ascending development was carried out in an HPTLC twin-trough glass chamber (CAMAG) saturated with mobile phase vapour. The optimized chamber saturation time was 20 min at room temperature the length of each chromatogram run was 8cm. Following the development, the HPTLC plates were dried in a current of air using an air dryer. Densitometric scanning was performed using a CAMAG TLC Scanner 3 in the reflectance-absorbance mode at 215nm for both AML and LSN operated by WINCATS software (Version 1.4.4.6337, CAMAG). The radiation source used was the deuterium lamp emitting a continuous uv spectrum between 190 to 800nm. Concentrations of the compound chromatographed were determined from the intensity of the diffused light. Evaluation was based on peak areas with linear regression.

Preparation of standard and stock solution:

Standard stock solution A: Accurately weighed quantity of AML (13.9mg, eq.to 10mg of AML) and LSN (10.0 mg) was transferred to 10.0ml volumetric flasks, dissolved and diluted up to mark with methanol.(Concentration: 1000µg/ml AML, 1000µg/ml LSN).

Standard stock solution B : Accurately weighed quantity of AML (20.8mg, eq.to 15mg of AML) and LSN (15.0 mg) was transferred to 25.0ml volumetric flasks, dissolved and diluted up to mark with methanol. (Concentration: 600µg/ml AML, 600µg/ml LSN).

Optimization of the HPTLC method:

HPTLC procedure was optimized with a view to develop assay method for simultaneous estmation of Amlodipine besylate and Lisinopril in pharmaceutical formulation. The standard stock solution B (5µL), containing AML and LSN 600µg/ml each, sample was spotted on TLC plate and run in different solvent system. Initially, n-butanol, ethyl acetate, toluene, methanol and ammonia were tried in different ratios to obtain acceptable Rf value with well separated bands. To ensure compact bands and good peak shape the chamber saturation was also varied from 10 minutes to 30 minutes. After observing the peak shape and separation of two drugs, the chamber saturation time was decided. The plates were developed to a distance of 8 cm; which takes approximately 20 min for complete development of TLC plate.

Method Validation:

Validation of the optimized HPTLC method was carried out with respect to the following parameters.

Linearity and range:

From the standard stock solution A, containing 1000µg/mL of AML and LSN each, 1 to 6µL solution was spotted on TLC plate (n=6). The plates were then developed under the optimized chromatographic conditions mentioned above under instrumentation. Peak areas were plotted against the corresponding concentration to obtain the linearity curves.

Limit of Detection and Quantification:

The limits of detection (LOD) and quantification(LOQ) were separately determined based on the calibration curves. The standard deviation of the y-intercepts and slope of the regression lines were used. The formula used to calculate LOD and LOQ were LOD = 3.3σ/S and LOQ = 10σ/S.

Precision:
The precision of the method was verified by intraday precision studies were performed by analysis of three different concentration (2,000, 6,000, 10,000ng/band) for both AML and LSN for six times on the same day. The inter-day precision of the method was evaluated, at three different levels in similar manner as under intra-day precision, by performing the analysis on three consecutive days.

Specificity:
The specificity of the method was determined by analyzing standard drug and sample. The band for AML and LSN in sample was confirmed by comparing the R_f and spectra of the band with that of standards. The peak purity of AML and LSN were assessed by comparing the spectra at three different levels, i.e. peak start, peak apex, and peak end position of the band.

Robustness of the method:
Robustness of the method was evaluated by introducing small changes in the method parameters such as mobile phase composition, mobile phase volume, duration of chamber saturation with mobile phase, spotting to development time variation (15, 20, 25 min), and stability of solution (20 min,1 hr,3hr,8hr and 24 hr). The effect on R_f values and peak shape of AML and LSN was examined.

Accuracy:
Accuracy of the method was determined by using standard addition method where known amounts of AML and LSN pure drugs corresponding to 80, 100, 120 % of label claim was added to the pre-analysed tablet powder. The samples were then treated and analyzed in similar manner as discussed under analysis of marketed formulation under optimized chromatographic conditions discussed above.

Analysis of marketed formulation:
To determined content of AML and LSN in conventional tablet (AMLOVAS™-L label claim 5 mg of Lisinopril and amlodipine besylate equivalent to 5 mg amlodipine), twenty tablet were weighed and crushed to obtain fine powder. The average weigh of tablet was calculated, tablet powered equivalent to 15 mg of LSN was transferred in 25.0ml volumetric flask dissolved in methanol and the content was kept in ultrasonicator for 5 min., the volume was then made up to the mark with methanol. The solution was then mixed and filtered through 0.2µ membrane filter. The filtrate (6 bands) and standard stock solution A (2 bands), 5µl each, was applied to TLC plate which was developed under the optimized chromatographic conditions. Six tablet power samples were analyzed in similar manner. The possibility of excipients interference with the analysis was examined.

Force degradation study:
Forced degradation of drug product was carried out under thermolytic, photolytic, acid/base hydrolytic and oxidative stressed conditions as per the ICH guidelines. Amount of tablet powder equivalent to about 15mg of LSN was individually transferred to five different 25.0 ml volumetric flask. To flask 1.0ml of methanol was added followed by addition of 3.0ml of 0.1 M HCl, 0.1 M NaOH and 3% H_2O_2. The contents of flask were heated in water bath for 3 hrs at 80^OC. For thermal stress, tablet powder sample was placed in a controlled temperature oven at $80^OC$for 24 h. For photolytic stress, tablet powder sample was exposed to UV radiation at 254nm for 24 h. After stipulated time interval all the flask were removed, cooled to room temperature and then the tablet sample were treated and analyzed in similar manner as described under analysis of marketed formulation.

RESULT AND DISCUSSION:

Among the different solvent combination tried in mobile phase for effective separation of AML and LSN, the mobile phase containing a mixture of N-butanol, methanol and ammonia in the ratio of 4:4:1 v/v/v was found to be optimum. The Rf values for both drugs was well within the acceptable range (Rf - 0.2-0.8), for LSN and AML the Rf values were 0.27 ±0.02 and 0.62 ± 0.02, respectively. The chromatogram obtained under optimized chromatographic conditions is depicted in Fig. 1.

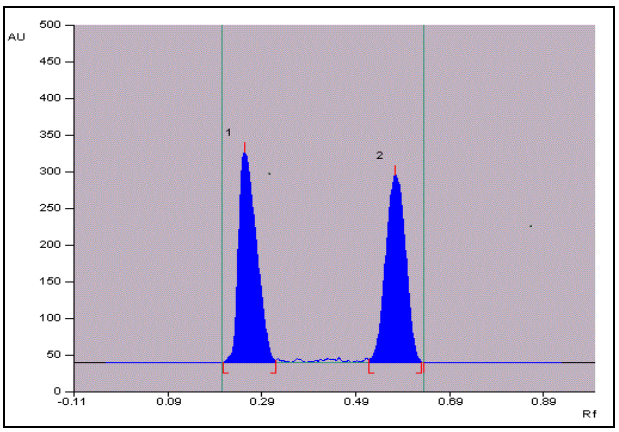

Figure 1: Densitogram of Lisinopril (Rf- 0.27) and Amlodipine besylate (Rf-0.62) in Tablet Formulation

Linearity:
Linear relationship were observed by plotting drug concentration against peak area for each compound. Amlodipine and lisinopril showed linear response in concentration range of 1000-6000ng/band. The corresponding linear regression equation was y=1.143x

+ 321.0 for Lisinopril and y=1.021x + 508.7 for Amlodipine besylate with correlation coefficient (R^2) of 0.998 for Lisinopril and 0.999 for Amlodipine besylate, respectively. The linearity curves for LSN and AML are depicted in Fig. 2 and 3, respectively.

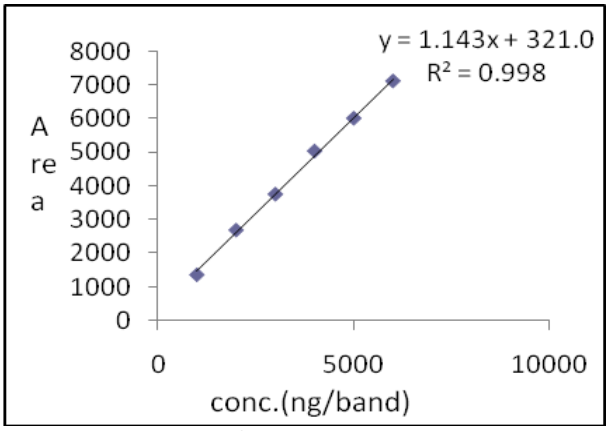

Figure 2. Linearity curve for LSN

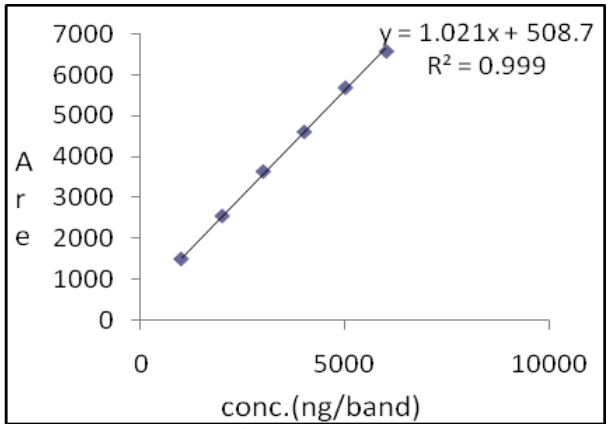

Figure 3: Linearity curve for AML

LOD and LOQ:

The Limit of detection and Limit of quantitation for both the drugs by proposed HPTLC method were calculated using 3σ/S and 10σ/S equations, respectively. Where σ is the standard deviation of Y intercept and S is the slope of calibration curve. The LOD and LOQ was found to be 1.437ng/band and 4.821ng/band for AML and 2.602ng/band and 7.686ng/band for LSN as shown in Table No. 1. In reported HPTLC[20] method LOD and LOQ was found to be 83.45ng/band and 252.87ng/band for LSN and 54.21ng/band and 164.28ng/band. The LOD and LOQ values for LSN and AML were lower than the reported method indicating the improved sensitivity of the developed method over reported HPTLC method.

Precision:

The repeatability and inter-day precision of the developed method is expressed in terms of percent relative standard deviation of percent label claim. In intra-day precision the percent RSD for LSN and AML was found to be 0.246 and 0.831. In inter-day precision the percent RSD for LSN and AML was found to be 1.293 and 1.616, respectively. The percent RSD less than 2 indicates the repeatability and reproducibility of the developed method.

Specificity:

The peak purity of LSN and AML was assessed by comparing their respective in situ spectra at peak start (s), peak apex (m) and peak end (e) positions of the bank. Good correlation among the spectra indicated the peak purity for LSN[Correlation r(s,m) = 0.9994 and r(m,e) = 0.9997] and AML [Correlation r(s,m) = 0.9999 and r(m,e) = 0.9996]. Hence, it can be concluded that there are no impurities or degradation products migrated with the bands obtained from solution of drugs.

Accuracy:

The accuracy of the proposed method was assessed by using standard addition method at three different levels viz. 80 %, 100 % and 120 %. The average percent recovery for AML and LSN was found to be 99.94 % and 100.29 %, respectively, indicating that there is no interference from the excipients used in tablet formulaton. Result of recovery studies are shown in Table No.2

Robustness of the method:

Robustness of the proposed method was assessed by deliberately varying the method parameters like mobile phase volume, mobile phase composition, chamber saturation time, time from spotting to development, and stability of solution. The effect of these variation on Rf value, peak area and resolution of peaks was assessed. The percent RSD for peak area of both the drugs under varied method parameters was less than 2. The resolution between LSN and AML peak was not significantly affected as there was no significant change in the Rf value of the drug peaks. The Rf value was within ±0.05 Rf units of standard values. The results of robustness studies are shown in Table No. 3. Hence, the developed method is robust for simultaneous determination of LSN and AML in fixed dose combination tablet.

Forced Degradation Studies:

In forced degradation studies, both LSN and AML were found to degrade under acidic (0.1M HCl), and oxidative (3% H_2O_2) stress conditions. However, both the drugs LSN and AML were stable under Alkaline (0.1 M NaOH), Neutral, Thermal (60^0C for 24 h) and Photo-degradation (exposure to UV-Light at 254nm) stress conditions. The results of stress degradation studies are shown in Table No. 4 and the typical

densitogram obtained under different stress conditions are depicted Fig. No. 4-9. The developed method was able selective quantitate LSN and AML in presence of degradation products confirming the stability power of the method.

Analysis of Marketed Formulation:

The percent label claim estimated by proposed HPTLC method for LSN and AML was found to be 100.24% and 99.94%, respectively. The results of analysis of marketed formulation are shown in Table No. 5.

CONCLUSION:

The developed HPTLC technique is compared with the reported HPLC method (Table No. 6) for estimation of Lisinopril and Amlodipie in fixed dose combination tablets. The results obtained by proposed HPTLC are comparable with that of the reported HPLC method. The proposed HPTLC method has distinct advantages over reported HPLC method like the amount of solvent required for analysis is very less, cost of HPTLC plate is very less as compared to the HPLC column, there is no need to filter and degas the solvents in HPTLC as necessary in HPLC, solvents of AR grade can be used instead of spectroscopic grade solvents, the chromatographic run is completed in 30 minutes (including chamber saturation time), no possibility of interference from previous analysis as fresh stationery phase and mobile phase is used for each analysis, different batches table samples can be analyzed in single run. Hence, the proposed HPTLC technique is economical and less time consuming than reported HPLC method. The proposed HPTLC method is more sensitive as compared to the reported HPLC method as the LOD and LOQ values are lower that the values obtained in reported HPLC method. Also, the results of method validation, carried out as per ICH guidelines, indicate that the method is accurate, precise, selective and reproducible.. Hence, the developed HPTLC method can be preferred as alternative to the reported methods for routine quality control of pharmaceutical formulations containing Lisinopril and Amlodipine.

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Table 1: Summary of Validation Parameter

Parameter	LSN	AML
Linearity range (ng/band)	1000-6000	1000-6000
Correlation coefficient	0.998	0.999
Limit of detection(ng/band)	2.602	1.437
Limit of quantitation(ng/band)	7.686	4.821
Precision (% RSD) Intraday Inter day	 0.246 1.293	 0.831 1.616
Robustness	Robust	Robust
Specificity	0.9991	0.9996

Table No. 2: Result of Recovery study

Drug	Level	Amount tablet powder taken (mg)	Amount of standard added (mg)	Amount recovered* (mg)	% Recovery ± %RSD (n=3)
LSN	I	739.2	12	12.69	99.91 ±0.082
	II	739.2	15	15.09	100.81±0.405
	III	739.2	18	18.00	100.16±0.208
AML	I	739.2	12	11.98	99.92± 0.685
	II	739.2	15	14.99	99.91±0.085
	III	739.2	18	18.04	100.0± 0.185

*denote the average of three determinations

Table 3: Result of Robustness Study

Parameter	Percent Relative Standard Deviation of Peak Area*		Rf Value*	
	LSN	AML	LSN	AML
Mobile phase composition (± 0.1 mL)	1.23	1.01	0.24	0.65
Total amount of mobile phase (± 1 mL)	0.51	0.15	0.29	0.60
Duration for chamber saturation (± 10 %)	1.37	0.61	0.30	0.66
Time from spotting to development				
5 min	1.50	0.82	0.27	0.64
30 min	1.43	0.89	0.26	0.63
1 h	1.65	0.96	0.29	0.61
Time from development to scanning				
5 min	1.13	0.97	0.25	0.63
30 min	0.94	0.88	0.28	0.66
1 h	1.07	1.02	0.31	0.64

*Mean of five determinations

Table 4: Result of Forced Degradation Study

Stress Conditions	Percent Assay of Active Substance	
	LSN	AML
Acidic (1 N HCl)	89.56	91.23
Alkaline 0.1 N NaOH	98.12	99.85
Oxidative (3.0 % H_2O_2)	88.25	89.60
Neutral (Water)	100.07	99.86
Thermal (60^0 C)	100.00	99.89
Photo-degradation (UV-254 nm)	100.03	100.0

Table 5: Results of Analysis of Marketed Formulation

Marketed Formulation	Drug	Label claim(mg/tab)	Estimated % of label claim ± SD*
Amlovas™-L	LSN	5	100.24 ± 0.328
	AML	5	99.84 ± 0.106

*denote the average of six determinations

Table 6: Comparison of Proposed HPTLC Method with Reported HPLC Method

Method Parameter	Proposed HPTLC Method		Reported HPLC Method	
Stationery Phase	Silica Gel 60F254 (10 X 10 cm)		Phenomenex C18 (250 X 4.6 mm, 5µ)	
Mobile Phase	N-butanol : methanol: ammonia (4:4:1 v/v/v)		0.02 M Phosphate buffer : methanol (75 : 25 v/v)	
Linearity	1000 – 6000 ng/band for both drugs		10 – 110 µg/ml for both drugs	
Drug Estimated mg/tablet	LSN- 5.01 AML- 4.99		LSN- 5.05 AML-5.03	
Precision (% RSD)	Less than 2		Less than 2	
Accuracy (% recovery)	LSN	AML	LSN	AML
Level- 80 %	99.91 ±0.082	99.92 ±0.685	100.54 ±0.536	100.13 ±0.853
Level- 100 %	100.81 ±0.405	99.91±0.085	100.06 ±0.626	100.57 ±0.514
Level- 120 %	100.16 ±0.208	100.0 ±0.185	100.04±0.127	100.51±0.405
Specificity	Specific		Specific	
LOD and LOQ	LOD and LOQ: LSN- 2.602 and 7.686 ng/band AML- 1.437 and 4.821 ng/band		LOD and LOQ: LSN- 0.025 and 0.075µg/ml AML- 0.029 and 0.090 µg/ml	
Robustness	Robust		Robust	

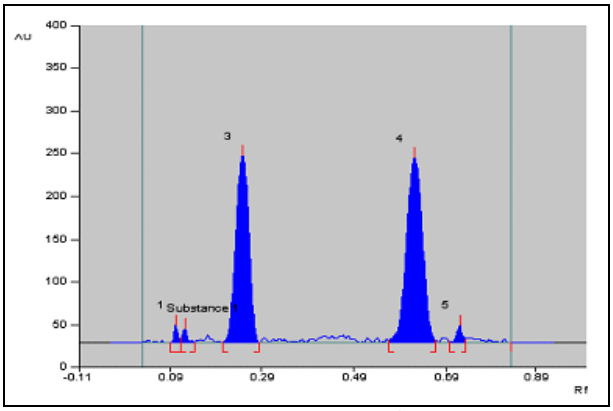

Fig. 4: Typical HPTLC Chromatogram of Acid (0.1 M) Treated Tablet Sample

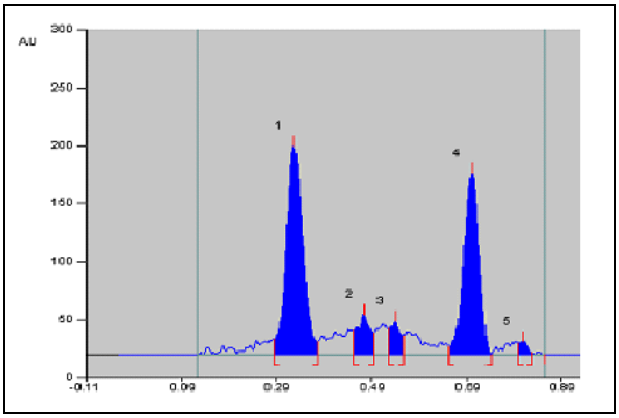

Fig. 4: Typical HPTLC Chromatogram of Oxide (3 % H_2O_2) Treated Tablet Sample

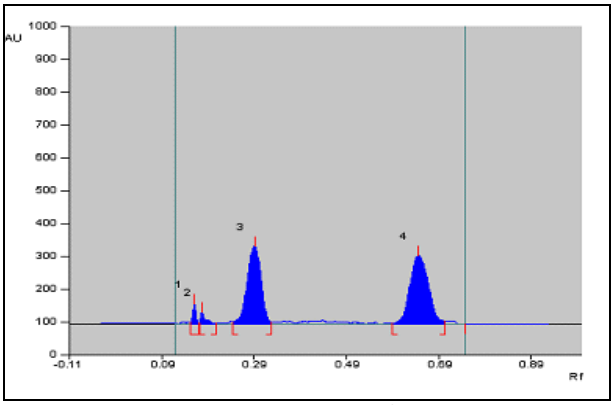

Fig. 4: Typical HPTLC Chromatogram of Alkali (0.1 M) Treated Tablet Sample

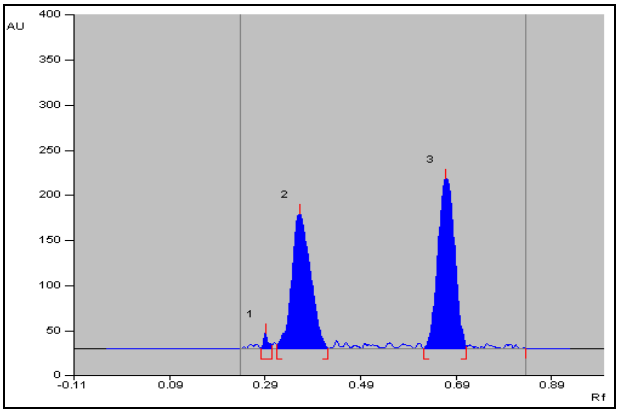

Fig. 4: Typical HPTLC Chromatogram of Tablet Sample Exposed to Neutral Hydrolysis

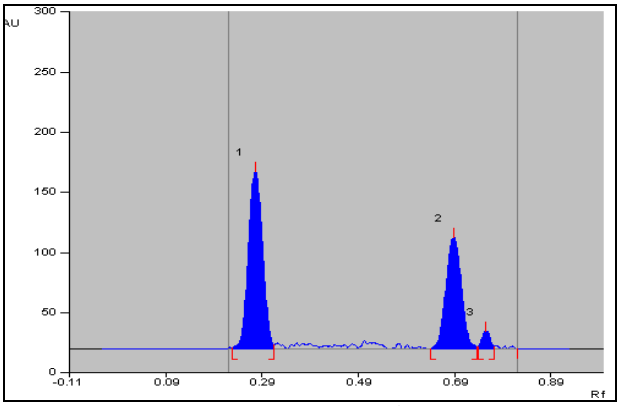

Fig. 4: Typical HPTLC Chromatogram of Heat (0.1 M) Treated Tablet Sample

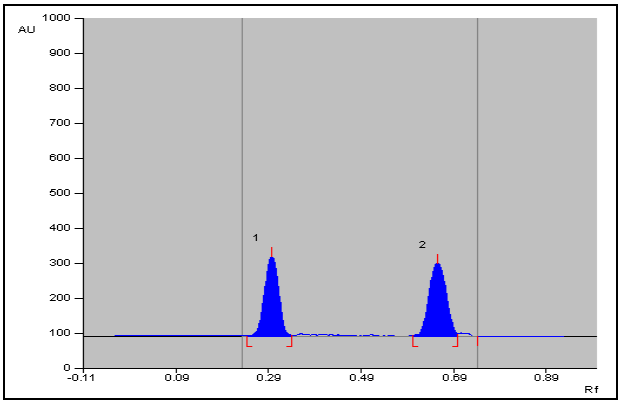

Fig. 4: Typical HPTLC Chromatogram Tablet Sample Exposed to UV Radiations

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RESEARCH ARTICLE

Comparative between Ammonia Ion Selective Electrode and Dye Binding Method to study effect of Processing Methods on Protein Content of Plain Yogurt

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ABSTRACT:
In this study, two analytical methods were used to determinate the protein, the ammonia ion selective electrode method and dye binding method using orange G and the spectrophotometer at λ_{max} 478 nm by determining the linearity, accuracy, precision, limit of detection and limit of quantitation of each. In comparison, the dye binding method was chosen for its accuracy, repeatability, sensitivity (LOD, LOQ) and speed of performance. After that, it was applied to samples of prepared plain yogurt to study effect of different properties (source, heat treatment and type) of used milk on protein content of plain yogurt.

KEYWORDS: Protein, Dye binding, Orange G, Ammonia ion selective electrode, Plain yogurt.

INTRODUCTION:

The protein content of milk is of outstanding importance. Milk proteins are of the highest quality, both in digestibility and in content of essential amino acids. The protein content of milk is a major factor in determining the nutritive value and palatability of nearly all other manufactured dairy products. It is also recognized that 80% of the nitrogen of milk is attributable to the caseins (αs1, αs2, β, κ) and 20% to whey proteins (α-lactalbumine, β-lactoglobulin, immune-globulins)1,2.

The original production of fermented milk products derived from the need to prolong the shelf life of milk instead of being disposed. Yogurt is defined as the product being manufactured from milk with a gel structure that results from the coagulation of the milk proteins, due to the lactic acid secreted by defined species of bacteria cultures3. Until a few years ago no simple and practical method was available for determining protein with sufficient accuracy to use it.

The Kjeldahl method, the official method of determining protein, is impractical for routine use, because it is so costly, complicated, and time-consuming. In the last 30 years, many approximate methods (based on diverse principles) have been proposed[2].

The dye binding method is an official AOAC procedure for determining protein in dairy products, including fluid milk, half-and-half, nonfat dry milk, ice cream mix, chocolate drink and buttermilk[4,5].

Udy first applied the dye binding procedure to the determination of flour proteins. Later, he applied the same procedure to the determination of milk proteins[6].

Orange *G* dye is a disulfonic acid which binds the basic groups of proteins near pH 2.0. Fraenkel-conrat and Cooper showed that in buffers at pH 2. 2 the acid dye, orange G, combined stoichiometric with these basic groups[7,8].

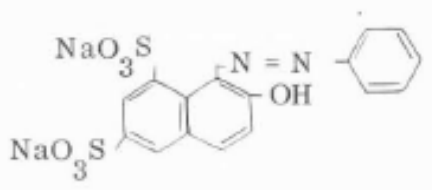

Figure 1: Structure of Orange G dye

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During the past few years there has been an active interest in potentiometry, and especially in ion-selective electrodes, to provide quantitative analytical techniques. An ammonia probe has been used to replace the distillation-titration procedure for estimating the ammonium content of Kjeldahl digest solutions. A procedure is described for estimating the total nitrogen content of barley, malt, wort and beer[9].

AIM OF STUDY:

Comparison between ammonia ion selective electrode method and dye binding method by using Orange G dye, and choosing the best one to be applied on the yogurt samples to study effect of different properties (source, heat treatment and type) of the milk used in the preparation on protein content of plain yogurt.

MATERIALS AND METHODS:

Reagents and solutions:

Dye binding method:

Orange G dye was purchased from Titan Biotech Ltd, Citric acid, Thymol. Orange G dye reagent is prepared by dissolving 1 g of the dye in a solution containing 21 g of citric acid and 2.5 ml of a 10% thymol solution in alcohol (used as a preservative). The final volume is made up to 1000 ml[6].

Ammonia ion electrode method:

Ammonium sulfate $(NH_4)2SO_4$ 99.5% (Panreac, Spain). Sulfuric acid H_2SO_4 (95-98%) (Panreac Quimica, Spain). Copper catalyst solution $CuSO_4.5H_2O$ we prepared solution (0.05 g/mL H_2O) (Rhodia, France). Sodium hydroxide solution NaOH 10 N (Panreac, Spain). Potassium sulfate K_2SO_4.

Samples: 3 samples of each cow, sheep, goat and soy milk.

3 samples of each pasteurized milk (Heating at 85 °C for 20–30 min/at 90–95 °C for 5 min)[3].

UHT milk (Heating at 145 °C for 1–2 sec)[3].

Sterilized milk (Heating at 110 °C for 30 min/at 130 °C for 40 sec)[3].

3 samples of each full fat and low fat milk

were obtained from many local markets and farms in Damascus.

Equipment:

Kjeldahl Digestion Apparatus (FOSS), NH_3 ISE Ammonia Sensing Electrode (ionode), Spectrophotometer UV-VIS model HTACHI U-1800, sensitive balance was produced by Sartorius, pipettes titration, balloons titration, filter paper 0.33 mm.

Experimental Procedures:

Preparation of Plain Yogurt:[10]

The milk samples was filtered of impurities using clean gauze. Then, the temperature of milk was gradually increased to 45 ºC for the purpose of adding 1.5 - 3% (w/v) of starter culture and stirring for 4 min, where they were kept at 42 ºC for 4 h until the completion of coagulation. Manufactured yogurt was stored in a refrigerator at 5 ºC.

Analytical methods:

1. Dye Binding method:

Polar groups in proteins can bind oppositely charged dyes to form insoluble protein-dye complexes. A known excess of dye is required, and the protein content is estimated from the amount of unbound dye. The dye concentration can be measured spectrophotometrically[7].

Determination the wavelength:

The absorbance spectrum of the Orange G solution (10 ppm) was scanned between (380-700) nm against the corresponding blank. The maximum absorbance was at 478 nm. The spectrum was shown in figure (2).

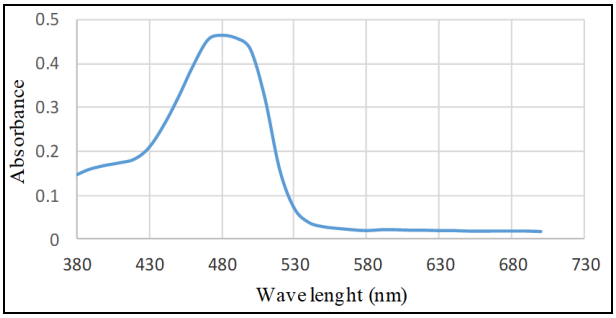

Figure 2: Determination of λ Max of Orange G dye.

Analysis procedure:[7]

A standard curve relating absorbency at 478 nm to concentration of orange G was established on serially diluted dye solutions. The operating range of this curve was between 2 - 10 ppm of dye concentration.

The yogurt sample (1 g) was mixed with 25 ml of the dye reagent. The mixture was stoppered and shaken for about 15 sec and allowed to stand for 30 min before filtering through a 0.33 mm filter paper.

The concentration of unbound dye was read from the standard curve. This value was multiplied by the total sample volume to give the total amount of unbound dye. The bound dye was found by subtracting this amount from the total amount of dye added (25 mg) .

The dye binding capacity was then calculated as the ratio of dye bound per unit of protein . From this ratio , its reciprocal was used as a factor for multiplying the amount of dye bound by unknown samples to find the amount of protein present.

The protein content of the yogurt was determined by the following formula:

Percent protein = $\frac{Q1-Q2}{DBC}$ ×100

Where: Q1 = original dye amount (25 mg)
Q2 = amount of unbound dye in filtrate (mg)
DBC = dye binding capacity (mg dye/ g protein)

2. Ammonia Ion Selective Electrode method:

A silver-silver chloride electrode is used as an internal reference electrode and the sensing electrode is a flat-ended pH electrode separated from the sample solution by a gas permeable hydrophobic membrane. The passage of ammonia through the membrane alters the pH of the thin film of ammonium chloride solution trapped between the membrane and the flat end of the pH electrode. The ammonia content of the sample will therefore determine the E.M.F produced by the cell. The response of the probe is logarithmic and follows the Nernst equation[9].

Analysis procedure:

The yogurt sample (1 g) was digested in a mixture containing sulphuric acid (20 ml) and potassium sulphate (12 g) with Copper (1 ml) as catalyst in order to convert the amino-nitrogen of the organic materials into ammonium hydrogen sulphate. The sample was then diluted with distilled water to 100 ml and made alkaline using sodium hydroxide 10 N (2 ml) and the ammonia released measured with the ammonia ion-selective electrode[11,12].

RESULTS AND DISCUSSION:

Validation of the two analytical methods:

Linearity:

Five-level protein series was established for known protein concentration diluted yogurt. The absorbance was measured. The absorbance is plotted against the protein amount.

Eight-level standard series was established for ammonium ion. The practical concentration was measured. The practical concentration is plotted against the theoretical concentration.

Both methods had good linearity as shown in (Fig. 3,4).

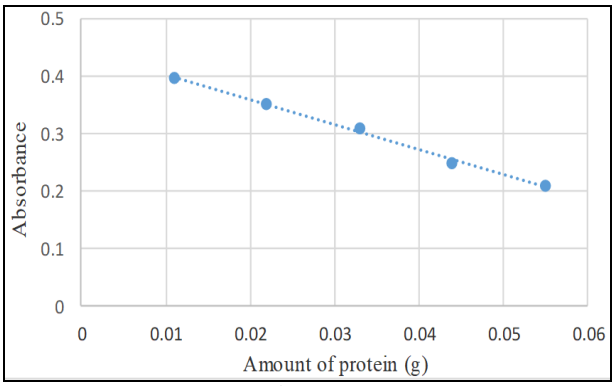

Figure 3: Calibration Curve of dye binding method

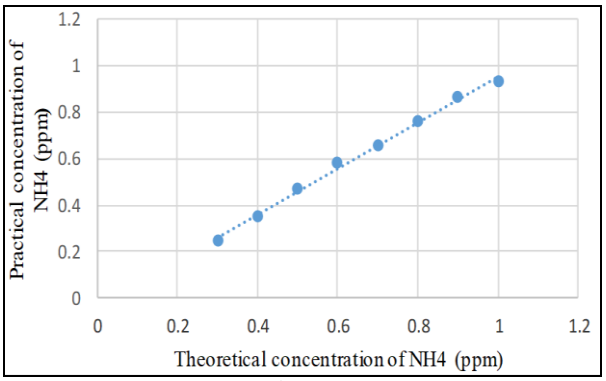

Figure 4: Calibration Curve of ammonia ion selective electrode

Accuracy:

Accuracy should be reported as relative error by measuring three known concentrations of protein (0.022, 0.033, 0.044 g) and ammonium ion (0.4, 0.6, 0.8 ppm).

Precision:

The relative standard deviation RSD% (coefficient of variation) were reported by measuring six solutions at the same concentration (0.022 g ptotein) and (0.6 ppm ammonium ion).

Table 1 shows all two validation results.

Dye binding method was chosen to apply it to samples because of its good linearity, accuracy and precision. It is also simple to operate. Other than being rapid (30 min), the dye binding method has the advantage of directly estimating the protein content in the sample rather than the ammonium content as measured by ammonia ion electrode procedure.

Table 1: Results of analytical methods validation

Parameters	Dye binding method	Ammonia ion selective electrode
Linearity	R^2 =0.9971 y = -4.3636 x + 0.448	R^2 =0.9957 y = 0.9909 x - 0.0345
Accuracy (Relative error)	2.02%	4.87%
Precision (RSD%)	0.97%	2.25%
LOD ppm	3.54 mg protein	0.28 ppm protein
LOQ ppm	10.74 mg protein	0.85 ppm protein
Time	30 min	2.5 h

Effect of source of used milk:
The average protein content of sheep, goat, cow and soy yogurt were 6.71, 3.04, 3.5 and 3.28%, respectively [Table (2)].

The differences in protein contents of yogurt may be due to the gross composition of milk shows large inter-species differences, Because the nutritional requirements of the neonate depend on its maturity at birth, its growth rate and its energy requirements, which depend mainly on environmental temperature[1].

Milk composition traits of sheep and goat milk from 10 studies show that sheep milk is richer in protein than goat milk[13].

Sheep yogurt has higher protein content and good textural characteristics (firmness and viscosity) than cow, goat and soy yogurt, while Goat yogurt has lower protein content and present poor textural characteristics (weak gel), which may be due to has lower amounts of αs1-casein, resulting in softer gel products, a higher water holding capacity and a lower viscosity[14].

Although soy yogurt has low protein content, it has good texture (hard gel). It may be due to soy proteins have many of the chemical and physical properties required for use in the dairy industry, which contributes to increased viscosity and gel strength[15].

Table 2: The protein content of samples

Source of milk	Sample 1	Sample 2	Sample 3
Sheep	6.71	6.7	6.72
Goat	3.06	2.98	3.1
Cow	3.5	3.52	3.48
Soy	3.25	3.29	3.31

Effect of heat treatment of used milk:
The average protein content of pasteurized, UHT and sterilized yogurt were 3.496, 3.49 and 3.473%, respectively [Table (3)].

The protein content of the different yogurt samples is generally close to each other, and this is due to The caseins (present 80% from milk protein) are very heat-stable. Milk may be heated at 100°C for 24 h without coagulation and withstands heating at 140°C for up to 20- 25 min. The heat stability of the whey proteins (present 20% from milk protein) is typical of globular proteins and they are denatured completely on heating at 90°C for 10 min. The remarkably high heat stability of the caseins, which is probably due to their lack of typical stable secondary and tertiary structure[1,16].

The reason may be to the protein content of sterilized yogurt samples are less because sterilization causes considerable changes in the proteins including casein and whey proteins, while pasteurization and UHT cause only denaturation in whey proteins[17].

Table 3: The protein content of samples

Heat treatment of milk	Sample 1	Sample 2	Sample 3
Pasteurization	3.51	3.5	3.48
UHT	3.49	3.51	3.47
Sterilization	3.47	3.49	3.46

Effect of type of used milk:
The average protein content of full fat and low fat yogurt were 3.49 and 3.65, respectively [Table (4)].

Low fat yogurt has higher protein content and has good texture (hard gel) than full fat yogurt, which is probably due to the cutting of the protein network, which form the gel, by the contained fat globules[16].

These results agree with the study of Guinee and O'callaghan which studied effect of fat level on properties of processed cheese product[18].

Table 4: The protein content of samples

Type of milk	Sample 1	Sample 2	Sample 3
Full fat	3.52	3.48	3.47
Low fat	3.65	3.66	3.64

CONCLUSION:
As shown in this study, both of ammonia ion selective electrode and dye binding method has good linearity, accuracy, precision and Sensitivity but the dye binding method is rapidly (30 min) determine protein and does not need additional steps like digestion which takes more than 2 h.

The protein content in yogurt is influenced by source and type of used milk, while it is not significantly affected by the type of heat treatment.

CONFLICT OF INTEREST:
The authors declare that there is not any conflict of interest related to this work.

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RESEARCH ARTICLE

Development and Evaluation of Herbal Mosquito Repellent Cream

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ABSTRACT:
Mosquito repellent is a product that is applied to the skin or other surfaces to deter insects (and arthropods in general) from landing or climbing on that surface. The interest in plant-based repellants has been revived seeing that the development of resistance, cross-resistance and possible toxicity hazards associated with synthetic insecticides and their rising cost. The present study aimed towards the development and evaluation of safe and efficient herbal mosquito repellent from leaf extracts of *Azadirachta indica*, *Vitex negundo*, and *Ocimum gratissimum*. Six different types of formulation (Type-1 to 6) were prepared using each plant extract and optimized ingredients. The mosquito repellent activity of the formulation was tested using the arm-in-cage method. One skin rubbed with a small quantity of formulation was exposed to 20 mosquitoes and the number of mosquitoes that aligned or biting the arm was recorded in each minute for five minutes. The most effective repellent activity of the Type2 formulation was 87.5% (Y-shaped Model) and 91.62% (Rectangular-shaped Model). The Type1, Type3, Type4, Type5 and Type6 formulation showed 66.62% , 58.25%, 68.75%, 70.75% and 62.5% repellent activity in Y shaped model and 66.62%, 62.5%, 70.75%, 83.25% and 62.5% repellent activity in Rectangular shaped model respectively. During these studies, we found that Hexane extract showed higher repellent activity. No allergic reactions were observed upon the skin. So, it is a safe product. The formulation was ecological, economical and highly efficient.

KEYWORDS: Herbal medicine, Mosquito repellent, Cream, *Azadirachta indica*, *Vitex negundo*, *Ocimum gratissimum*.

INTRODUCTION:
The world is eventually turning to herbal formulations which are known to be effective against a large number of diseases and ailments[1]. However, adding an appropriate recognition stating that plant remedies are efficacious without having adverse affects. Many of the herbs and shrubs are found to have medicinal as well as curative values along with mosquito larvicidal and mosquito repellent properties. Because the application of synthetic liniment has a poignant effect on the surroundings as well as non-target organisms; products of plant origin with insecticidal properties have been tried as an aboriginal method to control several insect pests and vectors in the recent past[2].

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Before synthetic ointments came into use, plants and their derivatives were being used to kill the pests of agriculture, veterinary and public health importance. The use of plant-based embrocation is encouraged as they are comparatively less detrimental to the environment, biodegradable and have minimal or no use of a synthetic chemical which may result in causing a health hazard to the user[3]. Moreover, many potent plants having biologically active constituents that can lead to the discovery of new insecticidal preperations[4]. Therefore plant materials have attracted significant interest in mosquito control programs in recent times[5].

Plant derivatives such as creams, essential oils, coils, incense, mist based products having plant-based derivatives have shown up in markets with high demand as compared to synthetic products. This has provided researchers with new scopes in the field to come up with more plants possessing mosquito repellent properties. Many polyherbal formulations having added qualities

have also become the central element for recent research in this sector.

A mosquito repellent is a substance applied to skin, clothing or other surfaces that rejects insects (and arthropods in general) from infecting that surface. As most of the mosquito repellent products and devices are either of synthetic origin or have an evident effect on human health leading to several complications, the need for a repellent that is more effective and less harmful has arisen[6,7].

Substantial efforts have been made to expound and persuade the use of environmentally friendly insecticides that are not enduring, have low toxicity against the fauna and are moderately safe. Since time immemorial, insect repellent formulations have been found to possess spirits of camphor[9], oil of tar[9], oil of Pennyroyal and castor oil[9] in their composition along with having effective results against the insects and arthropods.

From the Vedic era, various plants like *Vitex negundo*[10,11,12,13], *Ocimum species*, *Azadirachta indica*[14] are used as mosquito repellents or are used as mosquitocidal. Ramasamy R 2001 *et. al.,* performed the development of mosquito repellent fabrics using nanoparticles loaded with *V. negundo* leaf extract. The nanoparticles were loaded with *V. negundo* leaf extract using a traditional pad-dry method**.** The resultant fabrics showed 100% mosquito repellent effectiveness[15]. Ranasinghe M.S.N 2014 *et. al.*, reported that the hexane extract of *Azadirachta indica* seeds, hexane extract of *V. negundo* leaves, essential oils from *Ocimum sanctum* leaves, are having promising mosquito repellent activities[16].

Based on the present market scenario of mosque repellants and based on ethnomedicinal claims the present study was aimed to develop and evaluate an herbal mosquito repellent cream incorporating leaf extracts of *Vitex negundo, Ocimum gratissimum* and *Azadirachta indica.*

MATERIAL AND METHODS:

Collection of the plant material:

The *Vitex negundo, Ocimum gratissimum and Azadirachta indica* were collected from different village area of the Dhemaji District of Assam during the month of June-July, 2016. The fresh lives were identified by the Department of Botany, A.D.P. College, Nagaon.

Preparation of the plant extract:

The plant material of *Vitex negundo, Ocimum gratissimum and Azadirachta indica* were thoroughly washed with clean water and allowed for drying at 40ºC in a hot air oven for 3 days. The dried material was crushed in a mixture grinder to a fine powder.

Soxhlet extraction:

The leaf powder of *Vitex negundo* and *Azadirachta indica* were subjected to soxhlet extraction separately using solvent diethyl ether, hexane and ethanol according to their polarity. The extraction was carried out fora duration of 4 to 5 hours. This process was repeated third times and the obtained extracts were pooled for evaporation in a water bath at 60ºC for obtaining residue. The obtained residues were kept in a Petri dish and stored in the refrigerator at 40ºC. The leaf of *Ocimum gratissimum* was washed, shade dried and then chopped in to crush powder using a mixer. 40g of the powder was filled in the thimble and extracted successively with Water, Methanol and Chloroform using a Soxhlet extractor for 48hrs. All the extracts were concentrated using a rotary evaporator and stored at 4ºC in a refrigerator.

Preparation of optimized ingredients:

For the preparation of mosquito repellent cream, coal powder and cow dung[17] has been selected as a base and Flour (Maida)[17] have been selected as a binder. To impart porosity to the strongly bonded mixture and to ease continued burning, rice grain and sawdust have been used. The ingredients formula for the mosquito repellent was as expressed in Table 1.

Table1: The constituents in the mosquito repellent cream

Sl. No.	Ingredients	Weight Percentage
1	*Azadirachta indica* leaf extract	7.77%
2	*Ocimum gratissimum* leaf extract	3.0%
3	*Vitex negundo* leaf extract	3.0%
5	Rice dust	4.35%
6	Cow dung / Coal powder	40.4%
7	Flour (Maida)	6.35%
8	Peppermint oil	7.3%
9	Vegetable ghee	8.0%
10	Glycerin	10%

Preparation of Repellent testing Model:

A rectangular-shaped (43×15×18cm) and a Y shaped (21×10×10cm) mosquito repellent cages were prepared.

Preparation of the cream:

Table 1 summarizes the common heuristics of incorporation sequence and method used for the preparation of cream. As the majority of the heuristics are based on common practices, they can be derived from the basic information of the principal phenomenon of emulsion preparation. The extracts were mixed with cow dung and coal powder separately. The above-mentioned amounts of optimized ingredients are added where moida were used as a binder and peppermint oils were used as perfume. A small amount of Petroleum jelly was added to the formulation with continuous stirring at 70ºC. Then cream was estimated for various physical limitations[17]. Though the same plants were

extracted with different solvents thus plant extracts were named accordingly:

- DEEAI+DEEVN+MEOG
- HEEAI+HEVN+MEOG
- EEAI+EEVN+MEOG

(*****DEEAI= Diethyl ether extract of *Azadirachta indica*, DEEVN= Diethyl ether extract of *Vitex negundo*, HEEAI=Hexane extract of *Azadirachta indica*, HEVN= Hexane extract of *Vitex negundo*, MEOG=Methanolic extract of *Ocimum gratissimum*, EEAI= Ethanolic extract of *Azadirachta indica*, EEVN= Ethanolic extract of *Vitex negundo*.)

Six different formulations were prepared from the above extract which was based on cow dung and coal powder.

Table2: Types of Mosquito repellent formulations

Sl no	Types		Formulations
1	Type 1	Cow dung based	DEEAI+DEEVN+MEOG+ OI
2	Type2		HEEAI+HEVN+MEOG+ OI
3	Type3		EEAI+EAVN+MEOG+ OI
4	Type4	Coal powder based	DEEAI+DEEVN+MEOG+ OI
5	Type5		HEEAI+HEVN+MEOG+ OI
6	Type6		EEAI+EAVN+MEOG+ OI

Characterization and Optimization of Cream Formulation:

Different trial products of the cream formulation were characterized and optimized based on their aesthetic appearance, emulsification, pH, and consistency taking into account softness, greasiness, and stickiness. Sensorial observations including aesthetic appearance and consistency were assessed by ten observers[18,19,20].

Aesthetic Appearance:

The prepared cream must be aesthetically elegant in terms of its physical appearance, color, odor, and texture. Therefore, the cream formulations were subjected to sensorial observations[20].

Emulsification:

Indecent emulsification usually brings on phase separation/cracking and precipitation. Consequently, the batches were observed for fine emulsification, which leads to an elegant product[20].

pH Determination:

A distinct amount of cream (100 mg) was weighed, diluted in distilled water, and mixed well. The pH of the cream was recorded using Digital pH Meter (Mettler Toledo). pH evaluation was carried out for all experimental formulations. The measurement was carried out in triplicate[20].

Consistency:

Each batch of the cream was evaluated for its consistency by examining its softness, greasiness, and stickiness. The formulation should be of uniform consistency which could spread and soften easily when stress is applied. It must also be nongreasy and nonsticky.

Stability Studies:

In accordance with International Conference on Harmonization (ICH) guidelines, stability analyses of optimized formulations were performed. The optimized cream formulations were stored in tightly closed glass containers for 90 days at 25°C temperature and 60% relative humidity in a humidity chamber. At predestined intervals, 0, 30, 60, and 90 days, samples were collected and their physicochemical evaluation parameters such as color, consistency, phase separation, texture analysis, and pH were evaluated[20].

Spreadability:

Spreadability was determined by the apparatus which consists of a wooden block, which was provided by a pulley at one end[21]. By this method, spreadability was measured based on the slip and drag characteristics of cream. About 1 gm of cream was placed on the ground slide. The cream was then sandwiched between this slide and another glass slide having the dimension of the fixed ground slide and provided with the hook. The top plate was then subjected to a pull of 100gm. With the help of string attached to the hook and the time (in seconds) required by the top slide to cover a distance of 7.5cm be noted[22,23].

Spread ability was determined by using formula-

$S = ML/T$

Where, S = Spreadability,

M = Weight tied to upper slide,

L = Length of a glass slide and

T = Time taken to separate the slides from each other.

Evaluation of mosquito repellent activity:

Mosquito repellent activity was assessed by using the home-made test cage as described in the American Society for Testing and Materials (ASTM) standard E951-83 Laboratory testing of non-commercial mosquito repellent formulation on the skin. The formulations to be tested were applied to the skin.

The mosquitoes used in this experiment were caught using a net while biting humans between 7pm and 10pm. Mosquitoes were starved for 24hours and 20 mosquitoes were placed in the cage (43×15×18cm). Test timing was between 6pm and 9pm since the mosquitoes typically bite at night. The host-seeking behavior of the mosquitoes was tested before the experiment. This was done by introducing a pre-cleaned skin in the cage and counting the number of mosquitoes that allied within 10seconds. The skin which had been rubbed with 1 gm

of the formulated herbal repellent was exposed to the cage and the number of mosquitoes that aligned or biting the arm was recorded each minute for 5minutes. Mosquitoes were given an over one-hour interval and the above procedure was followed for each of the other plant extracts and essential oils. Each test was replicated thrice to get a mean value of mosquito repellent action.

For a repellent to be successful, it must first have a high percentage of protection against mosquito bites. Second, it should be toxicologically safe at the rate of application for which it is intended. Third, it should be easy to apply and pleasant on skin[24].

Percentage the mosquito repellency[25]

Percentage the mosquito repellency for the plant extract shown in Table 2 was calculated as below,

$$\% \text{ of mosquito repellency} = \frac{C - N}{C} \times 100$$

Where,

C = Number of Mosquitoes aligned/left and aligned/bit when the Standard was used.

N = Number of mosquitoes align/left and aligned/bit when the extract was used.

Statistical Analysis:

Data are presented as mean ± standard error mean (SEM). Data for different types were compared using one-way analysis of variance (ANOVA). All statistical analyses were performed using GraphPad Prism, Version 5.01 (GraphPad software. Inc., USA).

RESULTS:

Formulation Parameters:

Table 3: Formulation parameters of Cow dung based and Coal powder-based Herbal Mosquito Repellent (Type1 to Type6)

	Batch	Homogeneity	Skin irritation
Coal Powder Based	Type1	Good	Nil
	Type2	Good	Nil
	Type3	Good	Nil
Cow Dung Based	Type1	Good	Nil
	Type2	Good	Nil
	Type3	Good	Nil

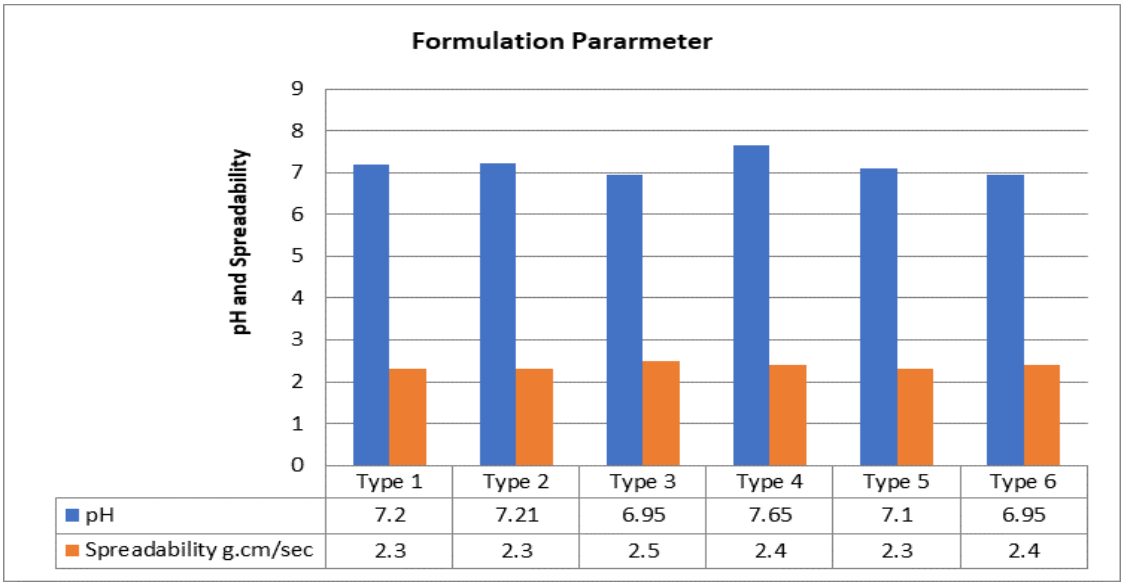

Fig 1: pH and Spreadability of formulated Herbal Mosquito Repellent (Type1 to 6)

Analysis of Mosquito Repellent activity

Table 4: Mosquito Repellency (Percentage) of the formulated Mosquito Repellent cream (Rectangular Shaped Model)

Batch	Replicate1 Total no of mosquitoes aligned/left and aligned/bite	Replicate2 Total no of mosquitoes aligned/left and aligned/bite	Replicate3 Total no of mosquitoes aligned/left and aligned/bite	The calculated mean value of aligned/left and aligned/bite	Percentage of Mosquito repellency
Type1	3	3	2	2.67	66.62%
Type2	1	0	1	0.67	91.62%
Type3	4	2	3	3	62.5%
Type4	2	3	2	2.34	70.75%
Type5	2	1	1	1.34	83.25%
Type6	4	3	3	3	62.5%

Table 5: Mosquito Repellency (Percentages) of the formulated Mosquito Repellent cream (Y-Shaped Model)

Batch	Replicate 1	Replicate2	Replicate3	The calculated mean value of aligned/left and aligned/bite	Percentage of Mosquito repellency
	Total no of mosquitoes aligned/left and aligned/bite	Total no of mosquitoes aligned/left and aligned/bite	Total no of mosquitoes aligned/left and aligned/bite		
Type1	3	2	2	2.67	66.62%
Type2	1	2	0	1	87.5%
Type3	5	3	2	3.34	58.25%
Type4	2	1	2	2.5	68.75%
Type5	3	2	1	2.34	70.75%
Type6	4	2	3	3	62.5%

Table 6: Mosquito Repellent activity (Percentages) of the Mosquito Repellent cream (Rectangular-Shaped Model)

Batch	Number of Mosquito Tested		No any action		Mosquito migrate away		Mosquito paralyzed		Remark
	Total	%	No.	%	No.	%	No.	%	Repellence
Type1	20	100%	08	40%	08	40%	04	20%	Low repellency
Type2	20	100%	02	10%	06	30%	12	60%	High Repellency
Type3	20	100%	08	40%	09	45%	03	15%	Low repellency
Type4	20	100%	05	25%	07	35%	08	40%	Low repellency
Type5	20	100%	05	25%	04	20%	11	55%	High Repellency
Type6	20	100%	08	40%	09	45%	03	15%	Low repellency

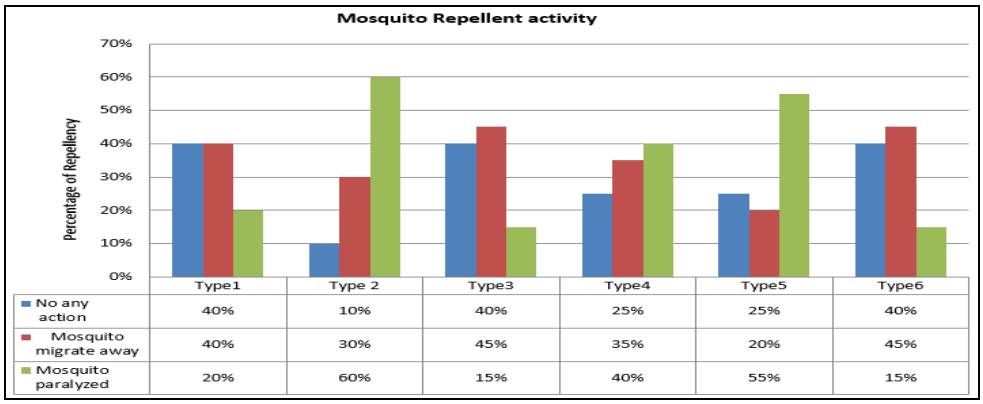

Fig2: Standard Curve for percentage of mosquito repellent activity in Rectangular Shaped Model

Table 7: Mosquito Repellent activity (Percentages) of the Mosquito Repellent cream (Y-Shaped Model)

Repellent Sample	Number of Mosquito Tested		No any action		Mosquito migrate away		Mosquito paralyzed		Remark
	Total	%	No.	%	No.	%	No.	%	Repellency
Type1	20	100%	07	35%	08	40%	05	25%	Low Repellency
Type2	20	100%	04	20%	04	20%	12	60%	High repellency
Type3	20	100%	08	40%	08	40%	04	20%	Low Repellency
Type4	20	100%	05	25%	07	35%	08	40%	Low Repellency
Type5	20	100%	05	25%	04	20%	11	55%	High Repellency
Type6	20	100%	07	35%	09	45%	04	20%	Low Repellency

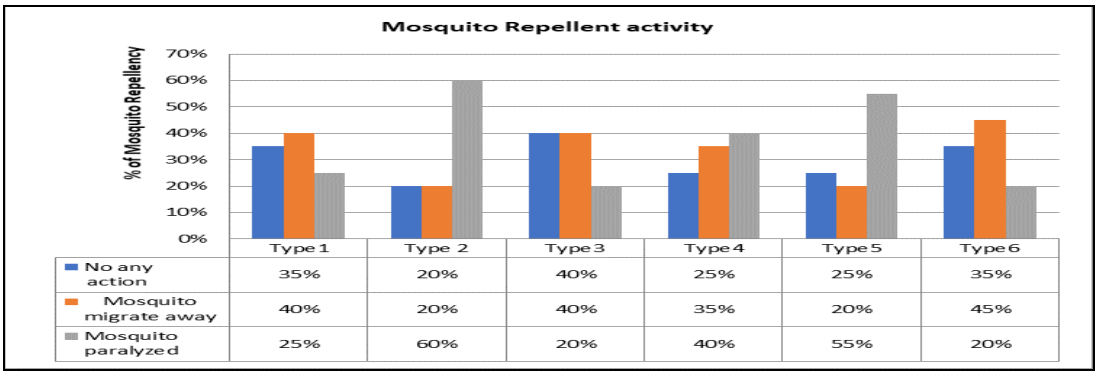

Fig3: Standard Curve for a percentage of mosquito repellent activity in Y Shaped Model

DISCUSSION:

Many researchers have found that plants *Vitex negundo, Ocimum gratissimum* and *Azadirachta indica* have better repellent efficacy rather than other plant materials[25,27]. However, a single plant extract obtained from plants contributes to poor longevity as mosquito repellents. Therefore, three plant extracts of the *Vitex negundo, Ocimum gratissimum* and *Azadirachta indica* are suitable to be used as a polyherbal mosquito repellent.

Preliminary batches of formulated Mosquito repellent cream were characterized for their elegancy, emulsification, spreadability, stickiness and consistency based on sensorial assessment. The cream was off-white in color and opaque with a homogeneous appearance (Table 3).

All prepared formulations had nearly constant pH, homogeneous, emollient, non-greasy and were easily eliminated after the application. The pH values of all developed formulations were found to be in the range of 6.95 to 8 (Fig 1). Under Stability Studies Color, consistency, viscosity, texture profile, and pH of the prepared creams were found to be consistent, and no separation was observed throughout a 90-day study, which revealed the reproducibility of the physical and chemical parameters which ensures the consistent quality of the developed cream formulation.

In the present investigation, repellent activities of Type1 to Type 6 formulations of *Azadirachta indica*, *Vitex negundo* and *Ocimum gratissimum* extracts were determined by using the Arm-in-Cage Model. In the arm-in-cage method experiment, although it appears that at 5minute exposure times at hourly intervals, the number of aligning and biting mosquitoes was generally reduced, the reduction is probably due to the satisfaction of the mosquitoes being sufficiently fed as time and exposure go up.

In this study, most of the formulated types of mosquito repellent have been shown to possess satisfactory repellency effects against insects. The remarkable activity was observed in the case of Type2 formulation. The most effective repellent activity of the Type2 formulation was 91.62% in Rectangular-shaped Model and 87.5% in the Y shaped Model. The Type1, Type3, Type4, Type5 and Type6 formulations showed 66.62%, 62.5%, 70.75%, 83.25% and 62.5% repellent activity in Rectangular shaped model (Table 4) and 66.62, 58.25%, 68.75%, 70.75% and 62.5% repellent activity in Y shaped model (Table 5) respectively.

Ranasinghe MSN *et. al.,* 2016, reported on the repellent activity of the different types of plant extracts and essential oils. They found that all the essential oil show more repellency than plant extracts. During our study, it was found that the Type2 formulation (cow dung based) have shown higher repellent activity than Type1 and Type3 formulation of cow dung based. Similarly, Type5 formulation (coal powder-based) has shown higher repellent activity than Type4 and Type6 formulation of coal powder-based (Table 6, Fig 2)[16]. Pandharbale AR 2007, evaluated the mosquito repellent activity of *Azadirachta indica* and *Ocimum gratissimum.* They found that Tulsi oil showed higher mosquito repellency than Neem leaf extract[24]. Gaddaguti V *et. al.,* 2016, reported about Potential mosquito repellent compounds of *Ocimum* species against 3N7H and 3Q8I of *Anopheles gambiae.* They have found that 12 out of 35 compounds of the two *Ocimum* species were found to be idyllic for the intend and development of possible mosquito repellents. And they have claimed that herbal ingredients of *Ocimum* species can be considered for formulation for mosquito repellents[27].

No skin irritations or rashes were detected on the arms of the test volunteers' with extracts, essential oils and the prepared mosquito repellent formulations. All formulations were safe concerning skin irritation and allergic sensitization as the primary irritation index was found to be zero, and there was no report of any edema or redness. However, a hot sensation was observed on the arms of the test volunteers with Type 1. Further improvements for the mosquito repellent formulations were redesigned by reducing the concentration of Type 1 ingredients to the level where no hot sensation was observed. The mosquito repellent formulations from plants are exceptionally fruitful due to the prosperity of insecticidal ingredients found in plants as defenses adjacent to insects[28]. Repellents have an important place in protecting man from the bites of insect pests. An effective repellent will be useful in reducing man vector contact and in the interruption of disease transmission[28,29]. The *Azadirachta indica*, *Vitex negundo* and *Ocimum gratissimum* not only has good mosquito repellent/mosquitocidal activity but also it has good odour characteristic and the effect was due to presence of various active ingredients in these plants[30,31].

CONCLUSION:

During this study, mosquito repellent activity of ingredients of polyherbal showed that the product was very efficient and safe to use. Statistically, it showed that the formulated product is very good and safe for use. During this study, we found that Hexane extract showed higher repellent activity. No volunteers complain about allergic consequences. So, it is a safe product. The formulation was ecological, economical and pocket friendly. Further investigations are needed to elucidate the efficacy of the herbal mosquito repellent

formulations against a wide range of mosquito species and also to identify active compounds responsible for mosquito repellent activity to utilize them if necessary, in preparing a commercial product to be used as a mosquito repellent.

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CONFLICT OF INTEREST:

The authors declare that they have no conflict of interest. All the tables and figures are self-made and original.

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RESEARCH ARTICLE

The Role of Andrographolide in *Andrographis paniculata* as a Potential Analgesic for Herbal Medicine based Drug Development

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ABSTRACT:

Background: *Andrographis paniculata* is a herbaceous plant in the Acanthaceae family, that is widely used as a traditional medicine in Asian countries and known to exhibit a wide range of pharmacological effects. Recent studies have provided an overview of the great potential of *A. paniculata* as an analgesic. The ethanol extract and ethyl acetate (EA) fraction of *A.paniculata* were shown to contain diterpene lactone compounds, which may be useful as a potential active ingredient in analgesic drugs. The development of a herbal medicine based drug requires an effective and high quality active ingredient. Therefore, this research was aimed to compare the analgesic activity of ethanol extract and EA fraction based on their andrographolide content and further to determine the more viable active substance for analgesic herbal medicine based drug development. **Method:** The andrographolide content in the ethanol extract and EA fraction was determined by High Pressure Liquid Chromatography (HPLC). Measurement of analgesic activity was performed by writhing test. The experimental animals were randomly divided into eight groups consisting of 5 mice in each. Group 1 (negative control) received 1% Tween-80 in normal saline. Group 2 (positive control) received a standard analgesic drug (diclofenac sodium) at a dose of 40 mg/kg body weight. Group 3, 4, and 5 received ethanol extract while Group 6, 7, and 8 received EA fraction, each at a dose of 12.5, 25, and 50 mg andrographolide/kg body weight, respectively. Each mouse was injected intraperitoneally with 1% acetic acid at a dose of 10 ml/kg body weight 30 minutes after oral administration of the treatments. The number of writhes were counted 5 min after acetic acid injection over a period of 45 min. **Results:** Andrographolide content in ethanol extract and EA fraction was 15.66±0.28 and 21.25±1.08 % w/w, respectively. Ethanol extract and EA fraction displayed analgesic activity of 67.68% and 70.91% respectively, at a dose of 50 mg andrographolide/kg body weight. The positive control at a dose of 40 mg/kg body weight showed an analgesic activity of 74.33%. Statistical analysis showed no significant differences between EA fraction at a dose of 50 mg andrographolide/kg body weight and ethanol extract at the same dose as well as the positive control ($P > 0.05$). The effective dose 50% (ED_{50}) of the ethanol extract and EA fraction was determined to be 29.49 and 25.55 mg/kg body weight, respectively. **Conclusion:** It was possible to use andrographolide content as an indicator for the analgesic activity of *A.paniculata*. Ethanol extract and EA fraction of *A. paniculata* at the same dose of andrographolide showed similar analgesic activity. The amount of ethanol extract which needed to reach similar analgesic activity was higher than EA fraction. Therefore, EA fraction likely has greater potential as an analgesic active substance due to its higher content of andrographolide; however further study is needed to develop it as a dosage form.

KEYWORDS: *Andrographis paniculata*, extract, ethyl acetate fraction, analgesic activity.

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INTRODUCTION:

Pain and inflammation are a defense mechanism of body to initiate the tissue healing process. It is a common nonspecific manifestation of many diseases[1]. Drugs

presently used for the management of pain and inflammatory conditions are narcotics (e.g., opioids), non-narcotics (e.g., salicylates) or corticosteroids (e.g., hydrocortisone). All of these drugs cause well known side effect, such as a low potency, and toxic effects, such as renal failure, allergic reactions, and occasionally hearing loss while also potentially increasing the risk of haemorrhage by affecting platelet function[2,3]. The use of nonsteroidal substances such as aspirin is also increasing due to its utility in reducing the incidence of a number of common disorders including stroke, myocardial infarction and cancer[4]. Therefore, the discovery of alternative substances to treat pain is crucial.

Plants are important sources of new drugs. Many plants were reported to have a potential analgesic activity including *Solanum surattense*, *Plumbago indica*, *Clerodendrum inerme*, *Lagenaria siceraria*, *Cuscuta reflexa*, *Alpinia conchigera*, *Sida Spp* and *Andrographis paniculata*. These plants extracts were showed inhibition on acetic acid induced writhing on mice compared to aspirin or diclofenac sodium as a positive control[5-12].

Andrographis paniculata is a herbaceous plant in the Acanthaceae family that is widely used as a traditional medicine in Asian countries[13]. This plant is known to have pharmacological effects, such as anti-inflammatory, antibacterial, antioxidant, anticancer, antidiabetic, antimalarial, hepatoprotective, immunostimulant, allergic, analgesic, and antipyretic activity[14-17]. Several studies have been conducted to determine the analgesic activity of *A. paniculata*. Previous investigations reported that the aqueous extract of *A. paniculata* at a dose of 100 mg/kg and 500 mg/kg per orally, significantly reduced (P<0.01) acetic acid induced writhing in both acute and chronic examinations. The test drug at a dose of 100 mg/kg and 500 mg/kg fared better than the standard drug (aspirin) at 150 mg/kg in acute cases[12]. The ethanol extract of *A. paniculata* is known to display an analgesic activity of 34%[18]. Andrographolide has also been shown to exhibit analgesic activity at a dose of 4 mg/kg, when given intraperitoneally[19]. These studies provide an overview of the significant potential of *A. paniculata* as an alternative analgesic drug.

In consideration of these results, *A.paniculata* has been identified as a prospective plant than could be used to address the current need for alternative analgesic drugs. Although many reports on the analgesic activity of *A. paniculata* have been published, little attention has been directed towards the development of analgesic herbal medicines based on effective and high quality active ingredients. The ethanol extract and ethyl acetate (EA) fraction of *A.paniculata* have been shown to contain diterpene lactone compounds and importantly andrographolide as a potential active substance. Therefore, this research was aimed to compare the analgesic activity of ethanol extract and EA fraction based on their andrographolide content and to further determine the more viable active substance for analgesic drug development.

MATERIALS AND METHODS:

Plant Material:

The plant material used in this research was *A. paniculata* dried powder containing 1.82% andrographolide (PT. Kimia Farma Tbk). Andrographolide standard was purchased from Sigma-Aldrich (Cat No.365645-100MG).

Preparation of the ethanol extract and ethyl acetate (EA) fraction:

A.paniculata dried powder was extracted by maceration method using ethanol 96% as a solvent. The liquid ethanol extract was then evaporated using a rotary evaporator. The concentrated extract was then further separated *via* liquid-liquid fractionation using ethyl acetate and water (1:1 v/v) to obtain the ethyl acetate (EA) fraction.

Determination of andrographolide content:

Analysis of andrographolide content in the extract and EA fraction was conducted by High Pressure Liquid Chromatography (HPLC) method. The analysis used an Agilent HPLC system, Poroshell RP-18 column (4.6x250 mm, 5µm), mobile phase methanol:water pH 3.05 (50%: 50% v/v) and a flow rate of 1 ml/min. The extract and EA fraction were weighed out (10 mg) and dissolved in 10 ml of methanol. Standard working solutions was made at concentrations of 100, 200, 400, 800, and 900 ppm in methanol, taking into account the 98% standard potential. The sample and standard solutions were filtered using a filter membrane with a pore size of 0.22 µm. The solutions were subsequently inserted into an auto sampler and injected into the HPLC system. Andrographolide content in the extract and EA fraction was then determined based on the chromatogram peak areas.

Animals:

This study used male mice BALB/C strain (25–30 g), were maintained on standard animal pellets and water *ad libitum* at the Animal Laboratory of the Institute of Tropical Disease, Universitas Airlangga, Surabaya. Animals were kept at constant temperature (25 ± 1°C) and underwent a regular 12/12 h light/dark cycle, while having free access to standard laboratory feed and water. Permission and approval for animal studies were obtained from the Faculty of Veterinary Medicine, Universitas Airlangga with the approval code 753-KE.

Evaluation of analgesic activity by acetic acid induced writhing test:

The mice were divided into eight groups (n = 5). Group 1 (Negative control) received 1% Tween-80 in normal saline. Group 2 (Positive control) received a standard drug (diclofenac sodium) at a dose of 40 mg/kg body weight. Group 3 to Group 8 were treatment groups. Group 3 to Group 5 received ethanol extract at a dose of 12.5, 25, and 50 mg andrographolide/kg body weight respectively. Group 6 to Group 8 received EA fraction at doses of 12.5, 25, and 50 mg andrographolide/kg body weight respectively. All treatments were administered orally. Each mouse was injected with 1% acetic acid intraperitoneally at a dose of 10 ml/kg body weight, 30 minutes after administration of the standard drug and test samples. The number of writhes (constriction of abdominal muscles along with the stretching of hind limbs) were counted 5 min after acetic acid injection over a period of 45 min. The percentage of analgesic activity was calculated as follows[20].

$$\% \text{ Analgesic activity} = \frac{\text{N negative control - N test}}{\text{N negative control}} \times 100$$

Where N is the mean number of writhes for the each group.

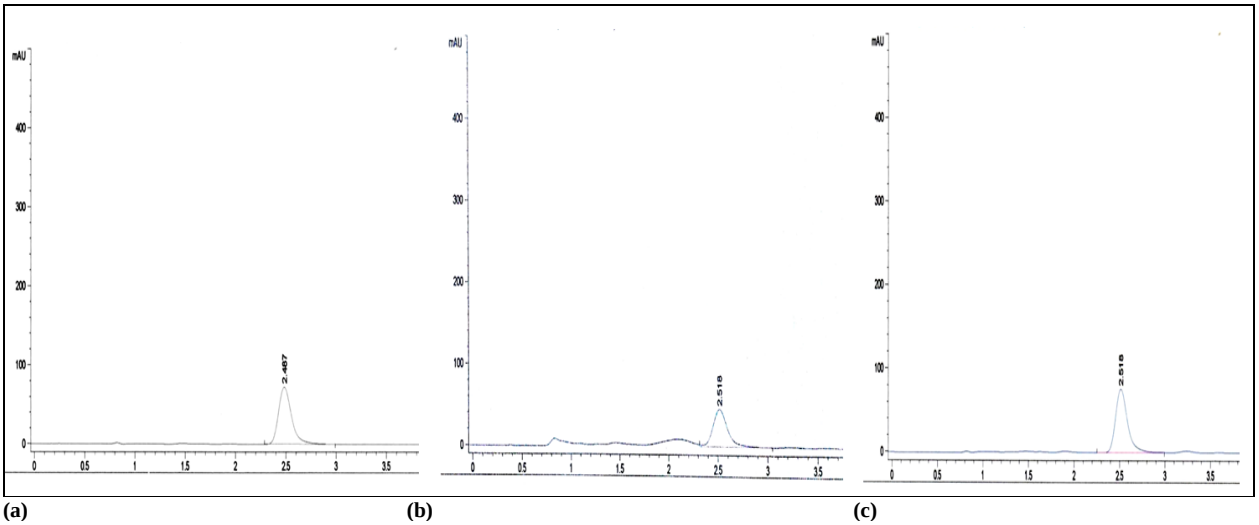
Statistical analysis:

Mean and standard error of mean (SEM) was calculated for the observed values in each experimental group (n=5). Statistical analysis was performed by one way analysis of variance (ANOVA) followed by Least Significant Difference (LSD). An effect was considered to be significant at the $P<0.05$ level. Effective Dose 50% (ED_{50}) was analyzed by probit analysis. SPSS 17.0 was used for the statistical analysis.

RESULTS:

Determination of andrographolide content:

The andrographolide content in extract and EA fraction was determined by HPLC method using andrographolide as an external standard. The analysis was done used a Poroshell RP-18 column (4.6x250 mm, 5µm), mobile phase methanol:water pH 3.05 (50%: 50% v/v) and a flow rate of 1 ml/min. Under these conditions, the andrographolide standard was observed at Rt 2.46 min. Meanwhile, andrographolide in extract and EA fraction was detected at Rt 2.51 min (Figure 1). The peak area of the andrographolide standard at concentrations of 100, 200, 400, 800 and 900 ppm was determined. The peak areas of the known standard concentration were used for linier regression analysis as shown in Table 1. Andrographolide content in extract and EA fraction was 15.66±0.28% and 21.25± 1.08% w/w as shown in Table 2.



(a) (b) (c)

Figure 1. HPLC Chromatogram of Andrographolide standard at a concentration of 200 ppm (a), ethanol extract of *Andrographis paniculata* (b), EA fraction of *Andrographis paniculata* (c).

Table 1. HPLC results of andrographolide standard (concentration vs area)

Concentration (ppm)	Area
100	209.84308
200	654.00830
400	1522.93140
800	3129.94482
900	3545.97778

$Y=4.1461x-177.57$, $R^2=0.9997$

Table 2. HPLC result of andrographolide content in extract and EA fraction

Sample	Area	Sample Concentration (ppm)	Andrographolide content (% w/w)	Andrographolide content (% w/w) Average±SD
Extract	486.47580	160.14	16.01	15.66±0.28
	461.17447	154.04	15.40	
	476.88068	157.83	15.78	
	463.30511	154.55	15.45	
EA Fraction	707.80560	213.53	21.35	21.25±1.08
	655.43182	200.90	20.09	
	668.67413	208.91	20.89	
	762.83105	226.80	22.68	

Table 3. Analgesic activity of extract and EA fraction of *A. paniculata* by acetic acid induced writhing in mice

Groups	Dose (mg/kg bw)	Number of writhes in 45 min (Mean±SEM)	Inhibition (%)
Negative control	-	105.2 ± 1.80^{abcd}	
Diclofenac sodium	40	27.0 ± 1.92^{abc}	74.33±1.83
Ethanol extract	12.5	85.8 ± 1.16^{acd}	18.44±1.10
	25	54.6 ± 1.94^{abcd}	48.10±1.84
	50	34.0 ± 1.14^{abd}	67.68±1.08
EA fraction	12.5	83.0 ± 1.61^{acd}	21.10±1.53
	25	47.4 ± 1.62^{abcd}	54.94±2.49
	50	30.6 ± 1.12^{ab}	70.91±1.07

Value are reported as Mean±SEM for all groups. The data was analyzed by ANOVA followed by LSD test. Letters (abcd) indicate statistically significant difference, $P<0.05$

Evaluation of analgesic activity by acetic acid induced writhing test:

The effects of the ethanol extract and EA fraction of *A. paniculata* on the acetic acid-induced abdominal constrictions in mice are presented in Table 3 and Figure 2. The results indicated that ethanol extract, EA fraction of *A. paniculata* (at a dose of andrographolide 12.5, 25, and 50 mg/kg body weight) and diclofenac sodium (40 mg/kg body weight) significantly ($P<0.05$) reduced abdominal writhing in mice when compared to the negative control group. Interestingly, the reduction occurred in a dose dependent manner. Maximum inhibition from treatment with ethanol extract (67.68%) and EA fraction (70.91%) was observed at a andrographolide dose of 50 mg/kg body weight. The effective dose 50% (ED_{50}) was analyzed by probit log analysis using SPSS. The results showed that the ED_{50} values of extract and EA fraction were 29.49 mg/kg body weight and 25.55 mg/kg body weight, respectively. Hence, the EA fraction displayed higher activity compare to the extract based on their ED_{50} value.

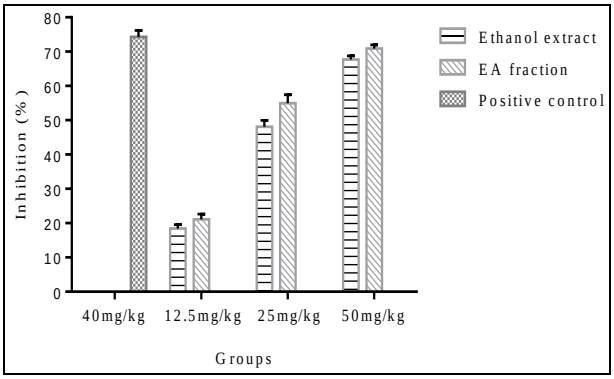


Figure 2. Percentage of inhibition of abdominal contractions of acetic acid induced writhing in mice.

DISCUSSION:

In order to facilitate the use of *A.paniculata* for its analgesic activity, this study aimed to determine the active substance involved and its potential for development into an analgesic herbal medicine based drug. This was undertaken by comparing the analgesic activity of the ethanol extract and EA fraction based on their andrographolide content.

The determination of andrographolide content in ethanol extract and EA fraction was conducted by HPLC method. Several methods to quantify the andrographolide content in extract and pharmaceutical dosage form were reported including High Performance Thin Layer Chromatography (HPTLC) and spectrophotometric method[21-23]. HPLC methods was adopted in this study because of its simple, precise and accuracy. The determination result reveal that andrographolide content in extract was 15.66±0.28% w/w. Meanwhile, EA fraction was contain andrographolide 21.25± 1.08% w/w, which was higher compared to the extract.

In this study, experimental animals in the form of mice (*Mus musculus*) were divided into eight groups consisting of a positive control group, negative control group, and six treatment groups. The number of animals used for each test group was determined based on the Federer formula[24]. The ideal number of animals per group according to the Federer formula was a minimum of four animals. The experimental animals were randomly divided into eight groups where each group consisted of five mice. Thus the total for all control and treatment groups amounted to forty male mice. The negative group was given CMC-Na 0.5%. The positive

control group was given the analgesic drug sodium diclofenac at a dose of 40 mg/kg body weight of mice. Group 3 to 5 were treated with 96% *A. paniculata* ethanol extract and Groups 6 to 8 were treated with *A. paniculata* EA fraction at an andrographolide dose of 12.5, 25, and 50 mg/kg body weight of mice.

The acetic acid-induced abdominal constriction method is widely used for the screening new analgesic drugs, because it is very simple and sensitive[25]. The working principle of this method is to observe the stretching response that occurs due to pain stimulation by an irritant. The irritants are administered by injecting acetic acid as an intraperitonial pain inducer in mice[26]. Administering acetic acid can cause a local inflammatory response because of the release of arachidonic acid from phospholipid tissue via Cox, prostaglandin production (PGE2 and PGE2α) and increased production of lipoxygenase which can increase intraperitoneal fluid. The production of prostaglandin and lipoxygenase causes swelling and release of endogenous substances that stimulate nerve endings in the visceral area, especially in the chest and abdominal cavity so that pain arises. This pain is characterized by the appearance of stretching which is classified as the foot being pulled back (stretched) and the abdomen touching the base of the form plate[27-29].

Based on ANOVA analysis, it was shown that significant differences occurred between the treatment groups (P<0.05). Hence, the administration of different test materials produced different amount of stretching. Regarding the results of the ethanol extract and EA fraction in the acetic acid-induced abdominal constriction assay, a prominent inhibition of the writhing reflex was observed. The ethanol extract and EA fraction of *A. paniculata* display analgesic activity as determined by significantly (P<0.05) reduced abdominal writhing in mice when compared to the negative control group.

Post hoc-tests were performed using the Least Significant Difference (LSD) analysis to reveal any differences between treatment groups. LSD test results showed that EA fraction at a dose of andrographolide of 12.5 mg/kg was not significantly different to the ethanol extract treatment at the same dose. Furthermore, the EA fraction at an andrographolide dose of 50 mg/kg did not differ significantly from the ethanol extract treatment at the same dose as well as from the positive control. From this it can be concluded that the administration of sodium diclofenac at a dose of 40 mg/kg and the EA fraction at a andrographolide dose of 50 mg/kg have reduce writhing in mice to a similar degree.

Diclofenac sodium, like other non-steroidal anti-inflammatory drugs, inhibits the biogenesis of prostaglandins, thus inhibiting the writhing in experimental animals like mice. The presence of phytochemicals in *A.paniculata*, such as diterpenes, lactones, and flavonoids has been reported in previous work[30]. Flavonoids were reported to have a role in analgesic activity primarily by targeting prostaglandins[31,32]. Moreover, andrographolide was detected in diterpene lactone compounds which are known to have analgesic effects[19]. Madav et al. (1995) reported that 300 mg/kg of andrographolide, administered orally, had significant analgesic activity on acetic-induced writhing in mice[33]. The results of our study showed that both ethanol extract and EA fraction at the same andrographolide dose of 50 mg/kg showed similar analgesic activity. Andrographolide, which was a major compound contained in the ethanol extract and EA fraction can be considered as a marker for analgesic activity. In agreement with previous work, *A.paniculata* analgesic activity was entirely explained by the presence of a high content of andrographolide[34]. The presence of other secondary metabolites did not influence the analgesic activity as shown by their similar activity. The EA fraction might be chosen as potential active substance in analgesic drug development based on its higher andrographolide content. The amount of EA fraction equal to 50 mg andrographolide (235.30 mg) needed to reach the similar analgetic activity was lower than ethanol extract (319.28 mg). Nevertheless, further study is needed to increase the analgesic activity of the EA fraction to enable practical applications. Specifically, formulating a study of the EA fraction wherein the solubility of active substance is increased will be of great interest study as well as working towards an understanding of the mechanism of action.

CONCLUSION:

Ethanol extract and EA fraction of *A. paniculata* at the same dose of andrographolide showed similar analgesic activity. Andrographolide could take a role as a marker for the analgesic activity of *A.paniculata*. The EA fraction contained a higher andrographolide content compared to the ethanol extract. The amount of ethanol extract which needed to reach similar analgesic activity was higher than EA fraction. Therefore, the EA fraction has great potential as an analgesic active substance, but further study is needed to elucidate the appropriate dosage form.

CONFLICT OF INTEREST:

The authors declare there is no conflict of interest.

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RESEARCH ARTICLE

Biochemical Investigation to Determine the Factors Involved in Renal Failure Formation for Dialysis Patients

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ABSTRACT:

Renal failure is on the top list of kidney diseases as being frequently reported in many medical facilities around the globe. Human kidneys play an important role in excreting, reabsorbing, secreting and filtrating substances in the body. Healthy kidney must excrete the waste products of the body in urine and preserve albumin, and other useful substances in the body. The current study aims to figure out the expected factors of renal failure formation in dialysis patients. Likewise, it is designed to search for any correlation between the imbalanced levels of electrolytes and kidney deterioration. The current study was conducted in Ramadi city-Iraq with the assistance of Al-Ramadi Educational Hospital (REH). The (14^{th}) samples of renal failure patients were collected from dialysis unit at (REH). (2-3 mL) of blood was taken from a patient's vein. The serum was separated from plasma with the use of a centrifuge. All samples were undergone centrifuging for (4-7 min) at (550 rpm). Afterward, samples were subjected to biochemical examinations to determine the levels and quantities of some biochemical elements and other substances in the selected cases. It was found that some patients had been recorded with a decrease in glomerular filtration rate (GFR), high HbA1c level (> 6.5 mg/dL) (21.5%), anemia (< 12.0 mg/dL) (%100) in women and (< 13.5 mg/dL) in men (%100), Uremia (> 45mg/dL) (%100), hypercalcemia (>10.5 mg/dL) (%7.14), hyperkalemia (> 5.1 mg/dL) (%92.8), and Hypernatremia (>145 mg/dL) (%28.5). Some results obtained, imbalanced levels of the mentioned substances, are either progressed with kidney deterioration, or they are the causes of renal failure. Changes in the levels of some blood substances were observed, and positive results were obtained regarding the aim of the study. Levels or quantities of some blood substances play an important role in preserving kidneys good performance. For instance, when the level of glucose in blood raises, a severe damage to the blood vessels of the kidney takes place and results in poor kidney's performance. While, an increment in the K serum level causes a sudden cardiac death. Changing in some levels and quantities of blood substances could be a core cause of renal failure formation, however, some of them develops when kidney deteriorates.

KEYWORDS: Glomerular Filtration Rate, Renal Failure, Kidney Functions, Hyperkalemia, Kidney Excretion.

INTRODUCTION:

Kidneys like other organs play an essential role in preserving human health including vitamin D metabolism (It produces 1, 25-hydroxycholecalciferol, and without this process, bone diseases are expected to occur), the production of erythropoietin (anemia emerges when the kidney fails to produce erythropoietin, preserving the fluid balance generally, as a filter to the minerals from blood, and filtering unwanted material as a result of food, toxic subjects, and medications. A human is basically born with a pair of

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kidneys that are located on both sides of the spine right above the waists. Kidneys make hormones that are utilized in the production of red blood cells, blood pressure controller, and boost bones functions[1]. Kidney diseases is one of the biggest worldwide health issues that could be emerged either with born or through a certain age due to numerous factors such as associated diseases, dietary system, and medications intake. However, the most fatal ones among these diseases is renal failure. Renal failure/end-stage kidney disease is defined as the disability of kidney to perform the excretory function that results in returning the waste to the blood instead of excreting it outside the body. The kidney is failed to work when it only performs less than %15 of its normal function[2]. Therefore, a human body will be unable to clean up the blood from the poisonous substances, dispose of the additional water in the blood, and regulating the blood pressure. In addition, it impacts the production of red blood cells that are very essential to maintain healthy body. When a kidney deteriorates, it poses the waste from blood and other toxic substance to other parts of the body which causes swelling in ankles, severe weakness, shortness of breath, nausea, and other disorders[3, 4]. Other reason for acute renal injury (ARI) is the side effect of some medications such as anti-tuberculosis medication. It has been proven to be one of the causes of (ARI)[5].Hypertension is also responsible for renal failure, which is formed when interaction of some environmental agents and genes take place. Arteries around the kidney may get affected, which become weak, hard, or narrow, due to hypertension. This result in a weak delivered blood to the kidney [6].

Renal failure is divided into two types, chronic and acute renal failure. Acute Renal Failure (ARF) is occurred when the glomerular filtration rate suddenly drops (hours to several days). The diagnostic of this type could be obtained by monitoring (increment) of creatinine within (48 hr.) about (0.3 mg/dL), as well as, an increment of creatinine within the last week to 1.5 times, or urine volume (about 0.5 mL/kg or less) per hour for (6 hr)[7]. Chronic Kidney disease is characterized by kidney's damage that leads to a disturbance in the glomerular filtration rate. Poisonous substances could not be properly excreted out of the body due to the kidney injury[8].

The excretory function of kidney fails to work withing a few hours or days where the levels of creatinine and urea in blood elevate. In some cases, the rate of urine production decreases. Anuria or oliguria are considered the signs of renal failure [9]. The term (ARF) has been altered to (AKI), acute kidney injury, due to that (AKI) signifies the abnormality of the level of serum creatinine until reaching a state where renal failure forms. This type of renal failure is a common problem recorded in most hospital around the globe. (ARF) incidence increases with age and about 150 cases out of million record in the UK. (Mehta RL; et al, 2007). While Chronic Renal Failure (CRF) is a continuous deterioration of kidney functions that is resulted in a significant elevation in serum creatinine for a continuous last three months, or the rate of glomerular filtration (GF) less than (60 per min / 1.73m2). (ESRD) end-stage renal disease is a stage where a patient reaches a therapy of renal transplantation. It usually comprises a gradual loss of the kidney's functions which may lead to either a kidney's transplantation or hemodialysis[10, 11]. Hemodialysis (HD) is a treatment that is designed for patients who suffer from end-stage renal disease. (HD) helps RF patients to filter unwanted fluid, waste, and various salt in the blood and send them out of the body[12,13]. CKDs are sometimes asymptomatic. The treatments are varied depending on the status. Early/mild kidney damage could be treated with certain medications, while end stage kidney damage is treated with either kidney transportation surgery or hemodialysis [14]. Bone disorders, such as osteoporosis, is initiated early or with kidney failure diagnosis. Abnormal bone tissue is a sign of most patients with CRF[15].

Stages of Chronic Kidney Disease:

Based on the classification presented by the National Kidney Foundation (NKF) , five stages of chronic kidney diseases are shown in table (1). These stages have been admitted by numerous clinical guidelines as an approach to deal with the (CKD)[16].

Table (1): (CKD) Chronic kidney disease Classification

Stage	GFR (ml/min per 1.73 m^2)	Kidney Condition
1	90 or more	Damage of the kidney with natural or (↑GFR)
2	60-89	Damage of the kidney with mild (↓ GFR)
3	30-59	Moderate ↓ GFR
4	15-29	Intense ↓ GFR
5	Less than 15	Renal Failure

Kidney's filtration rate per day of (water, sodium, glucose, and urea) are [180 $_{(L)}$, 630 $_{(g)}$, 180 $_{(g)}$, and 54 $_{(L)}$] respectively. While the quantity Excreted a day of the above substance are [1.8 $_{(L)}$, 3.2 $_{(g)}$, 0 $_{(g)}$, and 30 $_{(g)}$] respectively[17].

Aim of Study:

Aim of the study was to find out the reasons and factors that may have impacted the selected patients and ended up with renal failure. Likewise, to assess the levels of some biochemical elements in blood and corelate them to kidney's deterioration.

MATERIAL AND METHODS:

The current study was conducted in Ramadi city-Iraq with the assistance of Al-Ramadi Educational Hospital (REH). The (14^{th}) samples of renal failure patients were collected from dialysis unit at (REH). (2-3 mL) of blood was taken from a patient's vein. The serum was separated from plasma with the use of a centrifuge. All samples were undergone centrifuging for (4-7 min) at (550 rpm). Afterward, samples were subjected to biochemical examinations to determine the levels and quantities of some biochemical elements and other substances in the selected cases, which are explicated with their procedures as follow:

Urea Examination (Urease/ Glutamate Dehydrogenase):

(1 mL) of urea solution by (Biosystem) was placed into a white tube. (10 micron) of a patient's serum was transformed to the previous urea solution. The entire mixture was mixed up for (25 sec.) and left to settle at room temperature for (12 min). After that, the value of the resulted solution was obtained using (Apple Apparatus-Japanese Made) at (600 nm) wavelength. The consumption of urea by the sample was achieved by a coupling reaction as it is shown below where nicotinamide adenine dinucleotide hydrogen (NADH) had been measured by spectrophotometry.

$$\text{Urea} + H_2O \xrightarrow{\text{urease}} 2NH_4^+ + CO_2$$

$$NH_4^+ + \text{NADH} + H^+ + \text{2-oxoglutarate} \xrightarrow[\text{dehydrogenase}]{\text{glutamate}} \text{Glutamate} + \text{NAD}^+$$

Diabetic Examination (Glucose Oxidase/Peroxidase):

(1 mL) of a sugar solution by (Biosystem) was placed into a white tube. (10 micron) of a patient's serum was added to the previous sugar solution. The entire mixture was mixed up for (12 min) and left to settle at room temperature for (12 min). After that, the value of resulted solution was recorded by (apple apparatus-Japanese made) at (500 nm) wavelength. Glucose in the sample arises by a coupling reaction as it is explained in the following equations. Spectrophotometry was used to measure the resulted color complex of the coupling reaction.

$$\text{Glucose} + 1/2\ O_2 + H_2O \xrightarrow[\text{oxidase}]{\text{glucose}} \text{Gluconate} + H_2O_2$$

$$H_2O_2 + \text{4-Aminoantipyrine} + \text{phenol} \xrightarrow{\text{peroxidase}} \text{Quinoneimine} + 4H_2O$$

Other Examinations:

While the rest of the examinations, that will be explained in the result section, were conducted using (Reflotron Plus System). The Reflotron machine was zeroed using a provided zeroing strip. After that, (32 micron) of a patient's serum was placed on the surface of the strip, and eventually the strip was placed into the specified port of the machine. The values were obtained using the machine's screen[18].

RESULTS AND DISCUSSION:

In the current study, 14 renal failure patients were undergone biochemical examinations. The cases were mixed gender whose ages are ranged between (20-70 years old).

Biochemical examinations of the selected (RF) patients have been conducted (March-June, 2020) to figure out what factors that may have been impacted the patients' kidneys and resulted in renal failure formation based on the acquired results. The glucose level was among the conducted examination, and it was proven to be a core cause of a kidney damage. The following values depict the variations in glucose levels (HbA1c) of renal failure patients, where HbA1c is (glycated hemoglobin).

Table (2): HbA1c examination values of renal failure patients

Sample NO. (RF)	HbA1c mg/dL
p1	6.2
p2	8.2
p3	4.8
p4	5.1
p5	5.9
p6	6.8
p7	9.7
p8	4.8
p9	7.4
p10	5.6
p11	4.9
p12	6.2
p13	5.8
p14	4.6

Normal (HbA1c) level ranges from (4.0 – 5.6 mg/dL), while from (5.7 – 6.4 mg/dL) is considered prediabetes. However, any recorded value over (6.5 mg/dL) is a case with diabetes. Based on the obtained results in table (2), it is recorded that (21.5%) of the renal failure cases with a severe elevation of the (HbA1c) level (> 6.5mg/dL), whereas (7.14 mg/dL) was recorded slightly higher than the normal range. High glucose level in blood causes a severe damage to the blood vessels of the kidney, which leads to a poor performance of the kidney. As a result, the human health is affected. Due to the vessels damage, kidney losses its function of preserving albumin that possesses an essential role for maintaining the volume of extracellular fluid. Those kidney failure patients with abnormal HbAlc levels are expected to be developed with (RF) due to abnormal HbAlc levels since HbAlc examination records the level of the glucose in the blood for the past three months of the examination. The Albumin protein of the (21.5%) of the cases is anticipated to be high in urine as a result of blood vessels damage of the kidney (with an assumption as it is the reason of kidney failure of these patients). Urine Albumin examination needs to be conducted in future works as a complementary to the investigations[19, 20]. A study was reported by Senthilkumar and Dhivya, which

was found an inverse relationship between HbA1c level and GFR [21].

Urea values of all cases were recorded and shown in table (3). A significant variation was obtained of kidney failure patients as follow:

Table (3): Urea examination values for renal failure patients

Renal Failure		
S.NO	Creatinine mg/dL	Urea mg/dL
p1	6.0	211
p2	8.3	263
p3	8.9	287
p4	5.0	189
p5	8.2	224
p6	6.8	284
p7	8.8	191
p8	7.5	210
p9	8.8	170
p10	6.0	246
p11	5.8	192
p12	7.7	185
p13	7.7	294
p14	8.3	198

Uremia, high urea level in blood, urine in the blood, is a condition that is linked to kidney functions. Uremia is associated with a disturbance in the levels of hormones, electrolyte, and fluids in the body. This clinical condition is usually emerged with cases diagnosed with end-stage kidney failure or (AKI). Uremia is an indication of a kidney damage when the kidney transforms toxin, including urea and creatinine, and body's waste to blood instead of urine[13]. The normal range of urea and creatinine in blood are ranged between (25-45 gm/dL) and (0.5-1.2 mg/dL) respectively. Urea level is always accompanied with creatinine level when it comes to examine the performance of kidney functions. Creatinine values (ranged from 5.0-8.9 mg/dL) and urea values (ranged from 170-294 mg/dL) of the selected renal failure patients in table (3) indicate a severe damage to the kidneys as a result of either diabetes, heavy metals poisonous, non-steroidal anti-inflammatory drugs, imbalanced levels of some biochemical elements in blood, and many more. The kidneys of these patients may have been impacted by the mentioned factors according to the high values of creatinine and urea. In addition, the assessment of glomerular filtration rate (GFR) relies on the creatinine and urea measurements in plasma/serum levels. A healthy kidney correlates with a normal (Glomerular Filtration Rate) GFR (~125 mL/min). When GFR declines, an elevation in the blood concentration of urea and creatinine occurs, whereas their excretion decrease[22]. In a healthy kidney, approximately a half of urea quantity and the whole creatinine quantity are excreted to urine.

Even though Hemoglobin (HGB) levels vary in women and men due to the menstrual cycle in women, however, HGB examination was essential to be conducted for all cases in order to dig deeply into the investigation. All the HGB level values were listed in table (4) for both renal failure patients.

Table (4): HGB examination values for renal failure patients

Sample NO. (RF)	HGB g/dL
p1	7.8
p2	11.0
p3	9.8
p4	10.2
p5	10.2
p6	10.1
p7	11.4
p8	11.4
p9	7.4
p10	10.6
p11	8.5
p12	10.0
p13	10.9
p14	10.7

The normal range of hemoglobin in men is (13.5 to 17.5 g/dL), while in women is (12.0 to 15.5 g/dL). Anemia (decrease hemoglobin level) is a consequence of chronic kidney disease due to a decrease in the production of erythropoietin (EPO), which stimulates the production of RBC in the bone marrow. The role of (RBC) is to carry oxygen to the organs especially brain and heart. When less (RBC) produces, less oxygen delivers to organs, which eventually leads to poor performance of these organs including kidneys. Mortality rate increases of patients with a severe anemia. Anemia is commonly progressed with kidney disease; especially renal failure, when a kidney loss (%20-50) of its functions. Deficiency of Iron in the blood is another cause of anemia. The results in table (4) show an insufficient quantity of (RBC) in renal failure patients that ranges from (7.8 – 11.4 g/dL). This suggest that fewer (EPO) was produced by the damaged kidneys, which was resulted in anemia. It was also observed that (RBC) examination of two cases were close to the normal range. This could be attributed to either, those patients were treated with iron pills to raise up the hemoglobin level, or they were injected with erythropoietin injection via intravenously through the duration of dialysis[23, 24]. There is a significant correlation between the creatinine and Hb levels of patients with renal failure. RF patients were recorded with high levels of creatinine and a decrease in the Hb levels. The same result was also reported by Ghassan F. and co-workers [25]. Anemia is highly developed in diabetic patients rather than with those without diabetes. Deficiency in erythropoietin hormone production by the kidney is the major reason for the decrease in hemoglobin levels in chronic kidney patients [26].

Imbalanced levels of some biochemical elements (Na^+, K^+, and Ca^{++}) in blood affect human health and cause numerous organs' diseases such as cataract, renal failure, and bone diseases. The data in table (5) shows the levels of these elements in blood for renal failure patients.

Table (5): Biochemical element level for renal failure patients

Sample NO. (RF)	Calcium Ca^{++} mg/dL	Potassium K^+ mg/dL	Sodium Na^+ mg/dL
p1	10.1	5.6	140
p2	9.6	4.0	148
p3	9.1	6.5	134
p4	9.7	4.0	147
p5	9.1	5.2	138
p6	10.1	5.52	141
p7	9.6	5.1	139
p8	10.1	3.8	130
p9	8.8	5.21	148
p10	10.8	5.22	148
p11	9.8	5.02	142
p12	9.5	5.22	145
p13	8.9	4.52	137
p14	9.9	4.8	139

Hypercalcemia, or elevated serum calcium level, is a common clinical disorder that is accompanied with numerous changes in the body, as well as, it alters the functions of some organs. Calcium (Ca^{++}) must be preserved at a normal range to avoid many health issues such as bone loss in elderly that associates with vitamin D deficiency. The normal range of serum (Ca^{++}) level is (8.5-10.5 mg/dL). The values of mineral serum calcium levels of (RF) patients were within the normal range except one case, which was in the upper of normal range (10.5 mg/dL). The association of hypercalcemia and hypocalcemia for renal failure has not been well comprehended or illustrated due to the association of high and low minerals level in blood with many diseases. However, the (RF) case is a (62 years) women with serum (Ca^{++}) level of (10.8 mg/dL). This slightly high serum (Ca^{++}) level could be due to an inadequate dialysis that left some quantity of calcium in blood, excessive intake of food containing (Ca^{++}), or hyperparathyroidism condition that raised up the (Ca^{++}) level [27].

Many physiologic operations including preserving a regular cardiac conduction requires a normal potassium level in blood. Most chronic kidney disease (CKD) were observed with high serum potassium level compared to non-CKD, which could due to a reduction in potassium excretion, as well as, excessive intake of medications, such as diuretics, that block the kidney from receiving an enough quantity of potassium [28]. The normal serum potassium level is ranged from (3.5 - 5.1 mg/dL). The baseline (K^+) result for (RF) patients, hyperkalemia (> 5.1 mg/dL) was overwhelmingly predominant over hypokalemia. Sudden cardiac death could be attributed to an elevation in serum potassium level for people with renal failure. A periodic serum potassium level analysis should be conducted to CKD patients to eliminate the risk of developing sudden cardiac death [29].

Sodium is one of the three most important electrolytes in the body that controls the fluids as they exchange between tissues and cells. When the level of (Na^+) alters, blood pressure and acid-base balance are directly affected in the body. Vascular system and kidney are impacted when an elevation of serum sodium level occurs. The oxidative stress of the kidney elevates as sodium intake increases due to lowering the rate of breakdown interactive oxygen. The production of TGFβ1, HNO3, and a change in endothelium are resulted in glomerular and vascular fibrosis, which leads to a failure or a decrease in the kidney functions [30]. Hypernatremia is a disorder occurs when the serum electrolyte (Na^+) level elevates (>145 mg/dL). It is a reduction of the total water in the body proportionally to the sodium quantity. (%28.5) of (RF) was observed with up-normal serum sodium level. Kidney with failed functions is distinguished with a progressive development of isosthenuria and hyposthenuria, which turns eventually into a risk of hypernatremia development[31]. High serum sodium level could raise the rate of mortality of renal failure patients.

CONCLUSION:

Imbalanced levels and quantities of blood substances, such as electrolytes, HGB, Creatinine, urea, and uric acid, affect kidney's performance, which leads eventually to renal failure. Some of the changes of substances quantities initiate with kidney's deterioration, and some were recorded to be the anticipated causes of renal failure formation. High level of (HbA1c) and high levels of (Ca^{++}) and other substances damage the kidney, therefore, the waste and poisons return to blood instead of excreting them to urine and finally outside the body.

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CONFLICT OF INTEREST:

There was no conflict of interest.

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RESEARCH ARTICLE

Functionalization of Gold Nanoparticles with Monosaccharide Mannose

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ABSTRACT:

Gold nanoparticles have found a wide range of application in biomedical sciences. Unique properties of these metal nanoparticles include surface plasmon resonance and size dependent colour change. Various molecules have been functionalized on the gold nanoparticles surface but carbohydrates have garnered attention due to their properties and their role in living systems. However certain challenges make carbohydrate-gold nanoparticles association difficult to obtain and stabilize. This study was carried out to chemically remodel gold nanoparticles by adding a monosaccharide mannose to its surface. A modified phase transfer method was used to synthesize gold nanoparticles. The surface of the nanoparticles was fixed with cyanuric chloride to serve as a linker. Mannose was then linked to the linker molecule. All three stages of the process, gold nanoparticles, and gold nanoparticles with linker and gold nanoparticles with the carbohydrate were analyzed for size and stability. Zeta potential and UV-vis data exhibited stable gold nanoparticles dispersion, successful binding of linker molecule as well as the carbohydrate. This study shows a simple, cost-effective and robust method of glycomodification of gold nanoparticles surface which can further find use in wide ranging applications.

KEYWORDS: Gold Nanoparticles, Functionalization, Mannose, Cyanuric Chloride, Zeta Potential.

INTRODUCTION:

Gold, a soft and malleable metal, which falls under the category of transition metals, has very low chemical reactivity. But colloidal gold, unlike bulk gold, is highly reactive, the reactivity range of which generates newer application possibility.

Owing to the plasmonic properties of gold nanoparticle, observed as colour change during its change in size range, it has found its place in wide variety of applications like drug delivery, targeted delivery of peptides or DNA, clinical chemistry and molecular biology applications. [1-6]

Be it new drug screening, understanding biological processes, clinical diagnosis and treatment, carbohydrates have various functions. Physicochemical and biomedical applications of gold nanoparticles have attracted much attention. Since surface modification to attain functionality is a mainstay in attaining the intended objective, many molecules have been added on

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to surface of these gold nanoparticles. Of these, attachment of carbohydrate moieties has become a largely used target owing to their molecular properties as well as their action in living systems. [7, 8]

Two broad methods, namely, physical adsorption and chemical methods can be employed to functionalize gold nanoparticles with target carbohydrate. While physical adsorption has very less output due to low adsorbability chemical methods due to poor solubility and difficulty in interaction with metal nanoparticles make it challenging to functionalize gold nanoparticles with carbohydrates. [9] Furthermore, cyclization of carbohydrates attached causes it to lose the property that it aims to utilize. Other methods to functionalize gold nanoparticles with carbohydrates like photochemical reaction are expensive and have low yield.

Cyanuric chloride based reactions which employ the several hydroxyl groups of a carbohydrate which remain free, are a set of novel methods to functionalize gold nanoparticles with carbohydrates. Cyanuric chloride by the virtue of having chlorine atoms which can be replaced by hydroxyl or amino group, acts as a good linker. These methods employ lesser steps and are sometimes 'one pot' synthesis processes. The aim of this study was to attach monosaccharide mannose on to gold nanoparticles to which the linker cyanuric chloride has been added. [10]

MATERIAL AND METHODS:

Chemicals and reagents:

Cyanuric chloride, 11-mercaptoundecanoic acid (MUA), 11-mercapto-1-undecanol (MUOH) and HAuCl4 were obtained from Sigma Aldrich, USA. Tween 20, absolute ethanol, sodium citrate, mannose and other regents and solvents were of analytical grade and were obtained from Hi-Media Lab Pvt. Ltd, India. Aqueous solutions were all prepared with double distilled water.

Preparation of Mannose linked Gold Nanoparticles (GNP):

Gold nanoparticles were prepared based on a method described by Xiao Wang et. al.[10] An aqueous solution of $HAuCl_4$ (0.25 mM, 100 mL) was used to synthesize gold nanoparticles until colour of the solution became dark purple and finally light red.[9] To avoid aggregation, Tween 20 (0.1%, v/v) was used which was stored at 4°C. A mixture of MUOH and MUA in ethanol solution was added to the stored nanoparticles following which a harvest of the precipitate containing MUOH/MUA-modified GNPs was made. After re-suspending the prepared gold nanoparticles, cyanuric chloride was added. Further, mannose was added to the admixture. Post centrifugation, at 12000 rpm for 20 min, the precipitate was harvested, and washed which was followed by re-suspension using sonication. The nanoparticles were characterized using particle size, zeta potential, UV-visible spectroscopy and visible colour change.[11, 12]

RESULTS AND DISCUSSION:

Gold nanoparticle was synthesized by reducing the gold salt and then a modified phase transfer method was used to attach the hydroxyl group. This hydroxyl group was then replaced by linker cyanuric chloride and then the carbohydrate mannose was attached to the linker. Immobilization chemistry includes three steps. In the first step, hydroxyl terminal are created on GNPs. In the second step, cyanuric chloride is anchored onto these OH terminals to form Gold nanoparticle linked with Cyanuric chloride (GNP-CC). In the last step, the carbohydrate mannose was immobilized on the surface of cyanuric chloride layer to form GNP-CC linked with mannose (GNP-CC-Mannose). We synthesized gold nanoparticle of the average size 43.07 nm with zeta potential of - 11.2 mV. The addition of cyanuric chloride group increased the size and an average diameter of 57.02 nm was obtained, with zeta potential of -11.2 mV. On addition of mannose, the overall size of the molecule increased to an average of 257.7 nm, with a zeta potential of -28.9 mV, exhibiting good stability (Table 1, Fig 1 and 2). The successful introduction of each of these chemical is characterized by change in the UV spectra as shown in Fig 1.

Table 1: Size range and zeta-potential of gold nanoparticles and remodelled gold nanoparticles

Sample No	Size (nm)	Percentage Intensity	Zeta-potential (mV)
GNP	43.07	70.2	- 11.2
GNP- CC	57.02	59.8	- 11.2
GNP-CC-Mannose	257.7	100	- 28.9

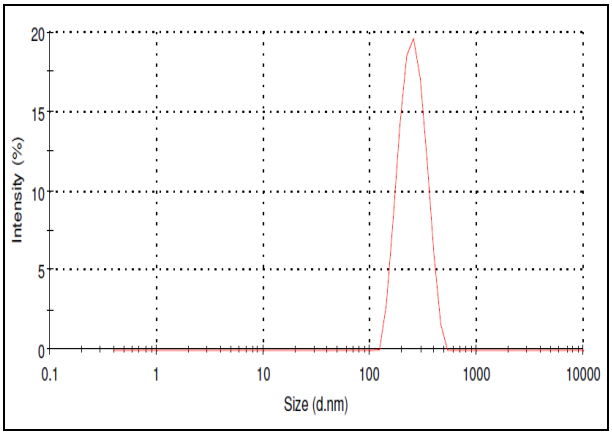

Figure 1: Average Particle Size distribution of GNP-CC-Mannose

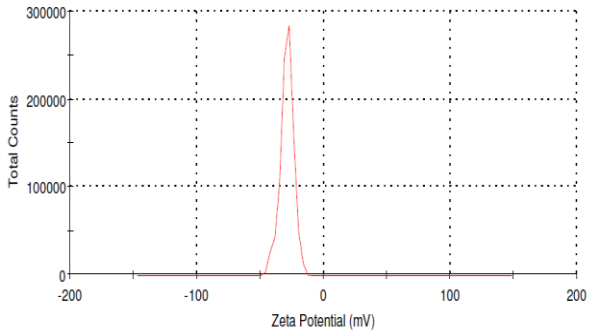

Figure 2: Average Zeta Potential of GNP-CC-Mannose

Attachment of OH terminals on GNPs causes a shift in absorption from 543 nm to 545 nm, while carbohydrate attached nanoparticles exhibited a maximum absorbance at 522 nm showing a significant hypsochromic shift. This shift to lower wavelength may be due to increase in hydrophilicity of the surface layer causing a decrease in aggregation (Fig 3). There is also a significant change in the colour of nanoparticles observed during these three stages, as shown in Fig 4. The visible colour change of the gold nanoparticle and its two subsequent modifications, from a shade of red to blue via violet, indicates shift in surface plasmon absorption to lower energies.

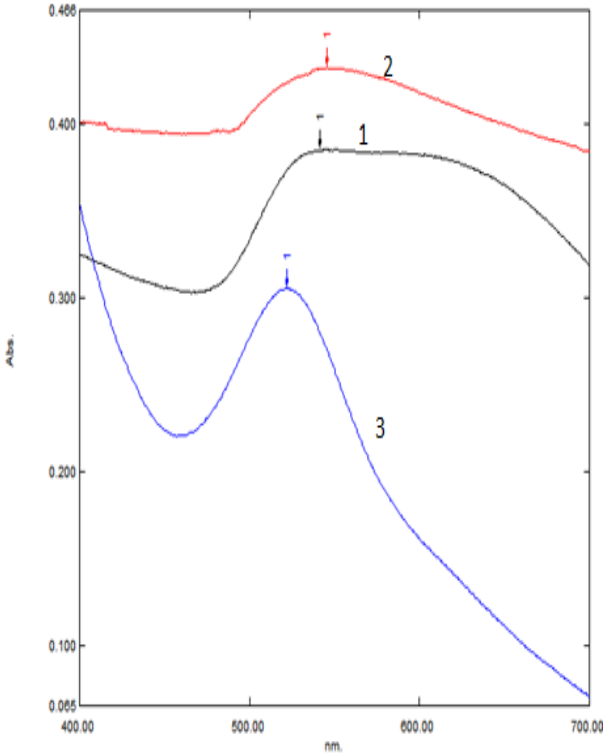

Figure 3: UV spectra of GNP at various stages of functionalization. 1 - Gold nanoparticle, wavelength 543 nm. 2 - Cyanuric chloride linked gold nanoparticle, wavelength 545 nm, 3 - Gold nanoparticle with mannose attached, wavelength 522 nm)

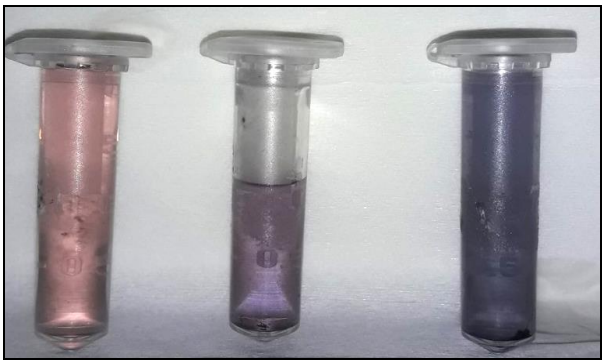

Figure 4: Colour of nanoparticles observed during the three stages (i: Gold nanoparticles, ii: GNP-CC, iii: GNP-CC- mannose)

CONCLUSION:

Metal nanoparticles have been used as a tether for carbohydrates since sometime and it has been applied in various detection techniques. However, these interactions are often unstable and less soluble. We have been able to bind mannose successfully and have a stable chemically modified gold nanoparticle by a cost effective and quick method. The inherent property of gold nanoparticles to exhibit colour change in congruence with size change encourages its applications in various detection techniques. Mannose tethered gold nanoparticles have been used to detect E Coli proteins, mannose receptor positive cancer tissue, etc, and has a potential to detect proteins in a carbohydrate-protein interaction.[13, 14] Its function as a frequency amplifier can be further explored. Limitation of the study includes analysis of the modified gold nanoparticles by scanning electron microscopy.

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CONFLICT OF INTEREST:

The authors declare no conflict of interest.

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RESEARCH ARTICLE

Solubility Studies and Validation of Lovastatin using High Performance Liquid Chromatography Method

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ABSTRACT:

This study aimed to determine the solubility of lovastatin (LV) in different oil, surfactant, and co-surfactant using the high-performance liquid chromatography method. LV was solubility studies in different vehicle. The different vehicle used almond oil, sunflower oil, oleic acid, olive oil, soybean oil, and corn oil, isoprophyl myristate, myoglyol, tween 80, tween 20, and cremophor R.H. 40, propylene glycol, and PEG 400. Each of them was added lovastatin until saturated. The mixtures were mixing, sonicating, putting in the water bath and standing for 24 hours, then centrifugated. Each of the aliquot 2 µL diluted with acetonitrile and determination of concentration lovastatin using HPLC, with detector ultraviolet at 237 nm. Before determinate LV validated, and curve calibration at range 2-16 µg/mL was made. This study using the HPLC method with detector UV 237 nm, Agilent C 18 (4.6 x 150 mm 5 µ) column, and acetonitrile: water (70:30 v/v) as mobile phase. Calibration curve of lovastatin at the range 2-16 µg/mL with linear regression 0.999. Accuracy and precision showed that. Lovastatin has high soluble in oleic acid, tween 80, and PEG 400.

KEYWORDS: lovastatin, solubility, vehicle, validated, HPLC.

INTRODUCTION:

In recent years, the formulation of poorly soluble compounds presented interesting challenge for formulation scientist in pharmaceutical industries and 50% of new chemicals entities exhibit poor aqueous solubility and the ones of drug is lovastatin[1] [2,3]. Lovastatin (LV) is a statin group, a specific and potent competitive inhibitor of 3-hydroxy-3 methyl glutaryl coenzyme A (HMG-CoA), hence a potent cholesterol-lowering drug[4]. Statin therapy represents the basis for the management of hypercholesterolemia and the prevention of cardiovascular disease [2]because of efficacy and safety. Statins are generally safe and well-tolerated[5].

Lovastatin is a drug that includes Biopharmaceutical Classification System class 2 with characteristic poorly soluble in the water and high permeability[6,7]. LV is a lipophilic statin, undergoes hepatic and enteric metabolism via enzyme CYT cytochrome P450 (P450 3A4)[8].

Lovastatin is an inhibitor of HMG CoA reductase as a key in the synthesis of cholesterol [5]. Absorption lovastatin after administration orally is 30% and exhibits low oral bioavailability estimated < 5% because of rapid metabolism in the gut and liver. Lovastatin and its active b-hydroxy acid metabolite have short half-life plasma between 1,1-1,7 hour, and steady-state concentrations are achieved within 2-3 days to the patient with a normal kidney. A formulation with a high degree of oral absorption and extended delivery potential would be highly desirable for lovastatin[9,10].

LV appears as a white, non-hygroscopic crystalline powder, a highly lipophilic drug [log P = 4,3] but poorly water-soluble ($0,4x10^{-3}$mg/mL)[4] [11,12]. Since the

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therapeutic efficacy of drug molecules depend on their solubility, it is important to enhance drug solubility in an aqueous environment to achieve the maximum therapeutic effect. Moreover, the aqueous solubility is the critical factor for evaluating the bioavailability of orally administered drugs with lower water solubility[13].

The figure of lovastatin can see below.

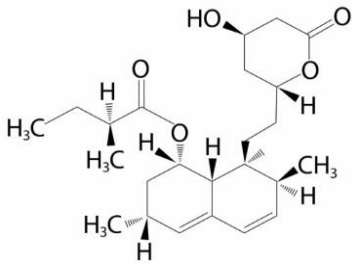

Fig. 1 Structure of Lovastatin

The Insolubility of lovastatin in the water needs to overcome increasing solubility lovastatin in the gastrointestinal tract and avoid the first-pass metabolism. Lipid-based formulations represent a unique solution to the delivery of poorly soluble compounds. A lipid dosage form typically consists of one or more drugs dissolved in a blend of lipophilic excipients such as triglycerides, partial glycerides, surfactant, or co-surfactant. Among the lipid-based system, the SMEDDS, SNEDDS is a promising technology to improve the rate and extent of absorption of poorly water-soluble drugs, which significantly facilities enhancing oral bioavailability[12] [14]. SNEDDS (Nano-Emulsifying Drug Delivery System) that composition is lipid, surfactant, and co-surfactant. The oil phase selection is based on the oil's ability to dissolve lovastatin on the gastric fluid. Anhydrous formulation's high solvent capacity generally decreases upon formulation dispersion and digestion in the gastrointestinal tract, thereby resulting in drug supersaturation. If a sufficiently long and stable period of drug saturation can be achieved, intestinal drug absorption can be increased [15] Various oils are used for SNEDDS formulation. Usually, long-chain triglycerides slightly digestible than medium-chain triglycerides, but long-chain triglycerides increasing lymphatic absorption so that they avoid first-pass metabolism [16]. This study aims to determine lovastatin's solubility in the different vehicle include oils, fatty acids, surfactant, and co-surfactant and to select higher soluble in lovastatin.

MATERIALS AND METHODS:

Materials: Lovastatin was purchased from Sanbe Farma Bandung Pharmaceutical Ind., Acetonitrile, and Methanol grade of HPLC Merck®, Aquabidest, almond oil, oleic acid, tween 20 Corn oil, olive oil, and sunflower oil, tween 80, PEG 400 and propylene glycol was purchased from CV Global Pratama.

Calibration of lovastatin:

Lovastatin stock solution was prepared by dissolving lovastatin 10 mg in acetonitrile 100 mL using the volumetric flask. Then diluted at ranging 2-16 µg/mL.

Preparation of sample:

The solubility of lovastatin was determined in all components (almond oil, sunflower oil, corn oil, soybean oil, olive oil, oleic acid, tween 20, tween 80, cremophor R.H. 40, propylene glycol, and polyethylene glycol. Each component was taken of 5 ml in vial 10 mL then added to all a few of lovastatin until saturated. The mixtures were mixing using a magnetic stirrer for 10 minutes, then using a sonicator for 10 minutes. Put in the water bath for 15 minutes at temperature 40°C. The mixtures were standing for 24 hours, and the combinations were centrifugated at 6000 rpm for 10 min. Filtered the varieties using membrane filter 0.45µm, then supernatant transferred to vials. An aliquot of sample @ 2 µL added acetonitrile until 5 mL, then moved to vial 1.5 mL and injected into the HPLC system.

High Performance Liquid Chromatography

Quantitative estimation of lovastatin analyzed using HPLC (Waters) with column C18 (ZOBRAX Eclipse XDB Agilent technologies, Santa Clara, USA) (4,6x 150 mm, 5µm) and U.V. at room temperature. A mobile phase using acetonitrile and water (70:30 v/v) and the flow rate was 1 ml/min and detection at 238 nm, and the injection volume of 5µL and run time was fixed at 6 minutes. Selection mobile phase modification from aqueous buffer (0,05 M ammonium phosphate and 0,01 M Phosphoric acid buffer and acetonitrile) (50:50) [11]

Method Validation

The method validated for precision, accuracy, and linearity, LOD, and LOQ. Linearity was evaluated using the average of nine concentration levels at range 2-16 µg/mL. For precision and accuracy studies, 3 three concentration levels (8,10, and 12 µg/mL). The method's precision expressed by the percent relative standard deviation (% RSD) of the regressed concentration was used. The accuracy of the process was determined by regression concentration represented as a percentage of the nominal concentration[17] [18,19].

Linearity:

The linearity of the calibration curve of lovastatin at concentration levels range 2- 16 µg/mL was found with a regression coefficient (0,999) and intercepted in figure 2.

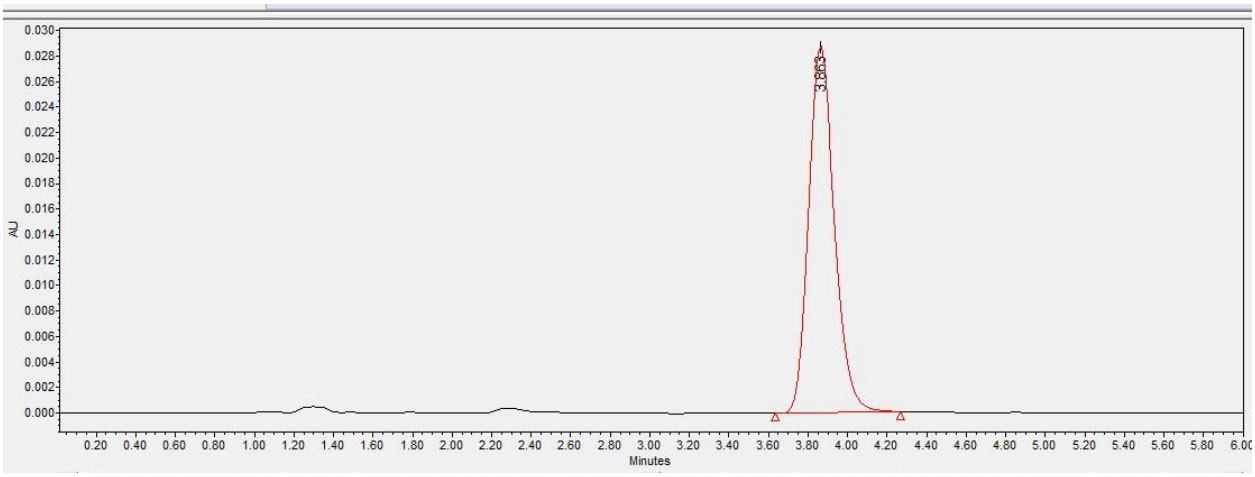

Fig. 1 Chromatogram of lovastatin with mobile phase acetonitrile: water (70:30) and flow rate 1 ml/ min

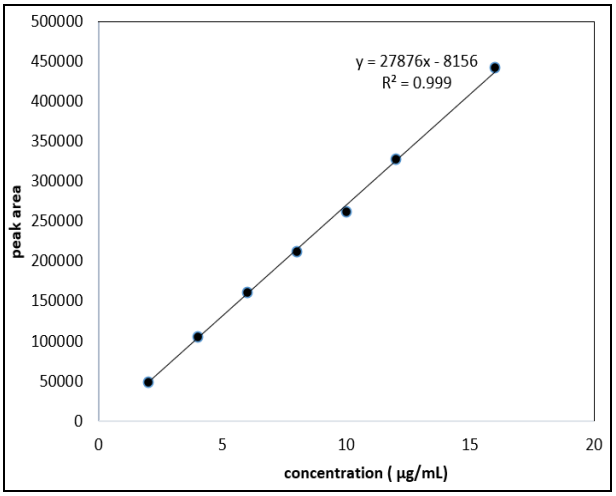

Fig.2. Callibration curve of lovastatin wit regression coefficient= 0,999 and intercept= 8156 and slope = 27876

Table 1. Validation Method of lovastatin

Validation method	Value
Linearity	R2= 0,999
Accuracy (% recovery)	95-108%
Precision (RSD)	0.3 and 1.38 %
LOD (Limit of Detection)	0.48µg/mL
LOQ (limit of Quantification)	1.60µg/mL

Table 2. Solubility lovastatin (LV) in different oils, surfactant, and cosurfactant

Component	Concentration (µg/mL)
Almond oil	2.04
Sunflower oil	1.40
Soybean oil	2.01
Olive oil	2.25
Oleic acid Myoglyol Isoprophyl myristate	3.67 2.47 1.70
PEG 400	20.5
Propilenglycol	5.54
Cremophor RH 40	9.85
Tween 20	16.99
Tween 80	19.04

DISCUSSION:

The Validation of lovastatin using HPLC (Waters) with column C18 (ZOBRAX Eclipse XDB Agilent technologies, Santa Clara, USA) (4,6x 150 mm, 5µm) at room temperature. A mobile phase using acetonitrile and water (70:30 v/v) and the flow rate was 1 ml/min and detection of UV at 237 nm, and the injection volume of 5µL and run time was fixed at 6 minutes. The retention time of lovastatin in 3.863 repectively[20,21].

Linearity of the calibration curve of lovastatin at concentration levels range 2- 16 µg/mL was found with a regression coefficient (0,999) and exhibit good linearity (r^2 >0,99) and the equation regression is y = 27876x- 8156[22].

Accuracy is one of the most critical parameters of an analytic methodology. It can be expressed as the percentage of recovery of the known amount of drug in the sample. The result of recovery showed that the mean accuracy level of lovastatin was 95-108%. The results are a good agreement with acceptable value for validation of an analytic procedure (recovery =80-120%)

Precision is the measure of the degree of repeatability of an analytic method under regular operation and expressed as the relative standard deviation. The result of accuracy with 3 level concentration (8, 10, and 12 µg/mL), showed that RSD 1, 38 respectively, indicating that the method presents an excellent precision because below 2% [16,23].

The detection limit (LOD) and Limit of Quantification (LOQ) test for the procedure are performed on samples containing very low concentration analytes. LOD is defined as the lowest amount of analyte that can be detected above baseline noise. LOQ is defined as the lowest amount analyte, which can reproducibly be quantitated above the baseline noise. In this study, LOD

for 5 µL injection of lovastatin was 0.48 µg/mL, and LOQ was 1,60 µg/mL. With a relative standard deviation lower than 10% [24].

Solubility studies are one of the critical parameters to achieve the desired concentration of drug in systematic circulation in order to elicit pharmacological respons [25]. Drug efficacy can be severely limited by low aqueous solubility. This study aims to select oils, surfactants, and co-surfactant that the extent of the solubility of the lipophilic drug (lovastatin)—this selection of components used for formulation SNEDDS. The result of solubility lovastatin (LV) in parts can see in Table 1.

The solubility of lovastatin is higher in unsaturated fatty acids than in triglycerides and the result showed that lovastatin high soluble in oleic acid than almond oil, olive oil, sunflower oil, myglyol, and isoprophyl myristat. As we know that almond oil contains more than 90% unsaturated fatty acid (62-76% oleic acid, 25-30% linoleic acid, palmitate acid 4-9%). The solubility of lovastatin in olive oil (oleic acid 55-83%, linoleic acid 3.5-21%), soybean oil (oleic acid 17-26%, linoleic acid 50-57%), and sunflower oil (linoleic acid 66%, oleic acid 21%) is lower than oleic acid[26]

This solubility of lovastatin in surfactant tween 80 higher than tween 20 and cremophor R.H. 40 because HLB of tween 80 is 15, HLB of tween 20 is 16.7, and HLB of cremophor R.H. 40 is 14-16. HLB value showed of lipophilicity of surfactant and HLB value lower, means more lipophilic, and HLB value higher means more hydrophilic. Tween 80 is more lipophilic than others surfactant, so has the ability to the solubility of lovastatin than others [26]

The solubility of lovastatin in co-surfactant showed than PEG 400 higher than propylene glycol. It showed that PEG 400 has a polarity index of 12.4, and propylene glycol has a polarity index of 32.1. The increasingly of index polarity, more hydrophilic and otherwise.

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RESEARCH ARTICLE

Screening of secondary metabolites, bioactive compounds, *in vitro* antioxidant, antibacterial, antidiabetic and anti-inflammatory activities of chia seeds (*Salvia hispanica* L.)

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ABSTRACT:

Recent research studies indicate the role of functional foods in preventing the development of complications associated with type 2 diabetes mellitus. Chia seeds are an excellent source of dietary fibre, essential fatty acids, micronutrients and non-nutritive components. The objective of the study was to evaluate the antioxidant, antibacterial, antidiabetic and anti-inflammatory potential of chia seeds. TPC and TFC were estimated using Folin-Ciocalteu Reagent and Alumininum Chloride method. The antioxidant activity was determined using DPPH$^{\bullet}$ radical, ABTS$^{\bullet+}$ radical, Superoxide (O_2^-) radical, Fe^{3+} reducing and phosphomolybdenum reduction assay. Agar well diffusion method was used to determine the antibacterial activity against *Escherichia coli*, *Proteus vulgaris*, *Shigella flexneri*, *Micrococcus luteus*, *Bacillus subtilis* and *Staphylococcus aureus*. Antidiabetic and anti-inflammatory activities were evaluated using alpha amylase inhibition assay and heat induced haemolysis method. Volatile functional compounds were identified using Gas chromatography mass spectrometry. Upon quantification, TPC and TFC were found to be 850.67±14.14µg/mg GAE and 171.21±12.86µg/mg QE. Free radical scavenging activity of chia seeds was ranked in the order of DPPH$^{\bullet}$ radical >ABTS$^{\bullet+}$ radical > Superoxide (O_2^-) radical. The capability of chia seeds to function as electron donors was evident through its strong reducing power. With regard to antibacterial activity, maximum inhibition was observed for *Staphylococcus aureus*, with a zone of inhibition of 31mm at 500µg/mL. Results of antidiabetic assay highlighted the alpha amylase inhibitory action of chia seeds with an IC_{50} value of 121.46µg/mL. The anti-inflammatory activity of chia seeds increased linearly in a dose dependent manner. GC-MS analysis showed the presence of functionally active compounds such as coumarine, napthoquinone, phytol, fatty acids, flavone and flavone derivatives. Findings of the study highlight that chia seeds have several essential therapeutic properties. Furthermore, clinical studies are required to validate the role of chia seeds in preventing the development of complications associated with type 2 diabetes mellitus.

KEYWORDS: Chia seeds, Bioactive compounds, Therapeutic properties, Functional foods, Diabetes mellitus.

INTRODUCTION:

Type 2 diabetes mellitus is a global public metabolic disorder that affects individuals of different age groups. Type 2 diabetes mellitus is associated with development of macrovascular and microvascular complications [1].

Macrovascular complications include cardiovascular diseases, peripheral artery disease and stroke. Microvascular complications include neuropathy, nephropathy and retinopathy[2]. Inflammation together with oxidative stress activates the pathway involved in development of macro and microvascular complications. Pro-inflammatory cytokines such as TNF-α, IL-6 and IL-1β plays a key role in the development of diabetic complications by promoting apoptosis, increasing vascular permeability along with increased production of reactive oxygen species[3]. Oxidative stress inactivates

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the action of nitric oxide synthase and prostacyclin synthase. It also increases the levels of protein kinase C polyol pathway activity and non-enzymatic glycation[4].

Hypoglycaemic drugs such as alpha glucosidase inhibitors, biguanindes, gliptins and sodium glucose cotransporter inhibitors are commonly prescribed for individuals with type 2 diabetes mellitus. Indefinite usage of these antidiabetic drugs is associated with side effects[5]. Usage of functional foods as a non pharmacological complementary therapy in treating and preventing diseases is gaining lot of interest. Mounting clinical evidence confirms the role of functional foods in preventing or delaying the development of complications associated with type 2 diabetes mellitus[6-8]. Bioactive compounds present in functional foods impart numerous health benefits apart from maintaining blood glucose homeostasis. Functional food enhances the action of endogenous antioxidant enzymes, maintains gut microbiota by inhibiting the growth of pathogenic bacteria, suppresses excess production of pro-inflammatory cytokines, and acts as a vasodilator[9].

Salvia hispanica which belongs to the family Lamiaceae is an annual herbaceous plant native to South Mexico. The seeds of this plant are oval in shape, very small in size and are either black, grey or white in colour. Traditionally, chia seeds were utilized as an anti-platelet agent, laxative, analgesic and anti-depressant agent[10]. Chia seeds are rich in bioactive peptides, dietary fibre, essential amino acids, phytonutrients and polyunsaturated fatty acids [11]. Previous studies indicate that chia seeds function as a laxative, cardiovascular protector, immune booster, vasodilator, anti-depressant agent, controls dyslipidemia and hyperglycaemia [12-13]. The growing demand for functional foods with multitudinous health benefits signify the need for evaluating different plant based products as an excellent source of antibiotics, antioxidants, antidiabetic and anti-inflammatory agents. Considering the aforementioned details, the objective of the study was to evaluate the antioxidant, antibacterial, antidiabetic and anti-inflammatory potential of chia seeds.

METHODOLOGY:

Preparation of extract:

Fifty grams (50g) of commercially available whole black chia seeds was coarsely crushed using a pestle and mortar. This powder was soaked in 100 mL of ethanol for 72 hours by maceration technique. The supernatant was filtered and concentrated using rotary evaporator.

Qualitative analysis of phytochemicals:

Chia seeds were screened for the presence of alkaloids, glycosides, saponins, phenols, flavonoids, terpenoids, steroids, quinones and tannins [14].

Estimation of total phenol content:

TPC was estimated using Folin-Ciocalteu reagent method[15]. One hundred µL of the extract was mixed with 1 mL of Folin Ciocalteu reagent. After 5 minutes, 1 mL of 20% sodium carbonate solution was added. The mixture was incubated at room temperature for 30 minutes and the absorbance was measured spectrophotometrically at 760 nm. TPC is expressed as µg/mg GAE.

Estimation of total flavonoid content:

TFC was determined using the aluminium chloride method[16]. Five hundred µL of the extract (1mg/mL) was mixed with 0.5 mL of 5% sodium nitrate solution. The mixture was allowed to stand for 5 minutes and 0.3 mL of 10% aluminium chloride solution was added. Finally, 1 mL of 1M sodium hydroxide solution was added and the mixture was incubated for 15 minutes at room temperature. The absorbance was measured spectrophotometrically at 510 nm. TFC is expressed as µg/mg QE.

Antioxidant activity:

DPPH$^{\bullet}$ radical scavenging activity:

Briefly, 1 mL of 0.1 mM of DPPH solution was added to various concentrations of the test sample. The setup was left in dark at room temperature and the absorption was measured spectrophotometrically at 517 nm after 30 minutes. Results are expressed in terms of percentage inhibition of DPPH$^{\bullet}$ free radical [17].

ABTS$^{\bullet+}$ radical scavenging activity:

Different concentrations of the test sample were mixed with 1 mL of diluted ABTS$^{\bullet+}$ solution and incubated for 10 minutes. The absorbance was measured spectrophotometrically at 734 nm. Results are expressed in terms of percentage inhibition of ABTS$^{\bullet+}$free radical[18].

Superoxide (O_2^-) radical scavenging activity:

Different concentrations of the test sample were mixed with 50 mM of phosphate buffer (pH 7.4), 200 µL of 1.5 mM of riboflavin, 200 µL 12 mM of EDTA and 100 µL 50 mM of NBT. The reaction was started by illuminating the reaction mixture in an UV illuminator for 15 minutes. After illumination, the absorbance was measured spectrophotometrically at 590 nm. Results are expressed in terms of percentage inhibition of superoxide (O_2^-) free radical [19].

Ferric (Fe^{3+}) reducing power assay:

Various concentrations of the test sample were mixed with 1 mL of phosphate buffer (0.2 M, pH 6.6) and 1mL of 1% $K_3[Fe(CN)_6]$. The mixture was incubated at 50^{o}C for 20 minutes. Later, 1 mL of 10% TCA was added followed by addition of 1 mL of 0.1% of freshly

prepared $FeCl_3$. The absorbance was measured spectrophotometrically at 700nm. Results are expressed as absorbance value measured at 700 nm[20].

Phosphomolybdenum reduction assay:
Various concentrations of the test sample were mixed with 1 mL of the reagent solution that contained 0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate. The tubes were capped and incubated in a water bath at 95°C for 90 minutes. The tubes were cooled to room temperature and the absorbance was measured spectrophotometrically at 695 nm. Results are expressed as absorbance value measured at 695 nm. For all antioxidant assays, ascorbic acid was used as standard [21].

Antibacterial activity:
Bacterial strains used in the study were *Escherichia coli* (MTCC 443), *Bacillus subtilis* (MTCC 441), *Staphylococcus aureus* (MTCC 96), *Shigella flexneri* (MTCC 1457), *Proteus vulgaris* (MTCC 426) and *Micrococcus luteus* (MTCC 1538). The antibacterial activity was determined using agar well diffusion method. Nutrient agar was poured into the petri plates and was allowed to solidify. The bacterial culture was streaked on the surface of the media using a sterile cotton swab. Wells were created in the petri plates using a sterile steel borer. Different concentrations of the test sample were poured into the wells and incubated. Results are expressed in terms of diameter of the zone inhibition in millimetres. Tetracycline was used as standard [22].

Antidiabetic activity:
Different concentrations of the test sample were added to 500µL of 0.02 M sodium phosphate buffer. Ten microlitres (10µL) of alpha amylase solution was added and the mixture was incubated for 10 minutes. Later, 500µL of soluble starch (1%, w/v) was added and incubated for 1 hour. Hundred microlitres (100 µL) of 1N dilute HCL was added to stop the enzymatic reaction, followed by addition of 200 µL of freshly prepared iodine solution. The absorbance was read spectrophotometrically at 565 nm. Results are expressed as percentage inhibition of alpha amylase. Acarbose was used as standard[23].

Anti-inflammatory activity:
Two hundred microlitres (200µL) of freshly prepared 10% RBCs suspension was added to different concentrations of the test sample. The volume was made to 1 mL using saline solution. All the tubes were incubated in a water bath at 56 ºC for 30 minutes. The reaction mixture was centrifuged at 2500 rpm for 5 minutes. Absorbance was measured at 560 nm. Results are expressed as percentage inhibition of haemolysis. Aspirin was used as standard [24].

GC-MS:
The test sample was injected into a HP-5 column (30 m X 0.25 mm id with 0.25 µm film thickness), Agilent technologies 6890 N JEOL GC Mate II GC-MS model. The following chromatographic conditions were used. Helium was used as the carrier gas at a constant flow rate of 1 mL/minute and the injector was operated at 200°C. The column oven temperature was maintained at 50-250°C at a rate of 10°C/min injection mode. Following MS conditions were used: ionization voltage of 70 eV, ion source temperature of 250°C, interface temperature of 250°C and mass range of 50-600 mass units. The database of National Institute Standard and Technology (NIST) having more than 62,000 patterns was used for the interpretation on mass spectrum of GCMS with was used for interpretation.

RESULTS AND DISCUSSION:

Phytochemicals or secondary metabolites exhibit several medicinal properties such as antioxidant, antimicrobial, antidiabetic, anti-inflammatory, anti-allergic, anti-thrombotic and vasodilatory effects. Preliminary phytochemical analysis showed the presence of alkaloids, glycosides, flavonoids, phenols, saponins, steroids, tannins and terpenoids in chia seeds. TPC and TFC were found to be 850.67±14.14µg/mg GAE and 171.21±12.86µg/mg QE. Phenols and flavonoids reduce oxidative stress by scavenging free radicals. Antioxidants or free radical scavengers neutralize or scavenge the harmful effects of free radicals. Free radical scavengers convert free radicals into stable end products by donating a hydrogen atom from their phenolic hydroxyl groups. Free radical scavenging activity of chia seeds is presented in (Table 1). The results are expressed as % inhibition of free radicals. Table 1 indicates that the ability of chia seeds to scavenge free radicals increased with increase in concentration. IC_{50} value refers to the sample concentration required to inhibit the action of 50% of free radicals. An inverse relation exists between IC_{50} value and antioxidant activity. Lower the IC_{50} value, greater is the antioxidant capacity. The IC_{50} value of $DPPH^{\bullet}$ radical, $ABTS^{\bullet+}$ radical and Superoxide (O_2^-) radical scavenging assay was 77.41, 18.11 and 14.10µg/mL. Based on IC_{50} value, free radical scavenging activity of chia seeds was ranked in the order of $DPPH^{\bullet}$ radical >$ABTS^{\bullet+}$ radical > Superoxide (O_2^-) radical. Ascorbic acid had higher activity when compared to chia seeds. Kumar *et al.* [25] reported the IC_{50} value of ethanolic extract of chia seed to be 160µg/mL. Sargi *et al.* [26] in their study reported that chia seeds were able to effectively scavenge $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ radicals in a concentration dependent manner. Reducing power assays are also used for measuring the antioxidant

potential. Fe^{3+} reducing assay is based on the reduction of Fe^{3+} complex to Fe^{2+} complex. Phosphomolybdenum assay is based on the reduction of phosphate Mo (VI) to phosphate Mo (V). Results of reducing power assays are expressed as absorbance value **(Table 1)**. Absorbance value refers to the concentration of Fe^{2+}/ phosphate-Mo (V) present in the solution. Higher the absorbance value, greater is the reducing power. The ability of chia seeds to function as electron donors and to reduce the production of oxidised intermediates in lipid peroxidation was evident through its strong reducing power. Similar trend of result was reported by Vijay *et al*. [27] for reducing power potential of chia seeds.

Table 1. Antioxidant activity of chia seeds (*Salvia hispanica* L.)

Free radical scavenging assays					
DPPH˙ radical		**ABTS$^{\bullet+}$ radical**		**Superoxide (O_2^-) radical**	
Concentration µg/mL	**% Inhibition**	**Concentration µg/mL**	**% Inhibition**	**Concentration µg/mL**	**% Inhibition**
50	32.53 ± 1.38	5	24.16 ± 3.93	20	45.63 ± 0.89
100	61.34 ± 0.62	10	37.68 ± 2.19	40	63.52 ± 1.30
150	72.08 ± 0.92	15	52.17 ± 3.79	60	63.18 ± 2.22
200	78.27 ± 1.17	20	59.17 ± 5.79	80	78.62 ± 2.33
250	81.52 ± 0.91	25	77.10 ± 3.29	100	81.50 ± 2.80
300	85.94 ± 3.01	30	89.27 ± 3.06	120	84.22 ± 2.43
Reducing power assays					
FRAP assay			**Phosphomolybdenum assay**		
Concentration µg/mL	**Absorbance @ 700 nm**		**Concentration µg/mL**	**Absorbance @ 695 nm**	
20	0.64 ± 0.03		20	0.25 ± 0.02	
40	0.77 ± 0.01		40	0.29 ± 0.01	
60	1.36 ± 0.03		60	0.31 ±0.01	
80	1.46 ± 0.01		80	0.34 ±0.01	
100	2.16 ± 0.05		100	0.41 ± 0.03	
120	2.21 ± 0.02		120	0.51 ± 0.04	

Values are the mean of triplicates

In order to reduce the burden of infectious diseases, use of natural products as antimicrobial agents are gaining interest. Tannins, alkaloids, flavonoids and phenolic compounds present in different parts of a plant contribute to antimicrobial activity. Table 2 indicates the antibacterial activity of chia seeds against gram positive and gram negative bacteria. The ability of chia seeds to inhibit the growth of micro-organisms was ranked in the order of *Shigella flexneri* > *Escherichia coli* > *Staphylococcus aureus*. Maximum inhibition was observed for *Staphylococcus aureus*, with a zone of inhibition of 31mm at 500µg/mL. Least inhibition was noted for *Proteus vulgaris* with a zone of inhibition of 19mm at 500µg/mL. According to Divyapriya *et al*. [28] chia seeds were effective in inhibiting the growth of periodontal pathogens such as *Porphyromonas gingivalis*, *Fusobacterium nucleatum* and *Aggregatibacter actinomycetemomitans*. GC-MS analysis showed the presence of flavones, derivatives of flavones and coumarine (Figure 1).

Table 2. Antibacterial activity of chia seeds (*Salvia hispanica* L.)

Bacterial strains	**Zone of inhibition (nm)**			
	Tetracycline	**250µg**	**375µg**	**500µg**
Escherichia coli	21	22	24	27
Proteus vulgaris	20	10	11	13
Shigella flexneri	20	15	16	17
Micrococcus luteus	22	20	22	23
Staphylococcus aureus	25	23	27	31
Bacillus subtilis	20	14	17	19

Antimicrobial activity of chia seeds could be due to the presence of these bioactive compounds. Structurally, flavones are phenolic compounds that have a carbonyl group. Flavones, flavonols and flavonoids exhibit antimicrobial activity against a wide range of pathogenic bacterial strains through their ability to complex with extracellular soluble proteins and by disrupting the cell membrane[29].

Controlling post prandial hyperglycaemia plays a vital role in treating type 2 diabetes mellitus. This can be achieved by inhibiting enzymatic breakdown of starch which in turn delays glucose absorption. The two main enzymes involved in starch digestion are α amylase and α glucosidase [30-31]. The ability of chia seeds to function as alpha amylase inhibitor was evaluated using alpha amylase inhibition assay. Chia seeds inhibited the action of alpha amylase in a dose dependent manner. IC_{50} value was 121.46µg/mL (**Table 3)**. Inhibiting the action of enzymes involved in starch hydrolysis depend upon the presence of compounds such as flavonoids, phenols, tannins and other bioactive compounds [32]. Maximum alpha amylase inhibition rate was 80.98 ± 0.33% at a concentration of 300µg/mL. The antidiabetic potential of chia seeds could be due to the presence of phenols and flavonoids. In the present study, TPC and TFC of chia seeds were found to be 850.67±14.14µg/mg GAE and 171.21±12.86µg/mg QE respectively. Phenolic compounds have a dual role of decreasing oxidative stress and inhibiting the action of enzymes involved in

starch digestion [33]. The latter function is because of non covalent interactions that occur between phenolic compounds and enzymes. Functional groups such as galloyl and hydroxyl groups present in phenolic compounds form hydrogen bond with enzymes.

Hydrophobicity property of galloyl groups enables phenolic compounds to bind with the enzyme by means of hydrophobic association. Thus, the hydrophobic effect of phenolic compounds plays a key role in preventing post prandial hyperglycaemia [34-35].

Inflammation is a natural defence response that occurs in the body. Conversely, uncontrolled inflammatory response is an important causative factor for allergies, type 2 diabetes mellitus, cardiovascular diseases and metabolic syndrome. Stabilization of lysosomal membrane is essential for limiting the inflammatory response. This can be achieved by preventing/reducing the release of lysosomal constituents by activated neutrophils which further aggravates inflammation and tissue damage[36]. Chia seeds exhibited anti-inflammatory activity at different concentrations (50-300µg/mL**)** by actively inhibiting hypotonicity induced lysis of red blood cell membrane. However, the anti-inflammatory action of aspirin was high when compare to chia seeds (Table 3). Coumarins and flavonoids present in chia seeds could have contributed to anti-inflammatory effect. Coumarins and flavonoids function as anti-inflammatory agents by inhibiting the action of cyclo oxygenase and 5 lipo oxygenase[37-38]. Furthermore, flavonoids inhibit the synthesis of prostaglandins that are involved in immunological responses [39].

Table 3. Antidiabetic and anti-inflammatory activity of chia seeds (*Salvia hispanica* L.)

Concentration µg/mL	% Inhibition of alpha amylase		Concentration µg/mL	% Inhibition of haemolysis	
	Chia seeds	Acarbose		Chia seeds	Aspirin
50	13.72 ± 2.98	59.26 ±1.02	50	13.93 ± 0.29	34.03 ± 0.12
100	53.51 ± 1.73	64.16 ± 1.33	100	15.23 ± 0.71	45.26 ± 0.12
150	73.94 ± 0.71	65.17 ± 1.34	150	15.83± 0.87	56.02 ± 0.09
200	75.87 ± 0.53	82.27 ± 2.83	200	17.42 ± 0.23	67.18 ± 0.07
250	76.98 ± 0.48	90.83 ± 2.35	250	30.73 ± 0.28	74.82 ± 0.09
300	80.98 ± 0.33	92.29 ± 1.79	300	46.35 ± 3.07	81.13 ± 0.06

Values are the mean of triplicates

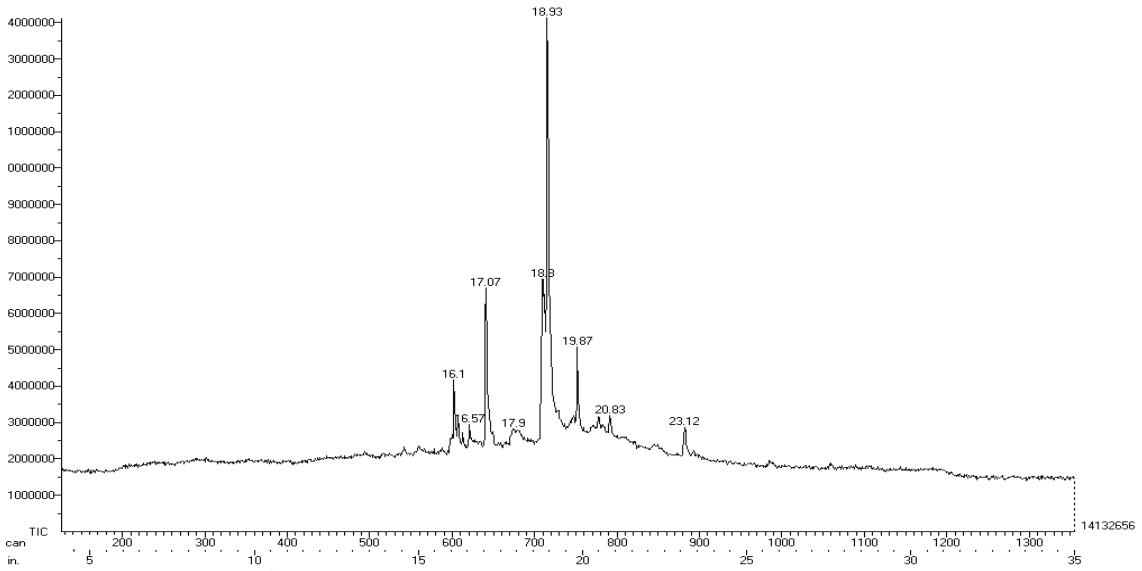

Figure 1: GC-MS chromatogram of chia seeds (*Salvia hispanica* L.)

CONCLUSION:

The study aimed at screening different bioactive compounds along with evaluating certain therapeutic properties of chia seeds. The results demonstrated that chia seeds were rich in phenolic and other functional compounds that contributed to antioxidant, antibacterial, antidiabetic and anti-inflammatory activities. The findings of the study thereby suggest the role of chia seeds as a functional food component in preventing the risk of developing various complications associated with type 2 diabetes mellitus. However, more *in vivo* studies are required to confirm the promising and contributory role of chia seeds in diabetes care.

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CONFLICT OF INTEREST:

The authors declare no conflict of interest

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RESEARCH ARTICLE

Application of Box–Behnken Design and Desirability function in the Optimization of Aceclofenac-Loaded Micropsonges for Topical Application

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ABSTRACT:

Background: The aim of the present investigation was to develop optimized Aceclofenac-loaded microsponges using Box-Behnken design (BBD) and desirability function. **Material and Method:** Aceclofenac-loaded microsponges were developed using ethyl cellulose, ethanol and polyvinyl alcohol (PVA). Initially, a trial batch was developed using quasi-emulsion solvent diffusion method, and by optimizing the drug-polymer ratio. A 3-level, 3-factor BBD was used to investigate the effect of PVA, ethanol and stirring speed on particle size and entrapment efficiency (EE). The models used for the optimization were analyzed through ANOVA and diagnostic plots. Finally, the desirability function was used for the selection of optimized formulation composition. **Results:** A drug-polymer ratio of 1.5:1 was taken as optimized ratio for all the formulations. The developed microsponges were of the spherical shape having size and %EE in the range of 22.54±2.85 µm to 49.08±5.01 µm and 70.57±4.19% to 86.43±2.58 %, respectively. The amounts of PVA, ethanol and stirring speed were noted to have a significant impact on particle size and %EE. Finally, an optimized formulation (size-22.69 and %EE-86.42) was developed with a desirability value of 0.9967. **Conclusion:** The BBD is a valuable tool for the development of optimized microsponges with desired properties.

KEYWORDS: Microsponges, Box-Behnken design, Quality by Design, Aceclofenac, Optimization.

INTRODUCTION:

Aceclofenac (ACF) is a nonsteroidal anti-inflammatory drug used in the management of pain and inflammation due to rheumatoid arthritis, osteoarthritis and ankylosing sponylitis.[1]Aceclofenac is chemically 2-[(2,6 -dichlorophenyl) amino] phenylacetoxyacetic acid, a non-selective COX inhibitor that stimulates glycosaminoglycan in human osteoarthritic. In addition, through suppression of metalloprotease production and proteoglycan release in rheumatoid synovial cells, it shows chondroprotective effects.[2]

A previous study showed that the ACF and its metabolite (4′-hydroxyaceclofenac) have a significant impact on COX-2 and have less effect on COX-1.[3] In comparison to Diclofenac, oral and parenteral administration of ACF is well tolerated with minimal gastrointestinal side effects.[4,5] However, ACF has a low water solubility and a low mean plasma elimination half-life (about 4 hours). Frequent dosing is needed which have been associated with various side effects including gastrointestinal irritation and gastrointestinal bleeding.[6,7]

To overcome these limitations, transdermal delivery of ACF via microsponges could be a promising strategy.[8,9]Microsponges are a sponge-like porous polymeric system with a size range of 5- 300 µm[10]. The microsponges prepared to date, showed excellent tolerability against a wide range of temperature (up to 130ºC) and pH (1-11). In addition, they are non-allergenic, non-mutagenic, non-irritating and have greater entrapment efficiency and excellent

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compatibility with a wide range of excipients.[11-14]As compared to conventional formulations, microsponges required less amount of API and able to release the entrapped drug in a sustained manner.[15,16]With respect to liposomal formulations, microsponges have greater entrapment efficiency, simple processing and extended stability.[17]

To date, the regulatory bodies have made quality by design (QbD) mandatory to maintain the desired quality in the end products[18,19]. The International Conference on Harmonization (ICH) covers QbD aspects in its three guidelines, namely ICH Q8 (pharmaceutical development), ICH Q9 (pharmaceutical risk management), and ICH Q10 (pharmaceutical quality systems).[20-23] In the present investigation, Box-Behnken design (BBD) was used to develop an optimized formulation of ACF-loaded microsponges. The BBD is response-surface deign used to illustrate the response function that is difficult to be described by a linear function. In comparison to other optimization designs, BBD is a more efficient and economical method having wide applications including energy application and for analytical method.[24,25]

In this study, initially, a trial batch was developed to confirm that the developed microsponges are spherical and of small size. The drug-polymer ratio was optimized in terms of size, shape and entrapment efficiency (%EE). A three-factor, three-level Box-Behnken design was used for the design of experiment (DoE). The effect of variables, such as amount of polyvinyl alcohol (PVA), ethanol and stirring time on different responses, such as particle size and EE was investigated. The models used for the response analysis were analysed using ANOVA, lack of fit, degree of freedom, sum of square and diagnostic plots. The variables effects were illustrated using 2D contour and 3D response surface plots. Finally, optimized formulation properties and composition were selected as per the desirability function value. The desirability value for the optimized formulation was illustrated via contour plots considering each variable.

MATERIAL AND METHODS:

Materials:

Aceclofenac was received as a gift sample from Orison Pharma International (Kala Amb, HP) Ethyl cellulose and polyvinyl alcohol were purchased from SD Fine Chemicals, Mumbai. All other chemicals and reagents used were of analytical grade.

Drug-excipients compatibility study:

Drug-excipients compatibility study was carried out by FTIR (Perkin Elmer Spectrum, BX II**)** spectrophotometer. The FTIR spectra of the drugs alone, physical mixtures (ACF+EC and ACF+PVA) and ACF-loaded microsponges were recorded using the potassium bromide (KBr) dispersion method. The test samples were added to KBr (1:1) and examined at the range of 400 to 4000 cm^{-1} with the resolution of 4 cm^{-1}.

Preparation of plain microsponges:

Initially, a trial batch of plain microsponges was developed using quasi emulsion solvent diffusion method.[26-.28] Briefly, the internal phase was prepared by dissolving ethyl cellulose (300 mg) in ethanol (10 ml) followed by bath sonication (5 min) for complete solubilization. For the external phase, polyvinyl alcohol (100 mg) was dissolved in distilled water (150 ml). The internal phase was then added dropwise to external phase with continuous stirring (1000 rpm) for 2.5 hr. Finally, the solution was filtered, and the microsponges were separated and dried at room temperature, kept in a glass vial and stored in a desiccator before further analysis. The developed microsponges were examined under a microscope for their shape and size.

Drug-polymer ratio optimization:

The ACF-loaded microsponges were developed with the same method that was used for the fabrication of plain microsponges. However, the internal phase was prepared by dissolving a predetermined ACF and ethyl cellulose ratios (1:1, 1:1.5, 1.5:1 and 2:1) in ethanol (10 ml). Finally, the drug-polymer ratio was optimized in terms of particle shape, size and percentage entrapment efficiency (%EE).

Formulation optimization:

In the present work, 3-factor, 3-level Box-Behnken design (total of 17 experimental runs with 5 center points) was used to assess the overall effect of the variables on the properties of ACF-loaded microsponges. The amounts of polyvinyl alcohol (A), ethanol (B) and stirring speed (C) were selected as independent variables. The particle size (Y1) and %EE (Y2) were selected as dependent variables or responses. The responses were analyzed using Design-Expert software (Trial version 11.0.5.0, Stat-Ease Inc., MN). The levels of variables and responses used for the optimization of ACF-loaded microsponges are illustrated in Table 1.

Table 1: Design of experiment

Independent variables			
	Low (-1)	**Medium (0)**	**High (+1)**
Polyvinyl alcohol	400 mg	500 mg	600mg
Ethanol	5 ml	10 ml	15 ml
Stirring speed	1000 rpm	1250 rpm	1500 rpm
Dependent variables			
Particle size (µm)	Minimum		
Entrapment efficiency (%)	Maximum		

Characterization of microsponges:

Particle size by optical microscopy:

The eye piece micrometer was calibrated with the help of a stage micrometer. The diameters of more than 100 microsponges were measured randomly. The average particle size was determined by using Edmondson's equation. D = ∑ nd /∑ n Where, n = Number of microspheres observed; D = Mean of the size range.

Surface morphology:

The surface morphology of the developed ACF-loaded microsponges was investigated using scanning electron microscopy (JSM-6100). ACF-loaded microsponges were mounted on the double-faced adhesive tape and coated with a thin gold palladium layer with the help of sputter-coated unit and were analyzed then for surface morphology.

Percentage entrapment efficiency:

The percent entrapment efficiency of drug entrapment for each batch was calculated using following formula,

%EE = (Practical drug loading/Theoretical drug loading) X 100

Theoretical drug loading was determined by assuming that the entire drug got entrapped in micropsonges and no loss occurs at any stage of preparation. Practical drug loading was determined by crushing 100 mg of Aceclofenac-loaded micropsonges in a dry glass mortar followed by addition of 50 ml of hydroalcoholic solution. Resultant dispersion was kept aside in a volumetric flask for 3 h and then sonicated for 1 h in a bath sonicator. The dispersion was filtered through Whatman filter paper (0.22 mm) and the filtrate was analyzed by using a UV/ Vis spectrophotometer at 256 nm.

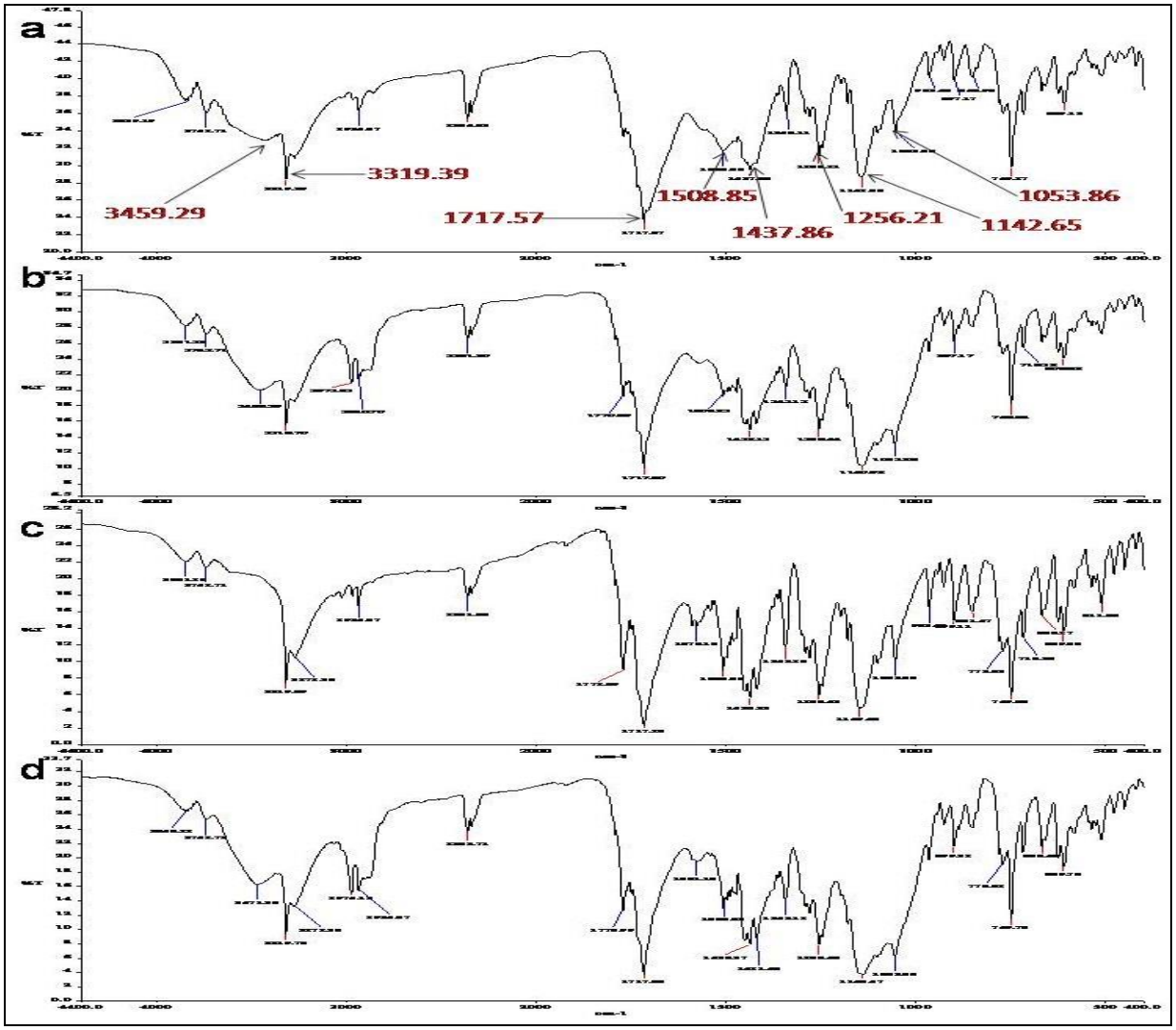

Figure 1: Drug: excipients compatibility studies; Spectra of Acelofenac(a), Acelofenac+ethyl cellulose (b), Acelofeac+polyvinyl alcohol (c), and Acelofenac-loaded microsponges (d).

Statistical analysis:

The BBD was used to investigate the effect of variables on the responses. The statistical model used for the optimization of the ACF-loaded microsponges was studied in terms of fit summary, lack of fit test, the sum of the square, correlation coefficient (R^2), adjusted R^2 and predicted R^2. The model used to evaluate the effect of variables on responses was analyzed by ANOVA, *F*-value, *P*-value, lack of fit, degree of freedom (df), Adjusted R^2 ($_{Adj}R^2$) and predicted R^2 ($_{Pred}R^2$). The goodness of fit of the proposed model was investigated by plotting diagnostic plots, such as externally studentized residuals vs. predicted plot, predicted vs. actual plot, normal probability plot, and externally studentized residuals vs. run number plot. The effect of variables on the responses was evaluated via response surface plots (3D) and contour plots (2D). The optimized values of the variables were estimated within the set criterion of desirability and further analyzed through overlay plots. Finally, optimized values of variables were selected for the development of final formulation.

RESULTS:

Drug-excipients compatibility studies:

The drug-excipients compatibility was evaluated using FTIR spectroscopy (Fig 1). The FTIR spectrum of ACF showed characteristic peaks at 3459.29 cm^{-1} (N-H stretching, amine group), 3319.39 cm^{-1} (N-H stretching, amine group), 1717.57 cm^{-1} (C=O stretch, carbonyl group), 1508.85 cm^{-1} (C-C stretch, aromatic), 1437.86 cm^{-1} (C-H stretch, alkane), 1256.21 cm^{-1} (C-O stretch, ester group), 1142.65 cm^{-1} (C-O-C stretch, ether group) and 1053.86 cm^{-1} (N-H bending, alkyl amine); Fig 1(a). These characteristic peaks of ACF were also prominent in the FTIR spectrum of physical mixtures and ACF-loaded microsponges; Fig.1(b-d). This confirms the compatibility of drug with the selected excipients and their suitability for their inclusion in the formulation.

Development of trial batch:

In order to confirm the formation of microsponges, an initial trial batch of plain microsponges was developed using quasi emulsion solvent diffusion method[29]. The microsponges with desired morphology and size were successfully prepared and harvested. The microscopic studies exhibited that the developed microsponges are of spherical shape with uniform size (Figure-2).

Drug-polymer ratio optimization:

The drug polymer ratios were optimized by evaluating the effect of different ratios on the shape, size and %EE of the microsponges (Table-2). A total of four formulations (MSP-1 to MSP-4) were developed having spherical microsponges with size and %EE in a range of 28.56 ± 2.8 µm to 35.22 ± 3.2 µm and 72.77 ± 4.3 % to 81.54 ± 2.7 %, respectively (Table-2). The formulation MSP-3 with a drug polymer ratio of 1.5:1 was considered as optimized formulation due to smaller size and highest entrapment efficiency.

Experimental design:

A 3-factor, 3-level BBD was used for the optimization of ACF-loaded microsponges. The independent variables selected were, amount of PVA (A), amount of ethanol (B) and stirring speed (C). The effect of these independent variables on particle size (Y1) and %EE (Y2) was assessed (Table-1).

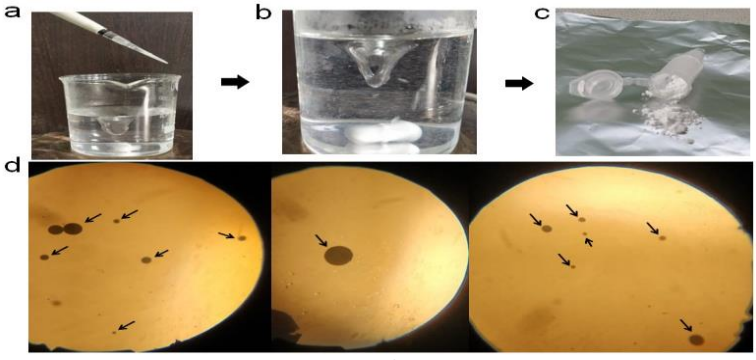

Figure 2: Development of the trial batch: (a) Dropwise addition of internal phase into external phase, (b) Continuous stirring, (c) Collection of dried microsponges and (d) Prepared microsponges

Table-2 Different formulations and their evaluation

Formulation code	Drug: Polymer ratio	Particle shape	Mean particle size (µm)	%EE
MSP-1	1:1	Spherical	32.42 ± 4.6	73.21 ± 1.7
MSP-2	1:1.5	Spherical	35.22 ± 3.2	77.12 ± 2.1
MSP-3	1.5:1	Spherical	28.56 ± 2.8	81.54 ± 2.7
MSP-4	2:1	Spherical	30.72 ± 3.5	72.77 ± 4.3

Table 3: Composition of different ACF-loaded microsponges formulations

Formulation code	Factor 1 A:PVA (mg)	Factor 2 B:Ethanol (ml)	Factor 3 C:Stirring (rpm)	Response 1 Size (µm)	Response 2 EE (%)
MS1	500	10	1250	29.95±3.41	84.32±1.75
MS2	500	15	1000	31.13±6.17	76.77±3.29
MS3	500	10	1250	29.92±4.41	84.51±1.81
MS4	500	10	1250	29.93±3.21	83.18±1.75
MS5	600	15	1250	27.54±4.11	81.29±3.78
MS6	600	10	1000	49.08±5.01	70.57±4.19
MS7	400	10	1500	24.37±4.32	85.62±1.38
MS8	500	10	1250	29.78±3.66	84.12±2.75
MS9	400	15	1250	25.67±2.66	85.78±4.52
MS10	500	5	1500	27.48±3.82	83.87±3.12
MS11	600	10	1500	29.32±4.59	86.43±2.58
MS12	500	10	1250	29.45±3.33	84.21±1.45
MS13	500	5	1000	34.73±5.36	70.72±4.79
MS14	400	10	1000	28.14±3.46	84.29±2.42
MS15	600	5	1250	30.85±4.65	81.05±3.11
MS16	500	15	1500	23.13±3.47	80.01±4.75
MS17	400	5	1250	22.54±2.85	84.11±2.84

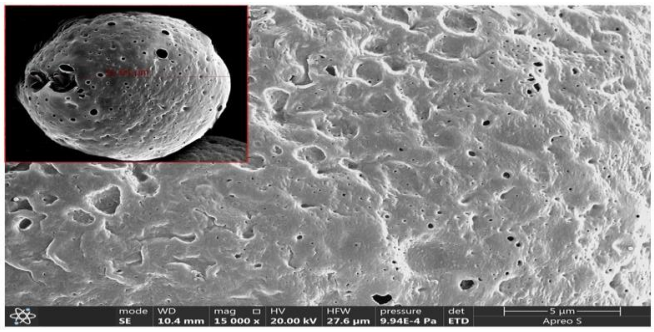

Figure 3: SEM images of the developed microsponges and the porous surface.

In total 17 formulations (MS1-MS17) were developed as per the BBD designed using quasi emulsion solvent diffusion method (Table-3). The developed formulations were characterized for particle size and %EE (Table-3).

Characterization of the ACF-loaded microsponges:

The developed formulations (MS1-MS17) were characterized for size, morphology and %EE. The microsponges were spherical in shape with a particle size range of 22.54±2.85 µm to 49.08±5.01 µm. The %EE of the developed formulations was found in the range of 70.57±4.19% to 86.43±2.58 %. The scanning electron microscopy images showed that the developed ACF-loaded microsponges were spherical shape with porous structure (Figure-3).

Model analysis:

Effect on size:

According to the fit summary, sequential model sum of square, model summary statistics and fit summary details (predicted R^2 ($_{Pred}R^2$), adjusted R^2($_{Adj}R^2$), *F*-value, *p*-value and degree of freedom), quadratic model was selected to evaluate the effect of variables on the size. The factors affecting the particle size, such as (A) quantity of PVA (B) volume of ethanol and (C) stirring time are included in the following equation,

Size (Y1) = +29.81+4.51*A-0.5162*B-5.35*C-1.61*AB-4.00*AC-1.19*BC-0.2730*A^2- 2.88*B^2+3.19*C^2

The model used to evaluate the effect on particle size was analyzed through analysis of variance (ANOVA). The ANOVA indicated the suitability andacceptance of the used model with F-value (12.14), p-value of 0.0017 (P<0.0500) and lack of fit values of 0.0001 (p<0.0500). The fit statistical analysis showed an adequate precision (ratio of signal to noise) value of 10.925 (greater than 4), which is desirable for the model to navigate the design space. The goodness of fit of the proposed model for the particle size was investigated using diagnostic plots; Fig 4 (a-d)). The normal plots of residuals showed a majority of the colored points indicating the value of particle size were located around the normal probability straight line. This further supports the normalcy of residuals and relevant analysis of response data; Fig.4 (a). The residual vs predicted values showed that the particle size lies within the set upper and lower limits. The plot showed a random distribution of the studentized residuals, which indicates that the assumption of constant variance is

true; (Fig.4 (b). The predicted vs actual plots showed that the experimentally observed values of particle size are in close agreement with predicted values; Fig.4 (c).

The effect of variables (A: amount of PVA, B: amount of ethanol and C: stirring time) on the particle size was evaluated using contour (2D) and response-surface (3D) plots. Contour and response-surface plots between amounts of PVA and ethanol, keeping stirring time constant showed that ethanol has no significant impact on the size. Whereas, increase in the amount of PVA resulted in an increase in the size; Fig.4 (d, g). Contour and response-surface plots between stirring speed and amount of PVA keeping the amount of ethanol constant showed that an increase in the stirring speed, decreases the particle size. Whereas, an increase in the amount of PVA increases the particle size; Fig.4 (e, h). Contour and response-surface plots between stirring speed and quantity of ethanol showed that an increase in stirring speed decreases the particle size. However, amount of ethanol had no significant effect on the particle size; Fig.4 (f, i).

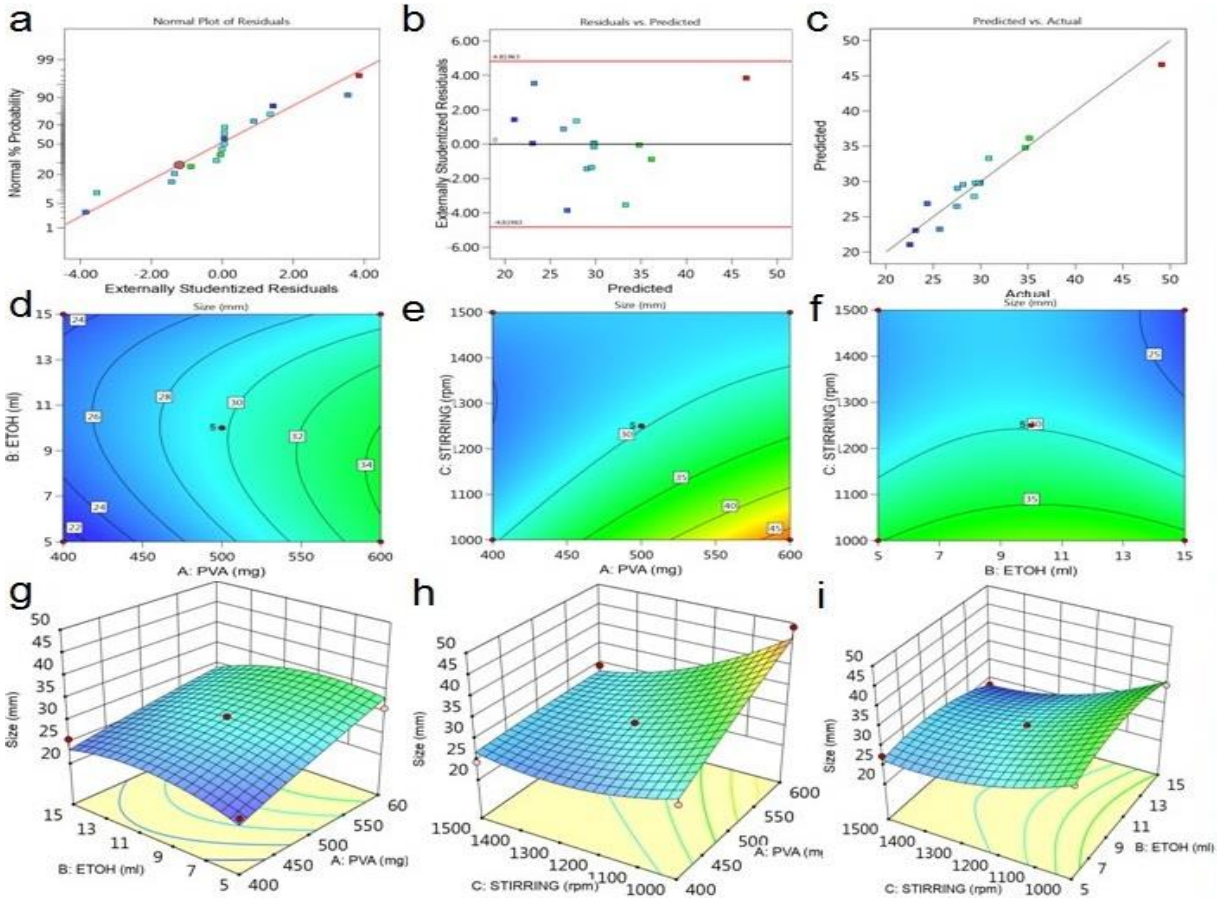

Figure 4: Effect of different variables on size. (a) normal plot of residuals, (b) residual vs predicted plot, (c) predicted vs actual plot, (d-f) 2D contour plots and (g-i) response surface 3D plots.

Effect on %EE:

According to the fit summary, sequential model sum of square, model summary statistics and fit summary details ($_{Pred}R^2$, $_{Adj}R^2$, *F*-value, *p*-value, PRESS and df), quadratic model was selected to evaluate the effect of variables on the %EE. The factors affecting the %EE are, (A) quantity of PVA (B) amount of ethanol and (C) stirring time as given in the following equation,

%EE (Y2) = +84.07-2.56*A+0.5125*B+4.20*C-0.3575*AB+3.63*AC-2.48*BC+1.44*A^2- 2.45*B^2-3.78*C^2

The model used to evaluate the effect on %EE was analyzed through analysis of variance (ANOVA). The ANOVA proved the suitability and acceptability of the used model with F-value (60.23), p-value of 0.0001 (P<0.0500) and lack of fit values 0.0212 (p<0.0500). The fit statistical analysis showed adequate precision (ratio of signal to noise) value of25.867 (greater than 4), which is desirable for the model to navigate the design space. The goodness of fit of the proposed model used to study the effect on %EE was investigated using diagnostic plots; Fig.5 (a-c). The normal plots of residuals showed a majority of the colored points indicating the value are located around

the normal probability straight line. This indicates the normalcy of residuals and relevant analysis of response data; Fig.5 (a). The residual vs predicted value plots showed that all the values indicating the % EE lie within the set upper and lower limits. The plot showed a random distribution of the studentized residuals, which indicates that the assumption of constant variance is true; Fig.5 (b). The predicted vs actual plot showed that the experimentally observed values of particle size are in close agreement with predicted values; Fig.5 (c).

The effect of variables (A: amount of PVA, B: amount of ethanol and C: stirring time) on the %EE was evaluated using contour (2D) and response-surface (3D) plots. Contour and response-surface plots between amounts of PVA and ethanol, keeping stirring time constant showed that ethanol has a significant impact on the %EE. Increase in the ethanol volume resulted in a significant increase in the %EE.

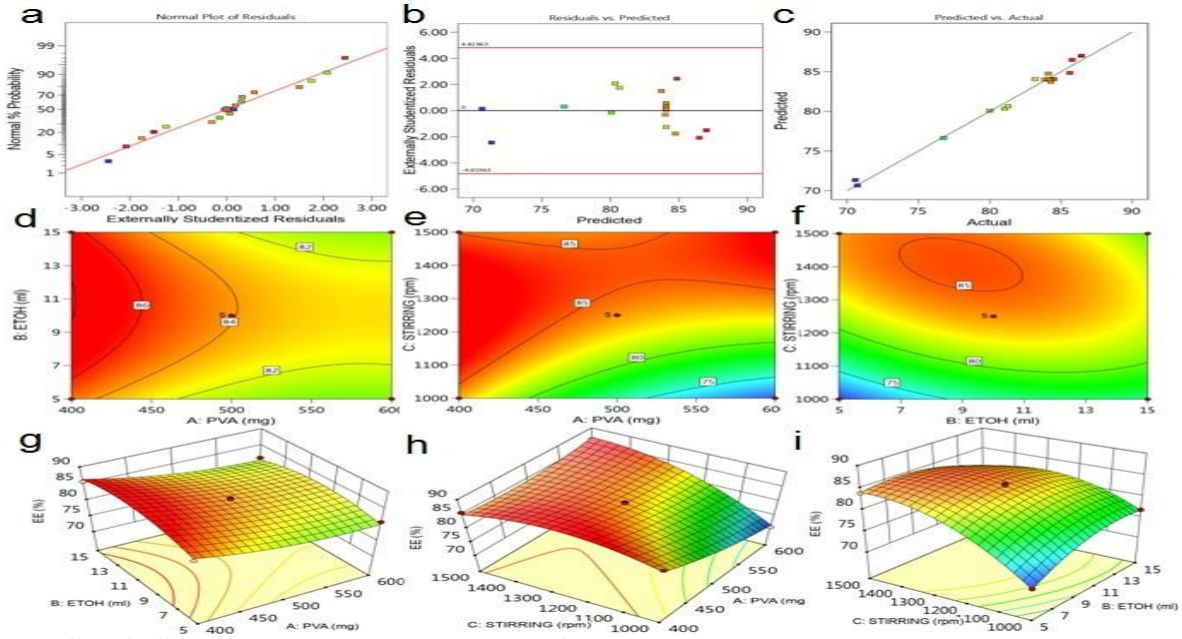

Figure 5: Effect of different variables on %EE. (a) normal plot of residuals, (b) residual vs predicted plots, (c) predicted vs actual plots, (d-f) 2D contour plots and (g-i) response surface 3D plots.

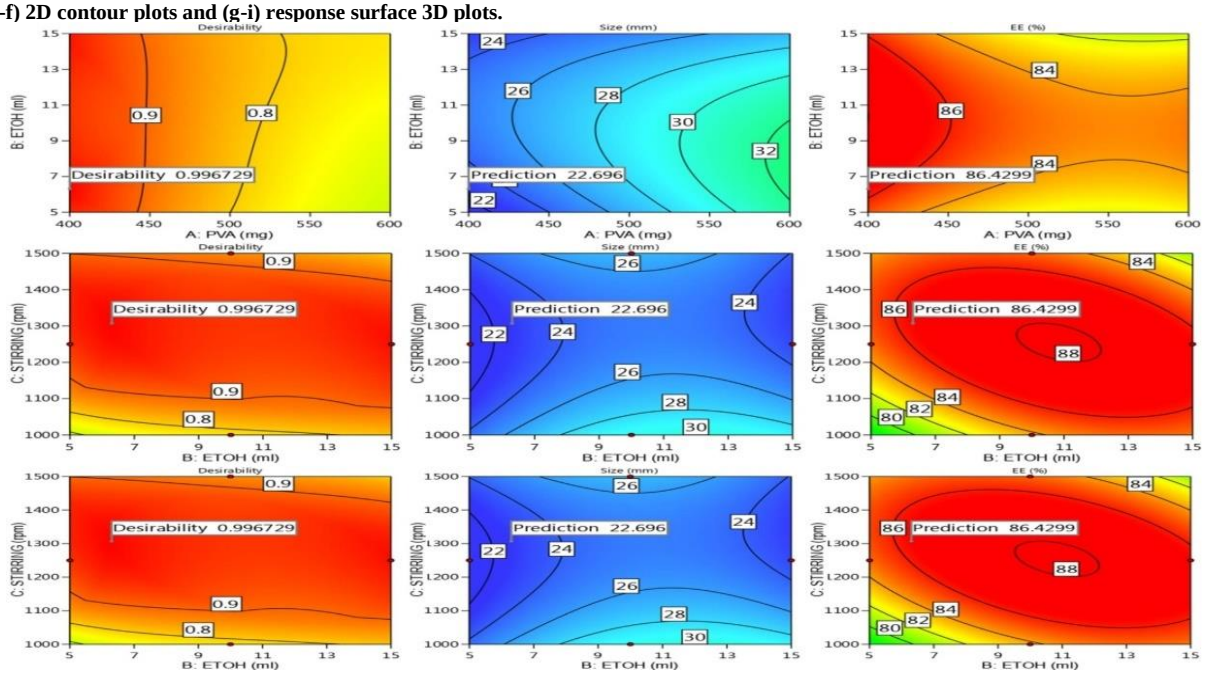

Figure 6: Contour plots showing desirability value and desired properties of the microsponges with different compositions

However, with an increase in amount of PVA, %EE was found to be decreased; Fig.5(d, g). Contour and response-surface plots between stirring speed and amount of PVA, keeping ethanol contents constant showed a rapid increase in %EE with an increase in stirring speed. Moreover, the increase in amount of PVA also increases the %EE in a linear manner; Fig.5(e, h). Contour and response-surface plots between stirring speed and ethanol contents showed a significant increase in % EE on increasing in stirring speed. There was a slight increase in %EE with an increase in ethanol content; Fig.5(f,i).

Optimization and desrability function:

Desirability function was analyzed to prepare an optimized formulation composition, which was obtained using the set predetermined desired quality parameters of the final product, such as small size and maximum %EE. A desirability value near to one is needed to obtain an optimized formulation composition. Desirability analysis helped in getting an optimized formulation composition (PVA- 400mg, ethanol-6.301 ml and stirring speed-1306 rpm) resulting in a formulation with the desired properties (size-22.69µm and %EE-86.43) with a desirability value of 0.997 (Fig. 6).

DISCUSSION:

The present investigation was aimed to utilize the QbD design models to evaluate the effect of variables on the quality attributes of the ACF-loaded microsponges. Moreover, desirability function was used to develop a formulation having desired properties. Initially, the drug-excipients compatibility was confirmed via FTIR spectroscopy (Fig. 1). Test formulations in the form of plain microsponges were developed to confirm the suitability of the selected method (Quasi-emulsion diffusion method). The developed plain microsponges were found to be spherical in shape and exhibited predominantly uniform size distribution (Fig. 2). Thereafter, the drug-polymer ratio was optimized for the development of desired formulations.

In total four formulations were developed with variable drug-polymer ratios and evaluated for size and %EE (Table-3). The results showed that with an increase in the polymer concentration, the microsponge size and % EE were also increased from 32.42µm to 35.22µm and 73.21% to 77.12%, respectively (Table-3). An increase in the drug amount (1.5:1) led to the decrease in size (28.56µm) and an increase in %EE (81.54%). However, further increase in the drug amount (2:1) resulted in an insignificant increase in particle size (30.72µm) and decrease in %EE (72.77µm), (Table-3). Finally, the drug-polymer ratio (1.5:1) was selected as an optimized ratio.

A 3-factor, 3-level BBD was used for the optimization of ACF-loaded microsponges. The effect of variables (amount of PVA andethanol and stirring speed) on microsponge properties, such as size and %EE was evaluated. Based on BBD, total seventeen formulations (MS1-MS17) were developed with variable compositions and at different stirring speed (Table-2). The developed formulations were evaluated for their size, %EE and morphology. The developed formulation showed the size and % EE ranging between 22.54±2.85 µm to 49.08±5.01 µm and 70.57±4.19% to 86.43±2.58 %, respectively. In addition, the developed microsponges were spherical in shape with porous structure(Fig. 3).

A quadratic model was selected to investigate the effect of the variables on the microsponge size. The ANOVA and fit statistical analysis showed that the selected model was significant ($P<0.0500$) and suitable for analyzing variables effects (signal to noise ratio-10.925). The diagnostic plots for analyzing the goodness of fit of the selected model showed the observed values of the developed formulations within the acceptable limits; Fig 4 (a-c). The contour and response plots showed an increase in size with an increase in PVA amount. However, with an increase in the stirring speed, the size decreased; Fig 4 (d-i). A high, stirring speed resulted in high turbulence that caused frothing and subsequent adhering of the microsponges to the container wall resulting in decrease in size. At low stirring speed, the microsponges tend to adhere to each other resulting in increased size.[30] Moreover, an increase in the amount of PVA increased the microsponge size. This could be due to an increase in an apparent viscosity which resulted in larger droplets and hence larger microsponges.[31]

The quadratic model used to investigate the effect of variables on % EE was found significant and the observed values were found under acceptable limits as per the diagnostic plots; Fig 5 (a-c). The contour and response plots showed a decrease in %EE with an increase in amount of PVA. However, there was an increase in %EE with an increase in ethanol concentration; Fig 5 (d-i). An increase in the amount of PVA resulted in a slight increase in viscosity. As a result, when the solvent diffused out, the dispersed phase converted into microsponges of large size with greater %EE.[31] The reduced diffusion rate of ethanol in a concentrated solution resulted in extended time for droplet formation and an increased precipitation of the drug. This led to an increase in %EE. A slow diffusion of ethanol from the concentrated polymeric solution resulted in a longer time for droplet formation which in turn may increase the drug precipitation in the microsponges and hence the %EE.[32] Finally, the optimized formulation composition with desired

properties was established through desirability function. The optimized formulation composition was noted to be PVA- 400mg, ethanol-6.301 ml prepared at a stirring speed of 1306 rpm. This formulation showed the desired properties (size-22.69µm and %EE-86.43) (Fig 6). The desirability function value for this formulation was found to be 0.997.

CONCLUSION:

A topical ACF-loaded microsponge formulation based on ethyl cellulose was successfully developed using quasi-emulsion solvent diffusion method. The formulation for topical use was developed in order to reduce the frequency of administration, irritation reactions and other side effects associated with conventional oral ACF formulations. It was noted that the amount of PVA and ethanol along with stirring speed significantly affect the microsponge size and % EE. These variables can be optimized to customize the formulation with desired properties. It can be concluded that the QbD approach is a valuable tool for the development of an optimized microsponge formulation with desired size and %EE for topical application.

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RESEARCH ARTICLE

Synthesis and Biological evaluation of some newer Pyrazole Derivatives

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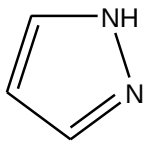
ABSTRACT:
Pyrazoline is a 5-membered heterocyclic moiety has two adjoining nitrogen iotas and three carbon particles inside the ring. Pyrazoline subordinate are related wide scope of pharmacological and restorative exercises, for example, antibacterial, antifungal, pain relieving, calming, hostile to parasitic, against malarial, against oxidant. The ongoing work of exploration is fundamentally engaged at the revelation and improvement of a progression of 1,3,5-trisubstituted pyrazoline. A progression of new 1-phenyl-3-(4-nitrophenyl)- 5-(subbed phenyl) pyrazoline subordinates (2a-2j) were blended by the response of subbed acetophenone and subbed benzaldehyde within the sight of fluid sodium hydroxide arrangement by Claisen Schmidt buildup system. The subbed chalcone were integrated which is additionally dense with phenyl hydrazine in ethanol and results in the definition of conclusive subordinates of pyrazoline (2a-2j). The response blend was observed by TLC and the last mixes were refined by recrystallization from wanted dissolvable. All the structures of blended mixes were affirmed by FTIR, 1H NMR, mass unearthly information and essential investigation. All the recently combined mixes (2a-2j) were assessed for antibacterial and antifungal movement. Mixes 2f, 2i and 2h showed powerful inhibitory impact against on strains of both bacterial and parasitic species.

KEYWORDS: Pyrazoline, Antibacterial, Antifungal, Chalcone, Substituted benzaldehyde.

INTRODUCTION:
Pyrazoline is a five-membered heterocyclic compound with atomic recipe $C_3H_6N_2$. Pyrazole contains two nitrogen molecules which are available at contiguous situations in regard to one another[1]. Among these two nitrogen particles, one has fundamental character and the different gets nonpartisan conduct in nature[2]. Pyrazoline is rich of pi-electrons and the fragrant conduct in pyrazoline shows up from the nearness of solitary sets present on the – NH nitrogen[3].



Pyrazole

Pyrazole core are available in a few driving medications, for example, Viagra, celebrex and so on pyrazoles is a raised plateform for a few examination programmes in agro compound just as pharmaceutical field[4].

Pyrazoline additionally containing two nitrogen particles however there is just single or one endocyclic bond[5]. Pyrazoline subsidiary are related wide scope of pharmacological and restorative exercises, for example, antibacterial, antifungal, pain relieving, calming, against parasitic, hostile to malarial[6]. Overview of some audit writing clarified that the pyrazoline subsidiaries have cerebro defensive impact and CNS depressant action[7]. We have integrated a progression of 1-phenyl-3-(4-nitrophenyl)- 5-(subbed phenyl) pyrazoline subordinates (2a-2j) by utilizing subbed chalcone subsidiaries (1a-1j). All the recently combined subsidiaries were assessed for antibacterial and antifungal exercises separately[8]. The antibacterial action was tried against Gram negative and Gram-positive microscopic organisms' on Nutrient agar media plates by paper circle dissemination strategy. Then again, the Antifungal action was completed against types of parasites strains on Nutrient agar media by circle dispersion strategy[9].

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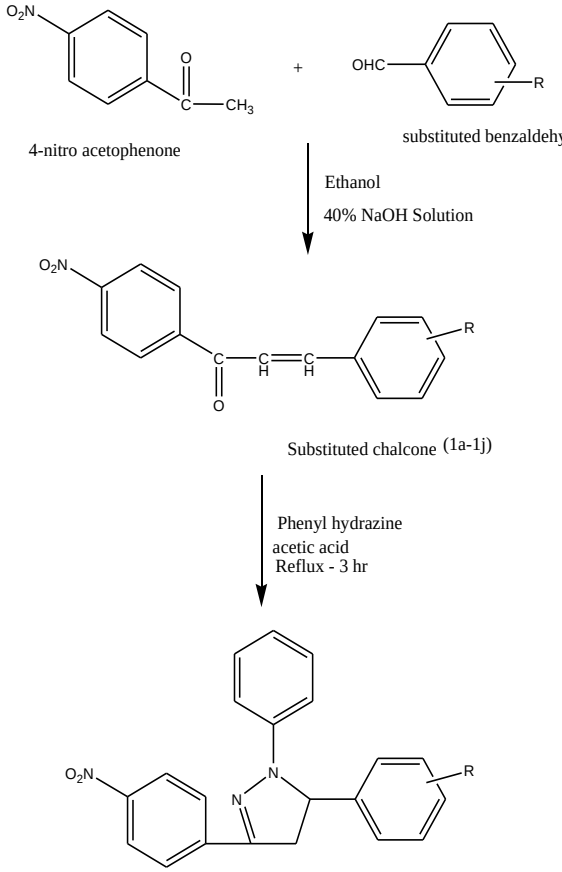
MATERIAL AND METHOD:

All synthetics utilized in the union were of engineered grade and were provided by CDH Delhi, Qualigens Fine Labs Mumbai, S d fine chem. Ltd. Mumbai, Loba synthetic concoctions ltd. Dissolving purpose of the orchestrated mixes was finished by open cylinder slender strategy. Cleaning of the mixes was checked by TLC by utilizing Chloroform: Methanol (1:1). Spots were seen under iodine chamber. IR spectra 1H NMR-spectra were recorded in DMSO or $CDCl_3$ utilizing TMS as the interior norm. (Compound move in ppm) and mass spectra were recorded on water OPLCTQDMS in positive mode[10].

Scheme of work:



1-phenyl-3-(4-nitrophenyl)-5-(substituted phenyl) pyrazoline (2a-2j)

Table 1: Substitution of Derivatives (2a-2j)

Compounds	R
1.	2-Chloro
2.	3-chloro
3.	2-nitro
4.	3-nitro
5.	4-nitro
6.	2-hydroxy
7.	3,4,5-trimethoxy
8.	4-methoxy
9.	4-fluoro
10.	4-dimethylamino

General procedure for Synthesis of 1-(4-Nitrophenyl)-3-(substitutedphenyl) prop-2-en-1-one (1a-1j):-

A blend of p-Nitroacetophenone (0.02mol) and fragrant subbed Benzaldehyde (0.02mol) was broken down in ethanol (40ml) and the arrangement was warmed to the bubbling. To this arrangement 20ml (40% NaOH) was included with consistent blending for 8 hour at room temperature to make the arrangement soluble. The strong mass was gotten which was kept for the time being and fermented by 10% HCl washed with NaHCO3 followed by water and recrystallized with ethanol[11].

Step:-2 Synthesis of 1-phenyl-3-(4-nitrophenyl)-5-(substituted phenyl) pyrazoline derivatives (2a-2j):-

A blend of substituted chalcone (0.05mol) react with phenyl hydrazine (0.05mol) in the presence of acetic acid (20ml) was refluxed for 3 hour. At that point filled ice cooled water. The hasten was isolated by filtration, washed liberated from corrosive to offered pyrazoline moiety and afterward dried and recrystallized from ethanol. The completion of reaction was monitored by TLC[12].

5-(2-chlorophenyl)-4,5-dihydro-3-(4-nitrophenyl)-1-phenyl-1*H*-pyrazole (2a):

Yield 55%, R_f value- 0.64 m.p. 193°C ; FTIR (KBr) $\nu_{max}cm^{-1}$: 3028.47 (Ar C-H, str.), 1674.31 (C=C, str.), 1528.12 (C=N, str.), 1322.18 (C-N, str.), 770.59 (C-Cl, str.), 1617.14 (C-C, str.), 1508 (Ar-NO_2, str.); ^{1}H NMR (DMSO-d_6): δ 1.017-1.134 (d, 2H, C-H of aromatic pyrazoline), 4.026-4.209 (t, 1H, aromatic pyrazoline C-H), 6.126-6.892 (m, 9H, Ar-H), 7.912-8.091 (dd, 4H, Ar-H); Anal Calcd. For $C_{21}H_{16}ClN_3O_2$: C, 66.76; H, 4.27; N, 11.12. Found: C, 66.74; H, 4.29; N, 11.13%.

5-(3-Chlorophenyl)-4,5-Dihydro-3-(4-nitrophenyl)-1-phenyl-1*H*-pyrazole (2b):

Yield 51%, R_f value- 0.62 m.p. 193°C ; FTIR (KBr) $\nu_{max}cm^{-1}$: 3073.86 (Ar C-H, str.), 1648.26 (C=C, str.), 1530.28 (C=N, str.), 1305.43 (C-N, str.), 768.95 (C-Cl, str.), 982.36 (C-C, str.), 1325.38 (Ar-NO_2, str.); ^{1}H NMR (DMSO-d_6): δ 1.512-1.662 (d, 2H, C-H of aromatic pyrazoline), 4.132-4.398 (t, 1H, aromatic pyrazoline C-H), 6.235-7.012 (m, 9H, Ar-H), 7.801-8.001 (dd, 4H, Ar-H); Anal Calcd for $C_{21}H_{16}ClN_3O_2$: C, 66.76; H, 4.27; N, 11.12 Found: C, 66.74; H, 4.28; N, 11.14%.

4,5-dihydro-5-(2-nitrophenyl)-3-(4-nitrophenyl)-1-phenyl-1*H*-pyrazole (2c):

Yield 51%, R_f value- 0.56 m.p. 193°C ; FTIR (KBr) $\nu_{max}cm^{-1}$: 3058.34 (Ar C-H, str.), 1630.32 (C=C, str.), 1550.81 (C=N, str.), 1317.14 (C-N, str.), 1623.36 (C-C, str.), 1373.15 (Ar-NO_2, str.); ^{1}H NMR (DMSO-d_6): δ 1.653-1.812 (d, 2H, C-H of aromatic pyrazoline), 3.879-4.011 (t, 1H, aromatic pyrazoline C-H), 6.612-7.398 (m,

13H, Ar-H); Anal Calcd for $C_{21}H_{16}N_4O_4$: C, 64.94; H, 4.15; N, 14.43 Found: C, 64.92; H, 4.16; N, 14.43%.

4,5-dihydro-5-(3-nitrophenyl)-3-(4-nitrophenyl)-1-phenyl-1*H*-pyrazole (2d):
Yield 65%, R_f value- 0.57 m.p. 191°C ; FTIR (KBr) $\nu_{max}cm^{-1}$: 3036.47 (Ar C-H, str.), 1648.23 (C=C, str.), 1536.64 (C=N, str.), 1223.65 (C-N, str.), 1617.55 (C-C, str.), 1436.58 (Ar-NO_2, str.); **^{1}H** NMR (DMSO-d_6): δ 1.698-1.818 (d, 2H, C-H of aromatic pyrazoline), 3.851-4.097 (t, 1H, aromatic pyrazoline C-H), 7.242-8.246 (m, 13H, Ar-H); Anal Calcd for $C_{21}H_{16}N_4O_4$: C, 64.94; H, 4.15; N,14.43 Found: C, 64.93; H, 4.12; N, 14.41%

4,5-dihydro-3,5-bis-(4-nitrophenyl)-1-phenyl-1*H*-pyrazole (2e):
Yield 65%, R_f value- 0.55 m.p. 191°C ; FTIR (KBr) $\nu_{max}cm^{-1}$: 3041.21 (Ar C-H, str.), 1642.52 (C=C, str.), 1538.42 (C=N, str.), 1248.51 (C-N, str.), 1619.25 (C-C, str.), 1398.38 (Ar-NO_2, str.); ^{1}H NMR (DMSO-d_6): δ 1.931-2.101 (d, 2H, C-H of aromatic pyrazoline), 3.856-4.001 (t, 1H, aromatic pyrazoline C-H), 7.233-8.012 (m, 13H, Ar-H); Anal Calcd for $C_{21}H_{16}N_4O_4$: C, 64.94; H, 4.15; N,14.43% Found: C, 64.91; H, 4.15; N, 14.41%

2-(4,5-dihydro-3-(4-nitrophenyl)-1-phenyl-1*H*-pyrazol-5-yl)phenol (2f):
Yield 65%, R_f value- 0.59 m.p. 191°C ; FTIR (KBr) $\nu_{max}cm^{-1}$: 3076.96 (Ar C-H, str.), 1649.59 (C=C, str.), 1610.03 (C=N, str.), 1305.48 (C-N, str.), 975.13 (C-C, str.), 1394.39 (Ar-NO_2, str.), 3293.76 (C-OH, str.); ^{1}H NMR (DMSO-d_6): δ 2.178-2.363 (d, 2H, C-H of aromatic pyrazoline), 4.131-4.399 (t, 1H, aromatic pyrazoline C-H), 7.135-7.912 (m, 13H, Ar-H), 9.891 (s, 1H, OH); Anal Calcd for $C_{21}H_{17}N_3O_3$: C, 70.18; H, 4.77; N, 11.69 Found: C, 70.20; H, 4.74; N, 11.68%

4,5-dihydro-5-(3,4,5-trimethoxyphenyl)-3-(4-nitrophenyl)-1-phenyl-1*H*-pyrazole (2g):
Yield 56%, R_f value- 0.72 m.p. 220°C ; FTIR (KBr) $\nu_{max}cm^{-1}$: 3066.21 (Ar C-H, str.), 1641.87 (C=C, str.), 1611.64 (C=N, str.), 1338.25 (C-N, str.), 1015.43 (C-C, str.), 1391.93 (Ar-NO_2, str.), 1243.28 (C-O, str.), 2921.30 (Aliphatic C-H, str.); ^{1}H NMR (DMSO-d_6): δ 2.475-2.593 (d, 2H, C-H of aromatic pyrazoline), 3.532-3.793 (t, 1H, aromatic pyrazoline C-H), 6.905-7.812 (m, 9H, Ar-H), 6.291 (s, 1H, Ar-H), 5.219-5.742 (s, 9H, OCH_3); Anal. Calcd for $C_{24}H_{23}N_3O_5$: C, 66.50; H, 5.35; N, 9.65 Found: C, 66.52; H, 5.32; N, 9.67%

4,5-dihydro-5-(4-methoxyphenyl)-3-(4-nitrophenyl)-1-phenyl-1*H*-pyrazole (2h):
Yield 56%, R_f value- 0.70 m.p. 220°C ; FTIR (KBr) $\nu_{max}cm^{-1}$: 3085.17 (Ar C-H, str.), 1663.36 (C=C, str.), 1534.54 (C=N, str.), 1323.98 (C-N, str.), 1614.63 (C-C, str.), 1466.23 (Ar-NO_2, str.), 1236.08 (C-O, str.), 2910.24 (Aliphatic C-H, str.); ^{1}H NMR (DMSO-d_6): δ 2.685-2.893 (d, 2H, C-H of aromatic pyrazoline), 4.421-4.693 (t, 1H, aromatic pyrazoline C-H), 5.246-6.246 (m, 9H, Ar-H), 3.501 (s, 3H, OCH_3), 6.972-7.386 (dd, 4H, Ar-H); Anal. Calcd for $C_{22}H_{19}N_3O_3$: C, 70.76; H, 5.13; N, 11.25 Found: C, 70.74; H, 5.15; N, 11.24%

5-(4-fluorophenyl)-4,5-dihydro-3-(4-nitrophenyl)-1-phenyl-1*H*-pyrazole (2i):
Yield 65%, R_f value- 0.68 m.p. 191°C ; FTIR (KBr) $\nu_{max}cm^{-1}$: 3058.83 (Ar C-H, str.), 1621.26 (C=C, str.), 1609.55 (C=N, str.), 1329.95 (C-N, str.), 1038.58 (C-C, str.), 1379.38 (Ar-NO_2, str.), 813.57 (C-F, str.); ^{1}H NMR (DMSO-d_6): δ 3.235-3.393 (d, 2H, C-H of aromatic pyrazoline), 4.629-4.823 (t, 1H, aromatic pyrazoline C-H), 5.496-6.546 (m, 5H, Ar-H), 7.289-7.991 (dd, 4H, Ar-H), 8.151-8.491 (dd, 4H, Ar-H); Calcd for $C_{21}H_{16}N_3O_2$: C, 69.80; H, 4.46; N, 11.63 Found: C, 69.78; H, 4.48; N, 11.60%

4-(4,5-dihydro-3-(4-nitrophenyl)-1-phenyl-1*H*-pyrazole-5-yl)-N,N-dimethylbenzene (2j):
Yield 65%, R_f value- 0.77 m.p. 191°C ; FTIR (KBr) $\nu_{max}cm^{-1}$: 2974.12 (Ar C-H, str.), 1637.59 (C=C, str.), 1603.59 (C=N, str.), 1326.09 (C-N, str.), 1108.81 (C-C, str.), 1328.85 (Ar-NO_2, str.), 2953.87 (Aliphatic C-H, str.); ^{1}H NMR (DMSO-d_6): δ 2.981-3.113 (d, 2H, C-H of aromatic pyrazoline), 4.571-4.793 (t, 1H, aromatic pyrazoline C-H), 9.112-9.998 (m, 5H, Ar-H), 6.321-6.413 (dd, 4H, Ar-H), 7.521-7.613 (dd, 4H, Ar-H), 5.140-5.290 (s, 6H, Ar-N(CH_3)$_2$); Anal. Calcd for $C_{23}H_{22}N_4O_2$: C, 71.48; H, 5.74; N, 14.50 Found: C, 71.46; H, 5.72; N, 14.52%

Antibacterial activity:
The antibacterial activity of as of late fused auxiliaries of pyrazole was evaluated by paper plate dispersion strategy using supplement agar medium against following microorganism: S. aureus, B. subtilis, (Gram positive) and E. coli (Gram negative).

In the method for paper plate dispersion, the paper hover impregnated with blends separate in DMSO at center 25, 50 and 100µg ml^{-1} were used. Hover impregnated with DMSO was used as dissolvable control for antibacterial development because of free dissolvability of test blends. The microorganism culture was spread over enhancement agar media in petri dishes, and a short time later the hover impregnated with the course of action was determined to the outside of the media inoculated with the bacterial strain[13].

The plates were brought forth at 35°C for 24 hrs for bacterial social orders. In the wake of incubating, the zones of restraint around the circle were viewed. The zones of hindrance show that the blends prevent improvement of microorganism. Each testing is done in triplicate.

Table 2: Antibacterial activities of synthesized compounds

Code of compound	Diameter of zone of inhibition (mm)[mean ±S.D. (n=3)]								
	B.subtilis			*E. coli*			*S. aureus*		
	25 µg ml^{-1}	50 µg ml^{-1}	100 µg ml^{-1}	25 µg ml^{-1}	50 µg ml^{-1}	100 µg ml^{-1}	25 µg ml^{-1}	50 µg ml^{-1}	100 µg ml^{-1}
2a	3.22±0.31	5.19±0.52	10.21±0.42	3.28±0.39	3.20±0.40	13.18±0.42	3.25±0.45	3.28±0.53	10.23±0.32
2b	4.51±0.52	6.61±0.43	11.56±0.50	3.20±0.40	5.76±0.61	11.08±0.44	4.62±0.63	7.36±0.26	10.66±0.75
2c	6.29±0.40	7.23±0.52	11.5±0.45	4.59±0.84	8.76±0.83	10.28±0.45	4.56±0.26	10.76±0.86	11.79±0.80
2d	4.23±0.52	6.26±0.38	10.36±0.50	6.16±0.35	9.96±1.00	11.3±0.40	0.00±0.00	5.36±0.40	10.62±0.82
2e	4.72±0.42	8.56±0.40	12.42±0.39	7.43±0.60	13.76±1.00	11.4±0.42	5.06±0.40	7.52±0.40	12.60±0.82
2f	4.36±0.40	9.56±0.41	14.62±0.42	4.38±0.40	6.55±0.45	10.28±0.60	4.83±0.70	6.76±0.78	14.16±1.00
2g	4.84±0.41	10.48±0.30	17.36±0.50	3.34±0.82	4.63±0.84	15.68±0.60	4.10±0.40	6.94±0.60	11.44±1.00
2h	6.58±0.30	11.62±0.40	12.92±0.40	4.63±0.41	12.96±0.45	13.0±0.40	4.55±0.60	6.91±1.02	16.04±0.60
2i	6.83±0.30	12.08±0.31	19.35±0.40	6.15±0.60	8.92±0.82	14.4±0.80	6.38±1.04	8.55±0.63	13.42±0.60
2j	4.62±0.20	6.38±0.41	14.38±0.40	4.83±0.21	11.42±0.20	13.69±0.30	3.75±0.30	5.58±0.42	11.90±0.31
Ciprofloxacin	-	-	19.48±0.30	-	-	20.52±0.40	-	-	19.68±0.90

Ciprofloxacin at fixation 100 µg ml-1 was used as standard medicine for antibacterial development. The recently integrated mixes 2g, 2i and 2j have greatest strength against B.subtilis. Mixes 2f and 2h displayed most extreme movement against *S.aureus*. Mixes 2g and 2i contain most extreme intensity against E.coli[14].

Antifungal activity:

The as of late mixed blends were pursued for their antifungal activity using circle dispersion method on supplement agar media. The in vitro assessment was cultivated for all the coordinated blends for the connection of antifungal activity. The goings with strains were used: *Aspergillus niger* and *Candida albicans*.

A spore suspension in ordinary saline was set up from the lifestyle of the test living beings on sabouraud's stock media. Each Petri dish was orchestrated into 5 identical portions along the estimation to put one plate. Three plate of test were put on three sections alongside one hover with reference sedate Fluconazole and a plate impregnated with the dissolvable (DMF) as a negative control[15]. Test were attempted at 25, 50, 100 µg/ml obsession in DMF. Fluconazole in the grouping of 50µg/ml was used as standard drug for antifungal activity.

The petri plates inoculated with infectious social orders were agonized at 25℃ for 48 hrs. Antifungal activity was directed by evaluating the separation across of the limitation zone for triplicate sets. The separations across got for the test was differentiated and that made by the standard drug Fluconazole. the recently integrated mixes 2a, 2h and 2i indicated great antifungal movement for both parasitic strains when given at fixation 50µg ml-1. Results were deciphered in distance across (mm) as far as zone of restraint[16].

RESULT AND DISCUSSION:

another arrangement of 1-phenyl-3-(4-nitrophenyl)- 5-(subbed phenyl) pyrazoline were inferred by the response of subbed acetophenone and subbed benzaldehyde within the sight of fluid sodium hydroxide arrangement by Claisen Schmidt buildup component[17]. The subbed chalcone were combined which is additionally consolidated with phenyl hydrazine in ethanol and results in the plan of definite subordinates of pyrazoline (2a-2j). The response blend was checked by TLC which is performed on silica gel G plates and the last mixes were separated and cleaned by recrystallization from wanted dissolvable[18].

Table 3: Antifungal activity of synthesized compound

Code of compound	Diameter of zone of inhibition in mm (mean± S.D... (n=3)]					
	C. albicans			*A. niger*		
	25 µg ml^{-1}	50 µg ml^{-1}	100 µg ml^{-1}	25 µg ml^{-1}	50 µg ml^{-1}	100 µg ml^{-1}
2a	6.24±0.21	12.72±0.22	21.28±0.30	6.05±0.12	9.05±0.24	14.30±0.10
2b	4.51±0.10	10.40±0.14	16.50±0.31	2.72±0.10	9.66±0.35	11.40±0.50
2c	8.40±0.20	8.18±0.21	20.18±0.26	5.70±0.10	7.31±0.32	16.35±0.33
2d	5.66±0.90	10.32±0.10	18.78±0.15	5.72±0.05	10.12±0.20	19.39±0.21
2e	3.88±0.60	7.70±0.06	13.08±0.08	1.66±0.05	7.45±0.10	10.58±0.36
2f	3.50±0.20	9.11±0.12	10.52±0.35	4.78±0.15	11.70±0.12	15.49±0.32
2g	2.36±0.10	9.65±0.20	15.45±0.72	4.76±0.08	10.34±0.17	13.35±0.10
2h	11.48±0.11	13.20±0.40	25.81±0.70	8.20±0.20	11.66±1.08	21.86±0.80
2i	7.40±0.20	13.56±0.18	24.46±0.10	2.35±0.05	12.26±0.50	11.54±0.21
2j	5.36±0.10	9.45±0.42	15.45±0.18	3.20±0.38	10.34±0.15	16.15±0.30
Fluconazole	-	16.65±0.06	-	-	16.85±0.16	-
Control	-	-	-	-	-	-

The structures of recently integrated subordinates (2a, 2b, 2c, 2d, 2e, 2f, 2g, 2h, 2i, 2j) were tried and affirmed by FTIR, 1H NMR, and Mass otherworldly information. The FTIR spectra of recently orchestrated subordinates demonstrated the nearness of trademark ingestion band in the district 3100-3000 cm^{-1} for sweet-smelling C-H str., 1610-1520 cm^{-1} for C=N extending, and 1350-1220 cm-1 for C-N extending, 1680-1530 cm-1 for C=C extending, 1360-1310 cm^{-1} for C-N extending (tertiary str). 1H NMR spectra of integrated subsidiaries showed the trademark tops in the area 6.673-8.035ppm for sweet-smelling protons, 3.501 for – OCH3 protons, 9.891ppm for – OH protons. The outcomes affirmed that recently orchestrated mixes 2g, 2i and 2j have most extreme power against B.subtilis. Mixes 2f and 2h showed most extreme movement against S.aureus. Mixes 2g and 2i contain most extreme power against E.coli. Mixes 2a, 2h and 2j were seen as moderate strength against E.coli[19]. Mixes 2a, 2h and 2i showed greatest strength against C.ablicans. Subsidiaries 2f, 2h and 2i have great action against A. niger. In last of the outcome, the electron withdwaring, electron giving and electronegative gatherings on benzene ring increment the strength of mixes.

CONCLUSIONS:

The blend os 1,3,5-trisubstituted pyrazoline by the above strategy brought about excellent yields and affirmed by FTIR, 1H NMR, Mass unearthly information, essential investigation, organic examinations indicated that mixes displayed huge antibacterial and antifungal movement.

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RESEARCH ARTICLE

In vitro analysis of synergistic effect of honey against chemically induced hepatic insult in Balb/c mice.

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ABSTRACT:

Human being knows honey since thousands of years for its nutritional and medicinal values. Traditional medicine systems like Ayurveda have elaborated honey as boon for health and patient care. Synergistic effect of honey was research but to a limited extend. Honey is advised with *Picrorhiza kurroa* for hepatic disorders or hepato-protection in Ayurveda. Here we have examined *in vitro* synergistic effect of honey when given with *Picrorhiza kurroa* in acetaminophen-induced hepatotoxicity in Balb/c mice model. We obtained Serum and liver lysate to check levels of hepatic markers, Alkaline phosphatase and Glutathione. The level of alkaline phosphatase raised while glutathione level is reduced during hepatic offence. We observed a compensation of above markers when *P. Kurrua*, honey and its combination were used in acetaminophen induced hepatic toxicity. We observed a better mice weight gain in combination group (Group IV) compared to control group. This study can pave a way for future research on honey as a better adjuvant for hepatotoxic drugs and other herb-drug interactions researches.

KEYWORDS: Ayurveda, *Yogavahi*, Synergism, hepato-protection, honey.

INTRODUCTION:

Ayurveda is a holistic health system. It is being recognized as a source of lead not only in the area of drug development but also in the field of therapeutic approaches. Its preventive and curative approaches are more nearer to nature and the drug formulations have minimal side effects.(Pushpalata et al., 2019, Srinivash et al., 2015) In traditional Medicine system, honey is extensive used as adjuvant or stand-alone therapy. The pH of honey ranges from 3.2 to 4.5. Honey has shown positive results for the control of pathogens or the improvement of human health (Samarghandian et al., 2017; Eteraf-Oskouei et al., 2013; Johnson et al., 2013; Pooja M et al., 2019).

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Inhibin, Hydrogen peroxide, high amount of catalase, and osmotic effect are few possible responsible for antibacterial activity of honey. It act was against many pathogenic organisms, like *Salmonella, Shigella* , *Staphylococcus aureus* and *enteropathogenic E. coli, Proteus mirabilis,* (Mandal et al., 2011; Mama et al., 2019; Almasaudi et al., 2017; Eteraf-Oskouei et al., 2013; Hashim et al., 2013). Honey is a good antioxidant, gastro-protective, wound healer and suitable sweetener for the diabetes.(Samarghandian et al., 2017; Eteraf-Oskouei et al., 2013). Honey is an effective measure to reduce the impact of radiation therapy in head and neck cancer cases. (Regupriya et al., 2020) (Kumbhar et.al., 2013). It is useful for many gynaecological, (Shikha S et al., 2009)Honey promotes wound healing (Wissam Z et.al., 2018).

Table 1- Studies on *Yogavahi* or synergistic property of honey

Study	Study model	Preparation	Results	Reference
In vitro study	15 bacterial strains (7 *Pseudomonas* and 8 *Klebsiella* species) isolated from bile, blood, pus, ascitic fluid etc.	Honey+ Gentamicin, Amikacin and Ceftazidime	Synergistic action seen in the case of *Pseudomonas* species, not with *Klebsiella species.*	Karayil S, et al., 1998
In vitro study	*Aspergillus niger* (ATCC 16404)	Five variety of honey+ Starch	Synergistic effect, MIC % five varieties of honey with starch ranged between 6% and 19.5%.	Boukraa L, et al., 2008)
In vivo study	Acetaminophen induced hepatotoxicity Balb/c mice model	Honey+ *Picrorhiza kurroa*	Enhanced hepatoprotective, hepatoregenerative ability	Gupta P, et al., 2016
Clinical study	30 subjects of pediatric age.	Honey + *Mandur bhasma* (herbo-mineral preparation)	Raised Hb gm% observed in honey+ *mandur bhasma* in comparison to mandur bhasma alone.	Gupta P, et al., 2015
In vitro study	2 clinical pathogenic bacterial isolates- *S. aureus, Strept. Pyogenes* and 2 reference strains- *S. aureus (ATCC 14775), Strept. Pyogens (ATCC 19615)*	Sidr and sommor honey+ Ofloxacin, Piperacillin, Amoxicillin+ clavulanic acid, Sulphamethoxazole + Trimethoprim,	Showed synergistic effect against tested bacterial strains, highest synergistic effect was observed against *Strept. Pyogenes* clinical isolate	Masoud E, et al., 2015

In Ayurveda, Honey (Madhu) is described significantly for its therapeutic value especially for *Yogawahi* property (Enhances/ complement potency of lead compound while not disturbing/reducing own potency) (Indu commentary on A.S. Su. 43/93). This *Yogavahi* property is equated with the synergistic effect of drug. Studies on *Yogavahi* or synergistic property of honey are summarised in table 1.

The oral administration of dried *Pircorhiza kurrua* rhizome and roots has been claimed as a cure for human viral hepatitis, Hepatic insult and Damage, Asthma, Allergy, Mushroom's Poisoning, Hypoxia, Vitiligo, Antidiabetic, Antioxidant, Heart ailment and abdominal pain.(Jadhao M et al., 2009)

Alkaline phosphatase (ALP) manifest as a critical biomarker of numerous diseases and a major tool enzyme of experiments. The majority of sustained elevated ALP levels are associated with disorders of the liver or bone, or both. Since production is increased in response to cholestasis, serum ALP activity provides a sensitive indicator of obstructive and space-occupying lesions of the liver.

Reduced glutathione, a tripeptide, serves as a vital antioxidant in animal, plant, fungi and bacteria by providing free thiol. In healthy human cells, majority (90-95%) of glutathione exist in reduced form and GSH/GSSG ratio is critical indicator for cell health. Oxidative stress deranges GSH/GSSG ratio by decrease in GSH and increase in GSSG (oxidized form of Glutathione). Antioxidant property of GSH is a prominent area of research and projects it as a possible therapeutic agent for several diseases.

Hence a study was planned to evaluate the synergistic effect of honey when administered with known hepato-protective like *Picrorhiza kurrua* against an acetaminophen induced hepatic insult.

MATERIAL AND METHODS:

A design was made to evaluate the synergistic effect (*Yogavahi)* property of honey by experimental study in mice by inducing the hepatotoxicity with Paracetamol (Acetaminophen) and therapeutic effect of honey as well as the synergistic effect with *P. Kurrua* were assessed by getting data of SGPT, SGOT, Alkaline Phosphatase enzymes and glutathione reductase and cytotoxicity assay in control, Group A (Acetaminaphen), GroupB (Acetaminophen and honey), Group-C (Acetaminophen and *P. Kurrua*) and the Group D (Acetaminophen + *P. Kurrua* + Honey). Due to huge data collection, a part of study was published earlier and referenced here (Gupta et al. 2016), the data related with levels of Alkaline phosphatase and Glutathione reductase in Serum and liver lysate are compiled in present study.

Animal care:

Screening, acclimatisation of mice was carried out from the Animal House, School of biotechnology B.H.U. Balb/c male mice, 5-6 weeks old, healthy, young age and having weight between (25-35 gm) were selected for the study. Standard mice chow and water were available *ad libidum.* Required treatment was given according to group distribution and mice were accordingly weighted and sacrificed on 2, 4, 6 and 8th day of experiment by cervical dislocation.

Group distribution and Dose calculation

Mice were divided randomly, and each group containing 5 mice.

Group I: Acetaminophen
Group II: Acetaminophen + Honey
Group III: Acetaminophen + *P. Kurrua*
Group IV: Acetaminophene + Honey + *P. Kurrua*
Group V: Control (Double distilled water)

A dispersible 1000mg/tab acetaminophen (paracetamol)(Apex Laboratory, India) was taken to produce hepatotoxicity in above four mice groups in a

sub-lethal dose of acetaminophen i.e. 625 mg/kg body wt. orally. Air-dried rhizome of *P. Kurrua Royle extract* was taken from Ayurvedic Pharmacy, Faculty of Ayurveda, Institute of Medical Sciences, Banaras Hindu University. Extraction (Aqueous) of *P. Kurrua* dried root and rhizome was concentrated in vacuum and dried in Department of *Rasa Shastra*, Institute of Medical Sciences, Banaras Hindu University and stored in a dark and dry place. *P. Kurrua* was given in the dose 200mg/kg P.o. in aqueous form. Honey was given in diluted to 1:1 (in D.D.W.) with dose 0.25 ml per oral.

Chemicals and Reagents:
Enzyme assay kit for alkaline phosphatase (Cat No. - K 753-100) was purchased from Hysel, new delhi and stored under advised temperature till further use.

Sample collection and tissue homogenate preparation:
Two mice from each group was sacrificed randomly at a set time interval by cervical dislocation, Blood samples were collected in centrifuge tubes/2 ml syringe via cardiac puncture and the serum was separated. The abdomen was then cut open and liver samples were removed. In order to prevent RBC contamination, samples were cut into small slices, rinsed thoroughly in ice-cold 0.9 % NaCl, and blotted with blotting paper, weighted and homogenised in ice-cold sodium pyrophosphate buffer (ph 8.3) and preserved in recommended temperature (4^0 C) till further use. Serum samples were frozen immediately in a deep freezer at – 20^0C and enzyme assays were performed on the same day or next day.

Enzyme assay:
The serum samples were subjected to assay for hepatic marker enzymes such as Alkaline phosphatase (ALP). Values were expressed as IU/dl ALP activity was measured using the method of Bower, G.N. and McComb. All six test tubes were taken and marked as blank, I, II, III, IV and V. Buffered substrate was taken in all test tubes except blank and brought it to 25^0 to 37^0 C then tissue lysate 0.02 ml was added in all test tubes, mixed it and after 1 minute initial absorbance was taken at wavelength 405nm at every 1 minute and alkaline phosphatase activity was calculated by multiplying change in absorbance per minute to the factor (2713).
(IU/Lt) = Δ A/min x factor (2713)

Method for Glutathione estimation:
The assay involves mainly using glutathione reductase and Ellman's reagent (DTNB). Glutathione reductase reduces GSSG to GSH. DTNB (5-5'- dithiobis [2-nitrobenzoic acid]) reacts with GSH to form yellow color chromophore, 5- thionitrobenzoic acid (TNB) with absorbance maxima at 415 nm and GS-TNB. GS-TNB is further reduced to GSH and TNB by glutathione reductase, thus this enzymatic recycling of GSH enhances the sensitivity of the assay.

All six test tubes were taken and marked as blank, I, II, III, IV and V. 0.2 ml of sample were taken in all test tubes except blank then 2 ml of substrate was added in all test tubes, mixed well and NADPH 1 ml was also added in all test tubes ,mixed and start times simultaneously then absorbance was taken at 1st minute , 2nd minute, 3 rd minute and 4th minute at wavelength 340nm, temperature 37^0 C and glutathione activity was calculated by multiplying change in absorbance per minute to 4983 (Factor).

RESULTS:

We estimated Alkaline phosphatase and Glutathione which showed that sub acute dose of paracetamol resulted in significant increased Alkaline phosphatase decreased Glutathione level. Honey, *P. Kurrua* and combination of both decreased the raised activity of Alkaline phosphatase while at the same time it increased the reduced activity of glutathione reductase.

Serum Alkaline Phosphatase activity:
Group I (acetaminophen feed) showed increased serum activity of ALP by 1.46, 2.36, 3.94 and 7.81 fold while in Group II (Acetaminophen + *honey*) it ranged increased serum ALP activity by 1.24, 1.87, 3.09 and 5.24 fold in comparison to control group on day 2 , 4, 6, 8th day respectively. Group III (Acetaminophen + *P. Kurrua*) expressed increased serum ALP activity by 1.16, 1.89, 2.06, and 3.94 fold while in group IV (Acetaminophen + *P. Kurrua* + honey) increased activity of serum ALP by 1.17, 1.29, 1.74, and 2.05 fold in comparison to control group on day 2, 4, 6, 8th day respectively. Results related with serum alkaline phosphatase are summarised in table 3 and figure 1.

Liver lysate Alkaline Phasphatase activity:
Group I (acetaminophen feed)showed increased liver lysate activity of ALP by 1.27, 2.06, 2.56 and 5.5 fold while Group II (Acetaminophen + *honey*) showed increased liver lysate ALP activity by 1.09, 1.56, 1.88 and 4.11 fold in comparison to control group on on day 2 , 4, 6, 8th day respectively. Group III (Acetaminophen + *P. Kurrua*) expressed increased ALP activity by 1.1, 1.45, 1.70, and 3.85 fold while in group IV (Acetaminophen + *P. Kurrua* + honey) increased activity of liver lysate ALP by 1.06, 1.09, 1.52, and 2.17 fold in comparison to control group on day 2, 4, 6, 8th day respectively. Results related with liver lysate alkaline phosphatase are summarised in table 3 and figure 2

Serum Glutathione Level:
Group I (acetaminophen feed)showed reduced serum

level of glutathione by 0.70, 0.37, 0.22 and 0.07 fold while in Group II (Acetaminophen + honey) it ranged decreased serum glutathione activity by 0.85, 0.54, 0.33 and 0.14 fold in comparison to control group on day 2 , 4, 6, 8th day respectively. Group III (Acetaminophen + *P. Kurrua*) expressed reduced serum glutathione activity by 0.94, 0.69, 0.25, and 0.15 fold while in group IV (Acetaminophen +*P. Kurrua*+ honey) decreased activity of serum glutathione was observed by 0.95, 0.73, 0.65, and 0.37 fold in comparison to control group on day 2, 4, 6, 8th day respectively. Results related with serum glutathione are summarised in table 3 and figure 3

Liver lysate Glutathione Level:

Group I (acetaminophen feed)showed reduced liver lysate level of glutathione by 0.52, 0.37, 0.35 and 0.30 fold while in Group II (Acetaminophen + honey) it ranged decreased liver lysate glutathione activity by 0.83, 0.60, 0.51 and 0.34 fold in comparison to control group on day 2 , 4, 6, 8th day respectively. Group III (Acetaminophen + *P. Kurrua*) expressed reduced liver lysate glutathione activity by 0.81, 0.72, 0.68, and 0.35 fold while in group IV (Acetaminophen +*P. Kurrua* + honey) decreasSSed activity of liver lysate glutathione was observed by 0.94, 0.83, 0.81, and 0.47 fold in comparison to control group on day 2, 4, 6, 8th day respectively. Results related with liver lysate glutathione are summarised in table 3 and figure 4

Table 2- Experiment observations of all group and all activities (Serum and Liver lysate)

		Sr. Alkaline Phasphatase (U/ml)	Liver lysate Alk. Phosphotase (U/ml)	Sr. Glutathione (U/ml)	Liver Lysate Glutathione (U/ml)
Group A	Day 0 Control DDW	31.99 ± 1.56	52.55± 1.32	142.52 ± 1.66	279.01 ± 1.75
	Day 2nd Acetaminophen	46.99 ± 1.12	66.98± 1.62	100.53 ± 1.01	145.50 ± 1.95
	Day 4th Acetaminophen	75.58 ± 1.54	108.52± 1.33	53.94 ± 1.09	103.77 ± 1.22
	Day 6th Acetaminophen	126.18 ± 1.62	134.72± 1.54	32.05 ± 1.08	97.66 ± 1.65
	Day 8th Acetaminophen	250.03 ± 1.92	289.53± 1.93	11.04 ± 1.02	85.75 ± 1.68
Group B	Day 0 Control DDW	30.62 ± 1.72	52.16± 1.62	143.36 ± 1.67	288.00 ± 1.78
	Day 2nd Acetaminophen + Honey	38.14 ± 1.97	57.21± 1.32	121.70 ± 1.62	239.68 ± 1.96
	Day 4th Acetaminophen + Honey	57.24 ± 1.26	81.49± 1.33	77.61 ± 1.11	173.40 ± 1.54
	Day 6th Acetaminophen + Honey	94.83 ± 1.53	98.183± 1.53	47.94 ± 1.18	148.29 ± 1.29
	Day 8th Acetaminophen + Honey	160.50 ± 1.62	214.76± 1.79	19.38 ± 1.47	99.11 ± 1.11
Group C	Day 0 Control DDW	31.66 ± 1.12	52.40± 1.67	141.11 ± 1.64	288.6 ± 1.12
	Day 2nd Acetaminophen + *P. Kurrua*	37.00 ± 1.36	57.87± 1.53	133.68 ± 1.66	234.00 ± 1.40
	Day 4th Acetaminophen + *P. Kurrua*	59.84 ± 1.73	76.31± 1.11	97.90 ± 1.36	210.12 ± 1.18
	Day 6th Acetaminophen + *P. Kurrua*	65.27 ± 1.54	89.42± 1.69	35.37 ± 1.54	196.96 ± 1.46
	Day 8th Acetaminophen + *P. Kurrua*	125.012 ± 1.66	201.87± 1.16	22.22 ± 1.66	101.31 ± 1.16
Group D	Day 0 Control DDW	30.62± 1.92	51.98± 1.77	143.36 ± 1.53	287.4 ± 1.72
	Day 2nd Acetaminophen + Honey + *P. Kurrua*	36.12 ± 1.73	55.54± 1.64	136.3 ± 1.03	242.88
	Day 4th Acetaminophen + Honey + *P. Kurrua*	39.79± 1.50	57.054± 1.12	105.88 ± 1.54	236.00 ± 1.32
	Day 6th Acetaminophen + Honey + *P. Kurrua*	53.51± 1.32	79.11± 1.79	94.42 ± 1.81	217.12 ± 1.38
	Day 8th Acetaminophen + Honey + *P. Kurrua*	62.914± 1.32	113.91± 1.27	53.26 ± 1.64	172.40 ± 2.54

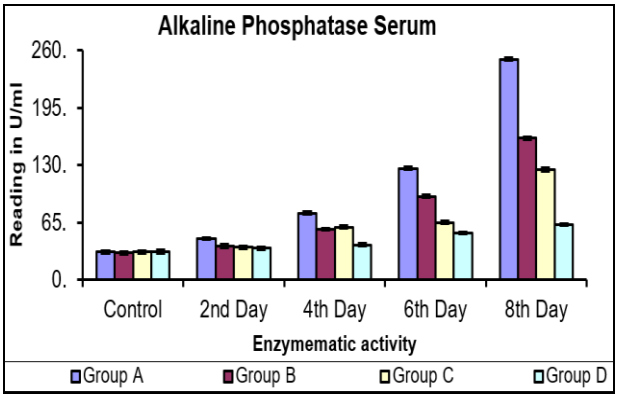

Fig. No. 1: Alkaline phosphatase serum enzyme activity on different days of treated mice.

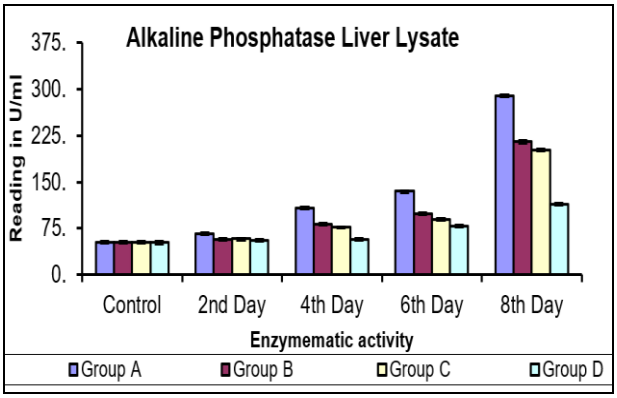

Fig. No. 2 : Alkaline phosphatase liver lysate enzyme activity on different days of treated mice.

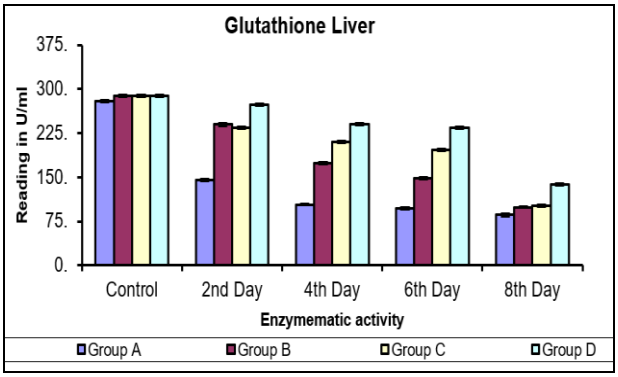

Fig. No. 3 : Glutathione liver enzyme activity on different days of treated mice.

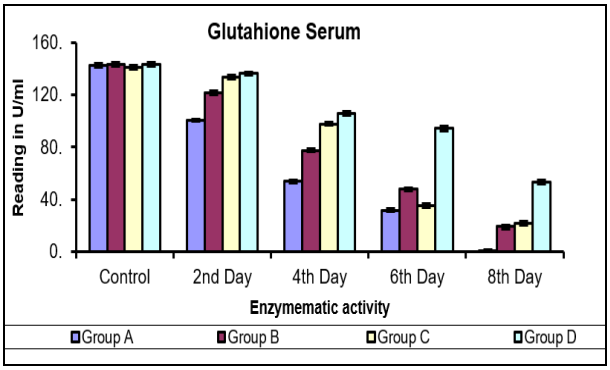

Fig. No. 4 : Glutathione serum enzyme activity on different days of treated mice.

DISCUSSION:

In traditional medicine therapeutics, Honey is popular for its various medicinal properties such as cardio-tonic, better for eye sights, hypo-lipidemic, appetizer etc. since ancient period but recently it has been found to possesses Antibacterial activity (Eteraf-Oskouei T, et al., 2013; Mandal, et al., 2011), hypoglycemic (Bobis et al., 2018; Erejuwa et al., 2012), antioxidant (Erejuwa et al., 2010; Ahmad et al., 2018), wound healing (Febriyenti et al., 2019; Oryan et al., 2016) and gastric ulcer protective, (Fazalda et al., 2018) properties.

The present study is aimed to evaluate the synergistic role of honey and *picrorrhiza* in combination against the paracetamol (Acetaminophen) toxicity in mice model.

Experimental data based on activities of Alkaline phosphatase (serum/liver) and glutathione level (serum/liver) suggest the protective role of honey against paracetamol (Acetaminophen) adverse effects to Heart and liver (Hazai et al., 2002). Sulfahydryl compounds are known as the most important endogenous antioxidants. NAPQEI binds with the GSH resulting in conversion of GSH to GSSG. Decreased GSH levels in cell resulting in increased cell damage. In such conditions, other proteins sulfhydryl groups present in the cells provide an alternative protection (Genet et al., 2000).

Antioxidant activity and hepatoprotective potential of honey has been described in a rat models against CCl_4. (Khadr et al., 2002). The CCl_4 administration causes increase in SGOT and SGPT at significant level in rat (Khadr et al., 2002). This agree with our observations in respect to alkaline phosphatase which increased in mice after administration of paracetamol (Acetaminophen) after 2 days, 4 days, 6 days and 8 days. We further observed that enzyme activity of alkaline phosphatase increased in serum maximum approximately 7 fold at day 8 in respect to day 2nd after paracetamol treatment. Honey when given with CCl_4 along with black seeds and silymarin (Khatr et al.) showed improvement in antioxidant status. Our data also suggest that honey, picrorrhiza and combination of both honey and picrorrhiza when administered along with paracetamol (Acetaminophen) resulted decrease in the activity of ALP at significant level on day 2nd, 4th, 6th, 8th. We can calculate that toxicity of liver and heart might be due to the elevated enzyme activity of ALP as well as decreased Glutathione reductase. In the current study, we estimated Alkaline phosphatase and Glutathione which showed that toxic dose of paracetamol resulted in significant increase in the activity of alkaline phosphatase tremendous decrease of the Glutathione level. Honey, picrorrhiza and combination of both decreased the activity of alkaline phosphatase while at the same time it increased the activity of glutathione reductase. At conclusion, honey and picrorhiza could be given along with paracetamol (Acetaminophen) to avoid liver and heart cells Damages.

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RESEARCH ARTICLE

Probiotic can Prevent Sepsis in Rats Induced by Lipopolysaccharide *Escherichia coli*

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ABSTRACT:

Escherichia coli bacterial infection (*E. coli*) is the cause of sepsis in critically ill patients. Consumption of probiotic is one attempt to increase endurance against bacterial infections. This study aimed to determine the effect of probiotic *Lactobacillus spp.* on pro-inflammatory cytokines (interleukin-1 (IL-1)), sepsis markers (procalcitonin (PCT) and white blood cell (WBC) count in rats (*Rattus norvegicus*) induced by lipopolysaccharide (LPS) *E. coli*. Twenty-one rats were divided into three groups: (1) group of healthy control rats, (2) group of rats induced by LPS *E. coli*, and (3) group of rats treated by *Lactobacillus spp.* and induced by LPS *E. coli*. The group of rats treated by *Lactobacillus spp.* and induced by LPS *E. coli* was treated with additional *Lactobacillus spp.* probiotic on day 1-14, then induced with LPS *E. coli* on day 15. All blood samples were taken on the 16th day. IL-1 and PCT levels were analyzed with the ELISA method, the WBC count was measured with improved Neubauer hemocytometer. The group of rats induced by LPS *E. coli* had the highest IL-1, PCT, and WBC count. The statistical analysis of IL-1 did not show a significant difference ($p > 0.05$), but PCT and WBC count showed significant difference when compared with group of rats treated by *Lactobacillus spp.* ($p < 0.05$). Probiotic *Lactobacillus spp.* can prevent sepsis in rats induced by LPS *E. coli.*

KEYWORDS: *Lactobacillus spp*., Lipopolysaccharide *E. coli*, IL-1, PCT, WBC count.

INTRODUCTION:

Nosocomial infection is one of the biggest causes of sepsis in critically ill patients admitted to intensive care unit. About 75% of the burden of nosocomial infections is present in developing countries. Most of these infections are caused by gram-negative bacteria originating from the gastrointestinal tract such as *Escherichia coli* (*E. coli*)[1-3]. Infections that develop into sepsis have been shown to increase the length of stay, cost of care, morbidity, and patient mortality[4].

An essential product in the occurrence of sepsis is lipopolysaccharide (LPS) which is the outer membrane component of gram-negative bacteria. LPS *E. coli* is a glycolipid complex, the main component of the outer wall of gram negative-bacteria with an endotoxin characteristic and capable of stimulating immune cells[5, 6].

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Induction of LPS *E. coli* in rats causes LPS to bind to specific proteins in the plasma through Lipopolysaccharide Binding Protein (LPB). Furthermore, LPS and LPB will integrate with Cluster Differentiation 14 (CD14) and Toll-Like Receptor 4 (TLR 4) to activate protein regulation (Nuclear Factor kappa Beta/NFkB). Activated cells secrete pro-inflammatory mediators, namely cytokines (TNF-α, IL-1, IL-6), chemokine (IL-8), prostaglandin and histamine. The mediator works on blood vessel endothelial cells, causing an inflammatory response, namely vasodilation, increased vascular permeability, and recruitment of neutrophils to tissues[7, 8].

The infection can develop into sepsis if the infectious agent secretes the toxin continuously. As a result, an inflammatory response persists through continuous activation of inflammatory mediators, cellular hypoxia, tissue damage, shock, and organ failure, and has the potential to cause death[9]. The detection of the infected condition and its development into sepsis can be seen from several parameters, namely levels interleukin-1 (IL-1), pro-calcitonin (PCT), and white blood cell (WBC) count[10-13].

The body responds to infection by secreting pro-inflammatory cytokines, including IL-1. IL-1 has a significant role in mediating innate and adaptive immune responses to various infectious diseases[14]. Studies by Pierrakos and Vincent show that pro-inflammatory cytokines, especially IL-1, is known to have increased in sepsis patients compared to non-sepsis patients[15]. The body also responds to infection by increasing WBC count which explains why WBC counts are used to indicate the presence of infection and to follow the development of infection into sepsis, which is when leukocytosis occurs (WBC count > 12,000 µL $^{-1}$) or when leukopenia occurs (WBC count < 4,000 µL $^{-1}$).[11] PCT is used to determine whether the condition of the infection has developed into sepsis. To mark sepsis in the acute phase, PCT is used due to its sensitivity and specificity in determining the prognosis of sepsis. Clinical trials have shown that PCT is a specific sign of severe bacterial infections, and can distinguish between patients with sepsis and patients with response systemic inflammatory syndrome (SIRS)[15, 16].

The inflammatory response is a body mechanism that aims to increase the immune response to eliminate pathogenic microorganisms. If the body's inflammatory response is adequate, the infection can be controlled and resolved. However, if the body cannot properly regulate the secretion of the mediator, sepsis will occur. One preventive effort to ensure that the infection condition does not occur and develop into sepsis is by increasing the body's immunity through the administration of lactic acid bacteria (BAL) which is isolated into probiotic[17, 18].

Lactobacillus spp. bacteria that are included in BAL have been widely consumed for its health benefits[19]. *Lactobacillus spp*. are able to "balance" unfriendly bacteria because when they produce lactic acid, they alter the intestinal environment, making it unsuitable for unfriendly bacteria[20]. Most research on the effect of probiotics focuses on preventing and treating gastrointestinal disease, even though probiotics have the potential to prevent infection from becoming sepsis. This study aims to determine the effect of probiotic *Lactobacillus spp.* on pro-inflammatory cytokines IL-1, and sepsis markers (PCT and WBC count) in rats induced by LPS *E. coli*.

MATERIALS AND METHODS:

This study was an experimental study. This was conducted in Microbiology Laboratory and Parasitology Laboratory, Faculty of Medicine, University of Brawijaya from December 2016 to February 2017.

Culture Preparation of *Lactobacillus spp*.:

Lactobacillus spp. was obtained from the Microbiology Laboratory, Faculty of Medicine, Universitas Brawijaya, Malang, Indonesia. The preparation procedure for the probiotic cultures of *Lactobacillus spp.* began by sterilizing a curved inoculating loop by heating the loop above the bunsen burner until it was red hot. After the loop had cooled, the *Lactobacillus spp.* was taken from the stock by touching the end of the loop to the stock, then it was scratched onto the surface of the media MRS-Agar with a streaking quadrant method to obtain separate colonies. It was followed by incubating the media for 37º C for 24 hours. The grown pure colony was re-identified to determine the bacterial species. After the colony was proven as *Lactobacillus spp.* species, a culture enrichment was done to produce a large amount, and dilution was done to reach the desired bacterial density[21]. *Lactobacillus spp* was administered a 0.5 ml dose of 10^9 CFU/Kg BW/day dissolved in the media dextrose 5% (D5%). Probiotic was administered through the gastric tube once a day for 14 days to achieve the optimal effect[4, 22].

Rats:

Rats *(Rattus norvegicus)* of Wistar strain, male, aged 10 - 12 weeks, weighed at 150 - 200 grams were obtained from the Faculty of Medicine, University of Brawijaya. Before being treated, the animals were acclimatized for seven days by being fed through an *ad-libitum* method. The use of experimental animals as a subject in this research has passed the ethical conduct from the Ethics Commission of Health Research, Faculty of Medicine, University of Brawijaya.

Induction of LPS *E. coli:*

Induction of LPS *E. coli* (Biological List, catalog number 201) was done orally. LPS was diluted with NaCl 0,9% solution, with a ratio of NaCl 0.9% : LPS *E. coli* of 10 : 1. The dose given was 1 mg/Kg BW dissolved in 0.5 ml NaCl 0.9% media[23].

Research Design:

Twenty-one rats were randomly divided into 3 groups as follows: group of healthy control rats (healthy group, n= 7); group of rats induced by LPS *E. coli* (LPS group, n= 7) with a dose of 1 mg/Kg BW on day 15; and group of rats treated by probiotic *Lactobacillus spp*. (10^9 CFU/Kg BW/day for 14 days), and induced by LPS *E. coli* (LPS + LB group, n= 7) at a dose of 1 mg/Kg BW on the 15th day. Intra-cardiac blood sampling was performed on day 16 (24 hours following induction of *E.* coli LPS).

Measurement of IL-1 and PCT levels:

The measurement of IL-1 and PCT levels was analyzed using enzyme-linked immunosorbent assay (ELISA) kit namely RayBio® Rat IL-1 beta ELISA Kit no. ELR-IL1b catalog, and MyBioSource no. catalog of MBS760081. The sensitivity of the IL-1 and PCT kits were 80 pg/ml and < 9.375 pg/ml.

Measurement of WBC Count:

The measurement of WBC count was calculated using counting chambers (improved Neubauer). The calculation of white blood cell count is carried out in four areas of counted chambers. The number of WBC per mm^3 of blood is obtained as follows: number of WBCs counted x blood dilution factor x chamber depth divided by the area of chamber counted= source of WBCs counted x 50 (Blood dilution factor= 20; Chamber depth= 10; Area of chamber counted= 4)[24].

Statistical Analysis:

Data were presented as mean value ± standard deviation (SD). Statistical tests were performed using SPSS statistical software (version 2.0, Chicago, IL, USA). One way ANOVA test was done to determine the effect of probiotic on the levels of IL-1, PCT, and WBC count in each group. Comparison of the effect of probiotic between groups was analyzed by the Post hoc test (Tukey's test).

RESULTS:

Effect of Probiotic on IL-1 Levels:

The difference of IL-1 levels among study groups was demonstrated in Table-1.

Table-1 The Difference of IL-1 levels in the group of healthy control rats, group of rats induced by LPS *E. coli*, and group of rats treated by *Lactobacillus spp.* and induced by LPS *E. coli*

Study Group	IL-1 Levels (pg/ml)			P-Value
	Mean ± SD	Range	Median (IQR)	
Healthy group	17.78 ± 2,64	(14.59-23.05)	17.57 (2.06)	0.952
LPS group	18.00 ± 5,56	(11.86-29.17)	16.55 (5.10)	
LPS + LB group	17.36 ± 2,91	(14.59-23.05)	16.55 (4.02)	

The highest level of IL-1 was found in the group of rats induced by *LPS E. coli.* However, there was no significant difference in IL-1 levels among healthy group, LPS group, and LPS + LB group ($p > 0.05$).

Effect of Probiotic on PCT Levels:

The difference of PCT levels among study groups was demonstrated in Table-2.

Table-2 The Difference of PCT levels in the group of healthy control rats, group of rats induced by LPS *E. coli*, and group of rats treated by *Lactobacillus spp.* and induced by LPS *E. coli*

Study Group	PCT Levels (pg/ml)			P-Value
	Mean ± SD	Range	Median (IQR)	
Healthy group	13.73 ± 0,14	(13.59-13.97)	13.67 (0.24)	0.000
LPS group	14.61 ± 0,38	(14.18-15.22)	14.51 (0.68)	
LPS + LB group	13.88 ± 0,23	(13.52-14.16)	13.91 (0.44)	

The highest PCT level was found in the group of rats induced by LPS *E. coli.* PCT level in the LPS group increased significantly when compared to the healthy group (14.61 ± 0.38 pg/mL vs. 13.73 ± 0.14 pg/mL; $p < 0.001$) (Figure-1). PCT level on the LPS group also increased significantly when compared with the LPS + LB group (14.61 ± 0.38 pg/mL vs. 13.88 ± 0.23 pg/mL; $p < 0.001$). The difference in PCT levels in the healthy group with the LP + LB group was not statistically significant (13.73 ± 0.14 pg/mL vs 13.88 ± 0.23 pg/mL, $p > 0.05$).

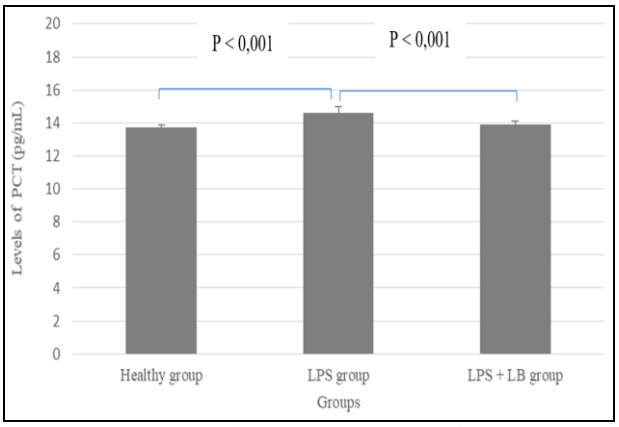

Figure 1. Comparison of PCT levels in group of healthy control rats, group of rats induced by LPS *E. coli*, and group of rats treated by *Lactobacillus spp.* and induced by LPS *E. coli*.

Effect of Probiotic on WBC Count:

The difference of WBC count among study groups was demonstrated in Table-3.

Table-2 The Difference of WBC counts in the group of healthy control rats, group of rats induced by LPS *E. coli*, and group of rats treated by *Lactobacillus spp.* and induced by LPS *E. coli*

Study Group	WBC Counts (cell/µl)			P-Value
	Mean ± SD	Range	Median (IQR)	
Healthy group	6,992.86 ± 1,110.34	(5,700-8,400)	6,800 (2,250)	0.000
LPS group	29,478,57 ± 3064,02	(25,500-34,650)	30,000 (4,200)	
LPS + LB group	26,535.71 ± 1,773.58	(24,000-29,000)	26,000 (3,150)	

The WBC count in both groups of rats induced by LPS *E. coli* showed an increase, with the highest number found in the group of rats which was only induced by LPS *E. coli* without treated by probiotic. The WBC count in the LPS group increased significantly when compared with the healthy group (29478.57 ± 3064.02 cell/µL vs. 6992.86 ± 1110.34 cell/µL; $p < 0.001$) (Figure-2). The WBC count in the LPS + LB group also significantly increased when compared with the healthy group (26535.71 ± 1773.58 cell / µL vs. 6992.86 ± 1110.34 cell/µL, $p < 0.001$). Although they both experienced an increase in WBC counts, the difference in the WBC counts in the LPS group with the LPS + LB group showed statistically significant difference (29478.57 ± 3064.02 cell/µL vs. 26535.71 ± 1773.58 cell/µL, $p < 0.05$).

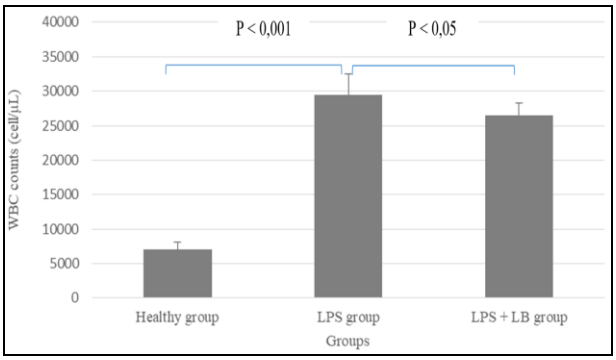

Figure-2. Comparison of WBC counts in the healthy control group of rats, the group of rats induced by LPS *E. coli*, and the group of rats treated by *Lactobacillus spp.* and induced by LPS *E. coli.*

DISCUSSION:

IL-1, PCT, and the number of WBC play an essential role in the inflammatory process and are widely used to identify the occurrence of sepsis. Sepsis induction can use an LPS *E. coli* dosage of 0.1 mg/Kg BW - 40 mg/Kg BW. In the use of low doses, the effect of sepsis persists for up to 48 hours. An oral dose of 1 mg/Kg BW will result in a maximum immune response within 24 hours after exposure[23, 25]. The pathological characteristics of sepsis is the inability of the body to maintain a balance of pro-inflammatory and anti-inflammatory mediators resulting in a systemic inflammatory response[26]. The results of this research show that the highest levels of IL-1, PCT, and WBC counts were found in the group of rats induced by LPS *E. coli*, compared to the group of healthy control rats, and the group of rats treated by *Lactobacillus spp.* and induced by LPS *E. coli*.

Induction of LPS *E. coli* in rats causes resident immune cells such as macrophages generate a pro-inflammatory state in response to pathogen-associated molecular patterns (PAMPs) from the LPS *E. coli*, and damage-associated molecular patterns (DAMPs) that are released by rats cells. Resident immune cells recognize these patterns mainly via Toll-like and lectin receptors and then release inflammatory mediators to attract other immune cells such as neutrophils and cells of adaptive immunity to the site of infection in order to eliminate the pathogen without harming the host. First responding cells are monocytes and macrophages, which through induction of early response genes such as TNF-α induce inflammatory cytokines and chemokines, amplifying the inflammatory response. This is followed by activation of lymphocytes as an adaptive immune response, as well as the coordination of later phases of the immune response. Additionally, nitric oxide (NO) that is produced by endothelial cells induces vasodilation and an increase in WBC delivery to the sites of immune-activity[6, 27].

IL-1 is one of the major resulting pro-inflammatory cytokines if the body experiences an infection in the acute phase, and also experience an increase in sepsis patients compared to patients of non-sepsis. In this study, blood plasma sampling for measurement of IL-1 levels was carried out 24 hours after the induction of *LPS E. coli*. The three groups showed almost the same value. This is probably due to a temporary increase in pro-inflammatory cytokine levels, such as IL-1, which reaches a peak of 1.5-4.5 hours after bacterial induction, and after that gradually returns to normal[23]. This is in accordance with the results of the McAllister et al. study, which reported the results of cytokine measurements in three patients with sepsis conditions after receiving erythrocyte transfusion (PRC) contaminated with gram negative bacteria. IL-1 in the two surviving patients were detected to reach the peak at four hours after exposure, then gradually returned to normal. Whereas in one patient who later died, IL-1 levels remained high for 22 hours[28].

WBC count in both LPS group and LPS + LPB group that received LPS *E. coli* induction both increased. This is caused by an inflammatory response to the induction of *LPS E. coli* in the body of white rats for 24 hours. This is consistent with research conducted by Penailillo et al. which showed that intravenous administration of LPS *E. coli* in rabbits caused a significant increase in total WBC and neutrophils count in samples taken at 12 and 24 h after the first injection of LPS[29]. *E. coli* infection will affect the hematologic system, one of which is the number of white blood cells as the body's first line of defense against infection and inflammatory conditions.

In most patients, the pro-inflammatory response is self-limiting, but in some patients the response becomes exaggerated, which leads to sepsis. PCT is a prohormone calcitonin produced by the liver, kidney, adipose, and parenchymal muscle cells in response to bacterial endotoxin, and is widely used as a biomarker of sepsis[30]. Numerous studies have investigated the diagnostic usefulness of PCT, comparing it with CRP. Initially, PCT was found more sensitive and specific than CRP for bacterial infection. PCT levels play a role in detecting sepsis while predicting the patient's prognosis. A study by Nanda et al. showed that patients with high PCT levels were more at risk of death when compared with patients with low PCT levels[31].

The results of this study also showed that PCT levels and WBC counts increased significantly in the LPS group, compared to the healthy group, and the LPS + LB group. However, there were no significant differences in PCT levels in the healthy group with the LPS + LB group. In addition, even though both experienced an increase, the WBC count in the LPS group increased significantly when compared with the LPS + LB group.

This shows that a worse infection occurred in the group of rats that were only induced by LPS *E. coli*. The *Lactobacillus spp.* probiotic positively influenced it and stopped the infection to become more severe/sepsis. When *Lactobacillus spp* probiotic were given to the rats for 14 days as an immunomodulator, it can suppress the inflammatory response and prevent it from going into a sepsis condition. The inflammatory response that occurred in the group of rats treated by probiotics and induction of LPS *E. coli* will be local and can be defended by the cellular immune system that has been strengthened by probiotic[32].

Some postulate can explain the mechanism of probiotics in preventing the development of infection into sepsis, ie: the ability to obtaining an attachment site, the ability to influence the intestinal pH, improve the function of the intestinal wall, microfloral modification with the synthesis of anti-microbes compounds, triggering immune response, disturbing communication between bacteria, and the ability to compete to obtain nutrition[33-37]. Probiotic strains of lactobacilli can attach to the epithelium and act as walls against pathogens (colonization barriers) by preventing pathogens from attaching to the gastrointestinal mucosa. Lactic acid and acetic acid produced by probiotics reduce luminal pH which results in a less conducive environment to pathogenic organisms. Probiotic can also improve the complex function of the intestinal walls and can control the stability of cytoskeletal cells through mucus production, chloride, and water secretion. In addition, many types of lactobacilli produce bacteriocins, compounds produced by bacteria with a portion of biologically active proteins and are bactericidal[38, 39]. Furthermore, probiotics are also known to increase immunoglobulin-A (IgA) secretion, increase the number of natural killer cells, or increase phagocyte macrophage activity. Increased IgA secretion can reduce the number of pathogenic organisms in the intestine thereby increasing the composition of the microflora. The type of lactobacilli is also known to secrete molecules that interfere with communication between bacteria and reduce the expression of virulence of *E. coli*. probiotic which also use the necessary nutrients for the growth of pathogenic bacteria that can inhibit the growth of pathogen bacteria[17, 40, 41].

The mechanism of *Lactobacillus* in inhibiting the action of infection-induced LPS is probiotic not only alleviates inflammation, but also has an immunostimulatory effect on intestinal epithelial cells. Previous study demonstrated that *Lactobacillus rhamnosus* GG alleviates inflammation in LPS-stimulated porcine intestinal epithelial cells by modulating Toll-like receptors (TLRs) and inhibiting mitogenactivated protein kinase (MAPK) and nuclear factor kappa B (NF-kB) signaling[42].

The conclusion of this study is; probiotic *Lactobacillus spp.* can prevent sepsis in rats induced by LPS *E. coli.* The administration of probiotic *Lactobacillus spp*. can be beneficial in preventing increased PCT levels and WBC counts in rats induced by LPS *E. coli*, where PCT levels and WBC counts are markers of sepsis. Further studies are needed to determine the effective dose of probiotic *Lactobacillus spp*. in preventing sepsis caused by infection of *E. coli* bacteria.

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CONFLICT OF INTEREST:

The authors declare no conflict of interest.

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RESEARCH ARTICLE

Atom based 3D QSAR and Fingerprint based 2D QSAR of Novel Molecules as MmpL3 receptor inhibitors for *Mycobacterium tuberculosis*

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ABSTRACT:

Tuberculosis is one of the leading cause of increase in mortality rate in today's health care scenario. Due to increase frequency of drug resistant TB it is prudent to find new targets and promising targets for anti-tubercular activity. MmpL3 (Mycobacterial Membrane Protein Large 3) is one of the most effective and promiscuous targets for development of new drug for anti-tubercular therapy due to its cross resistance inhibition property. In this study we have presented atom based 3D QSAR and finger print based 2D QSAR models to study different structural and functional groups of Adamantyl urea derivatives and their action in MmpL3 inhibitory activity which will provide us the insight for designing better and far more effective anti TB drugs.

KEYWORDS: MmpL3 inhibitors, Atom based 3D QSAR, Fingerprint based 2D QSAR.

INTRODUCTION:

Tuberculosis (TB) is an infectious disease which primarily affects the lungs. This contagious disease is caused by the microorganism *Mycobacterium tuberculosis.* Tuberculosis is one of the leading cause of death in today's health care scenario.[1]

According to Global Tuberculosis Report by WHO in 2018 nearly 10 million people suffered from Tuberculosis with an overall mean of 130 cases per 100,000 population per year.[2]

Hence, with the increasing prevalence of MDRTB (Multi Drug Resistance TB) and TDRTB (Total Drug Resistant TB) it is of utmost importance to develop new drugs with novel mechanism of action targeting MTb disease.

One of most effective and important target for anti TB drugs is the mycobacterial cell wall. Due to its unique structure it plays a very important role in Tuberculosis pathogenesis. The cell wall mainly contains mycolic acid which is responsible for cell viability.

This cell wall is extremely hydrophobic and is responsible for its survival and virulence. The outer cell wall includes a long chain of mycolic acid (MAs) which are transported from the inner membrane as Trehalose Monomycolates (TMMs) with the help of a transporter to outside the cell wall where it gets incorporated as Trehalose Dimycolates (TDMs), along with noncovalently binded lipids. This distinct structure of the outer cell wall of Mtb is very rigid and extremely impermeable to many anti TB Drugs. Hence, TMMs transport and biosynthesis is essential for the survival of Mtb.[3, 4]

MmpL3 (Mycobacterial Membrane Protein Large 3) which belongs to RND family (Resistance Nodulation and cell Division) is a lipid transporter that binds with the Trehalose Monomycolates and transfer it to the outer cell wall from the cytoplasm where it is required for cell wall integrity and biosynthesis. MmpL3 is one of the most recent targets for development of new drug for

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anti-tubercular therapy. Several compound which act as inhibitors of MmpL3 have been reported so far inhibitors like BM 212, SQ109, adamantyl urea derivatives that have shown promising inhibitory activity in vitro.

Over the years, the study of QSAR (Quantitative Structure Activity Relationship) has emerged as an adept technique which used as an important and efficient tool in for drug discovery and development.[5]

QSAR is a mathematical model of statistical correlation between biological activity and deviation structural properties in a series of chemical compounds. The QSAR helps in predicting the activity of unknown compounds using a structurally similar QSAR model. The molecular descriptors (the molecular properties used in QSAR) can be either 2D (Hydrophobicity, Bond length, bond angle, dipole moments, steric effects, electronic properties, pKa in ionic compounds etc.) or 3D (involves stereochemistry, optical activity, active site interaction etc.)[6,7,8,9].

Here, a set of 1-Adamantyl-3-Heteroaryl Urea derivatives with MmpL3 inhibitory activity were selected from the literature to study and design 3D and 2D QSAR models and structural features required for MmpL3 inhibitory activity were identified. The data reported by the various QSAR models provides guidance for the designing of structurally new adamantyl urea inhibitors with potential activity against MmpL3 of M. tuberculosis.

Experimental:

MATERIALS AND METHODS:

All the in silico experiments were conducted using the Schrodinger Software. The chemical structures were drawn and prepared using Marvin Sketch from ChemAxon. The alignment of the ligand, generation of atom based 3D QSAR models and fingerprint based 2D QSAR were performed using Maestro version 11.4 (Schrodinger Inc.) and Canvas software from the Schrodinger Package in using an Intel Core i3-4160 processor with 4GB RAM and Intel Haswell graphics card using a Linux Ubuntu 18.04.1 LTS operating system[10,11,12,13]..

Molecular Dataset:

A set of 48 structures **Table 1.** Of 1-Adamantyl-3-Heteroaryl Urea derivatives having MmpL3 inhibitory property in M. tuberculosis H7RV strain were selected from the literature to generate QSAR models. The structure variation and the relation to its wide range of biological activity served as an ideal data for generating suitable QSAR models for predicting activity. The MIC activity data [MIC H37RV (μM)] for the above derivatives was used after being converted to the logarithmic scale pMIC H37RV as the depending variables in 3D and 2D QSAR studies[14, 15,16,17,18.].

Ligand Alignment:

It is one of the most crucial step to ensure the generation of most precise and accurate 3D QSAR model. It helps in comparing and studying the relation between the deviations of the structures in the dataset. The structures in the data set are aligned in such a way that they are superimposed over each other, this was achieved using flexible ligand alignment in the maestro program of the Schrodinger software. [**Fig.1.**]

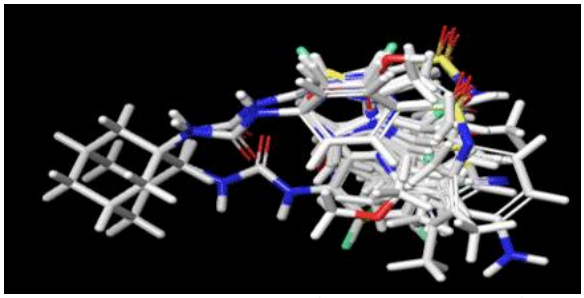


Fig.1. Flexible Ligand Alignment of the selected dataset from the literature

Building Atom based 3D QSAR model.

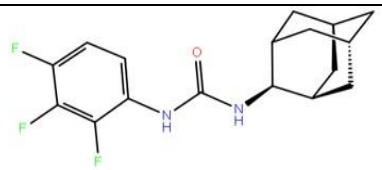
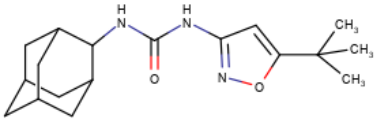
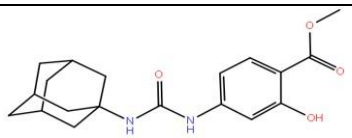
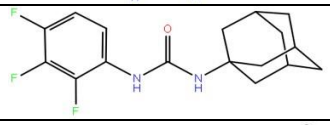
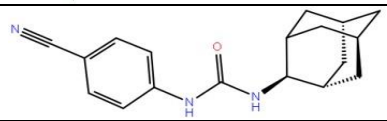
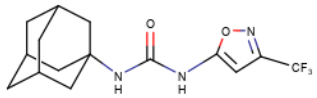
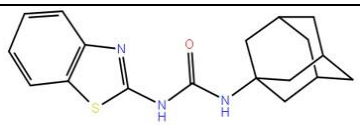
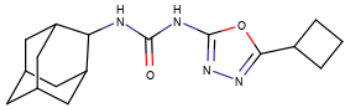
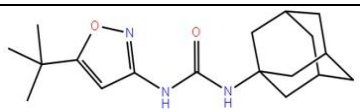
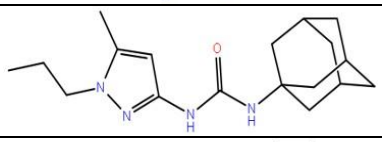
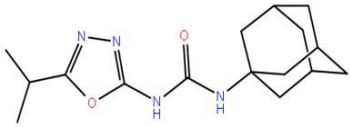
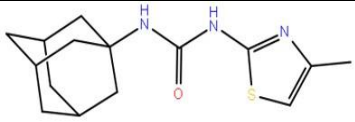
Hereby, using the above dataset atom based 3D QSAR was built. From this dataset 70% were chosen as training set and 30% as test set to generate the best possible QSAR model with 6 PLS (Partial least square) factors to obtain a more precise cross validated coefficient R^2 with minimum standard deviation (<0.3-0.2). The QSAR model with the value of R^2 >> 0.9 or equal to 1 is usually preferred. This in turn helps in generating a better and more accurate cross validated value of q^2 (>>0.7). This was carried out using Atom Based QSAR in Phase application of Maestro (Schrodinger).[19, 20, 21] Refer Table1.

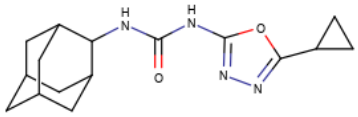
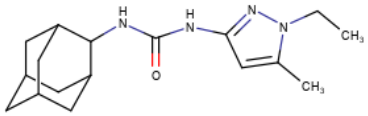
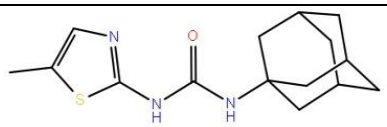
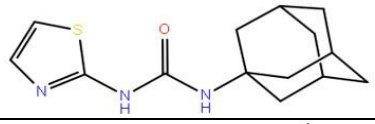
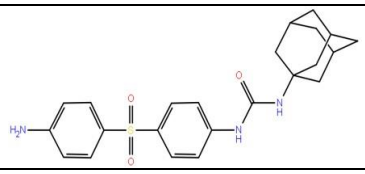
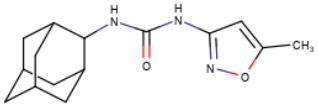
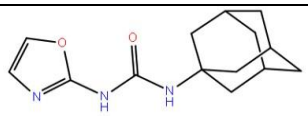
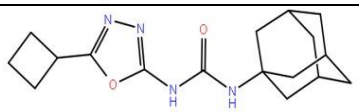
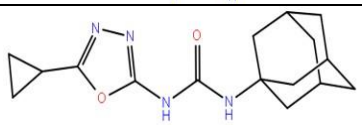
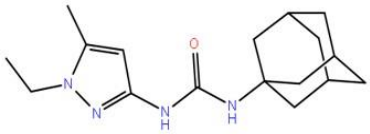
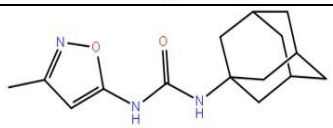
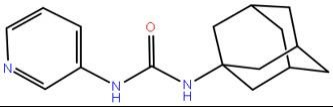
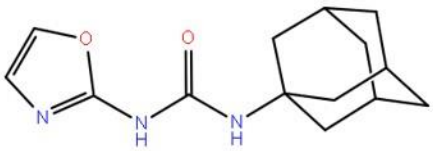
Building Fingerprint based 2D QSAR Models:

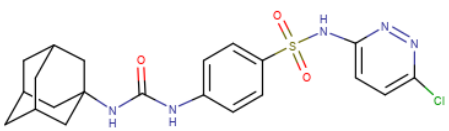
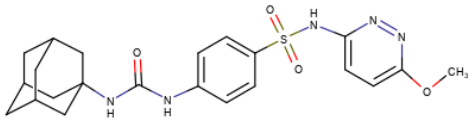
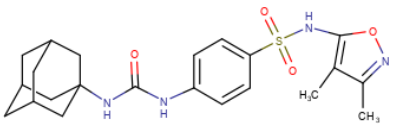
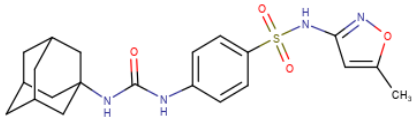
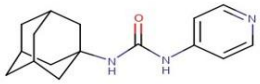
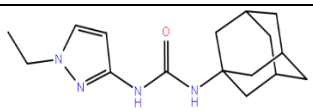
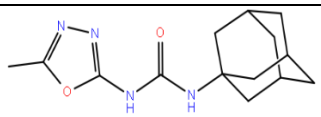
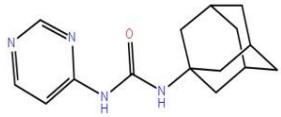
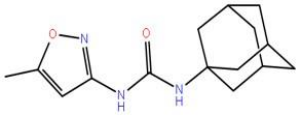
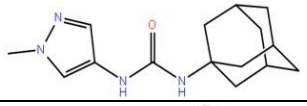
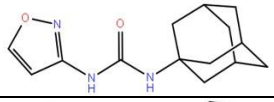
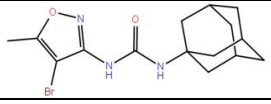
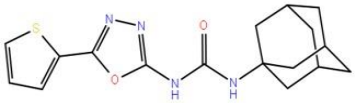
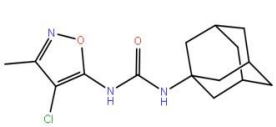
The Canvas provides seven 2D fingerprint based models. They are explained briefly as follows: Atom pair, Atom Triplet, Dendritic, linear, molprint, radial and topological fingerprint models.

Here the 2D canvas fingerprints are associated with Kernel based PLS in order to generate precise and suitable QSAR models that represents the atoms in the structure and give information about the overall beneficial and non-beneficial attributes of the given dataset. The same set of test and training used in atom based 3D QSAR was utilised in the fingerprint based 2D QSAR model generation. 2D QSAR analysis see Table 2.

Table 1. Structure, Predicted and experimental activity of 1-Adamantyl-3-Heteroaryl Urea derivatives with anti-tubercular activity utilised in the test and training set in QSAR model.

Compound	Structure	MIC H37RV (µM)	pMIC H37RV	QSAR Set	Predicted Activity	Predicted Error
1		0.00003	10.511	Training	10.2366	-0.274413
46		0.00032	9.502	Test	8.63846	-0.863198
6		0.00116	8.935	Training	8.91062	-0.0244011
2		0.00123	8.909	Training	9.25025	0.341301
4		0.00135	8.868	Training	9.16503	0.295597
28		0.00237	8.626	Training	8.40991	-0.215621
35		0.00238	8.623	Test	0.096095	0.437895
51		0.00247	8.608	Training	7.96568	-0.642469
24		0.00491	8.309	Training	8.63846	0.329927
43		0.00493	8.307	Test	8.21433	-0.0928476
37		0.00512	8.29	Training	7.89887	-0.391441
33		0.00535	8.271	Training	8.31264	0.0412451

50		0.01035	7.985	Test	7.55453	-0.430476
52		0.01035	7.985	Training	7.65847	-0.326596
34		0.01074	7.969	Training	7.91737	-0.0516071
32		0.01128	7.948	Test	7.90751	-0.0400336
13		0.01469	7.833	Training	7.61688	-0.216196
47		0.0227	7.644	Test	6.80983	-0.834181
21		0.02392	7.621	Training	7.35433	-0.266974
39		0.03951	7.403	Training	7.96568	0.562346
38		0.04134	7.384	Training	7.55453	0.170889
42		0.04133	7.384	Training	7.65847	0.274769
27		0.0454	7.343	Training	7.02542	-0.317557
15		0.04606	7.337	Test	7.24852	-0.0881216
45		0.04784	7.32	Training	7.35433	0.0340912

7		0.05412	7.267	Training	7.38997	0.123288
8		0.05464	7.263	Training	7.18819	-0.074317
12		0.05624	7.25	Training	7.23276	-0.0172287
9		0.05807	7.236	Training	7.19772	-0.0383465
14		0.06633	7.178	Training	7.00804	-0.170238
41		0.08669	7.062	Training	7.25519	0.193142
36		0.09047	7.044	Test	6.94194	-0.101564
18		0.09179	7.037	Training	6.75326	-0.283934
23		0.09806	7.009	Test	6.80983	-0.198697
44		0.18224	6.739	Training	6.8286	0.0892343
22		0.19133	6.718	Training	6.77585	0.0576337
25		0.28228	6.549	Test	6.81061	0.261293
40		0.29033	6.537	Training	6.29932	-0.237789
29		0.32279	6.491	Training	6.58683	0.0957547

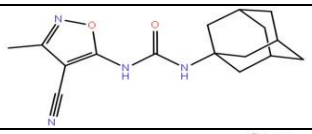
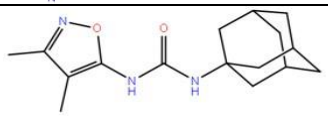
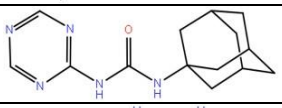
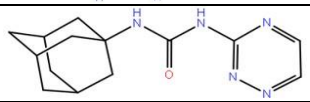
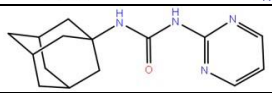
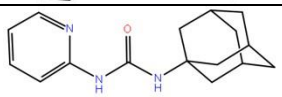
30		0.33293	6.478	Test	6.54856	0.0709141
31		0.34557	6.461	Training	6.47593	0.0144623
19		0.45731	6.34	Test	6.37966	0.03987
20		0.73169	6.136	Test	6.70511	0.569433
17		0.73434	6.134	Training	6.43449	0.300391
16		0.73701	6.133	Training	6.80809	0.675562

Table 2. Fingerprint based 2D QSAR data set.

Ligand molecule	MIC H37Rv (µM)	pMIC	fp linear 1	fp radial 2	fp dendritic 3	fp molprint 2 d4	fp atompairs	fp atomtriplets 5	fp topological 7
1	0.0000	10.511	266	67	188	15	106	767	22
46	0.000	9.502	237	61	180	15	99	539	22
6	0.001	8.935	292	70	226	19	133	893	29
2	0.001	8.909	249	61	188	15	103	682	22
4	0.001	8.868	184	66	127	14	95	545	15
28	0.002	8.626	237	61	180	15	99	538	22
35	0.002	8.63	352	69	224	16	90	569	28
51	0.002	8.608	279	66	184	16	103	617	23
24	0.005	8.309	237	61	180	15	99	539	22
43	0.005	8.290	277	70	207	17	120	723	26
37	0.005	8.290	229	60	168	15	96	532	20
33	0.005	8.271	218	57	157	14	84	432	20
50	0.010	7.985	269	59	177	15	94	518	21
52	0.010	7.985	261	64	198	16	105	617	25
34	0.010	7.969	218	57	157	14	84	432	20
32	0.011	7.948	197	52	37	13	66	315	18
13	0.015	7.833	219	72	158	16	142	1084	17
47	0.023	7.833	219	55	157	14	84	432	20
21	0.024	7.621	197	49	137	13	66	315	18
39	0.040	7.403	279	66	184	16	103	620	23
38	0.041	7.384	269	59	177	15	94	521	21
42	0.041	7.384	261	64	198	16	105	617	25
27	0.045	7.343	218	55	157	14	84	432	20
15	0.046	7.337	196	59	136	14	72	981	18
45	0.048	7.320	197	49	137	13	66	315	18
7	0.054	7.267	310	84	230	22	203	1629	27
8	0.055	7.263	325	84	237	2	217	1835	28
12	0.056	7.250	331	81	270	22	207	1627	33
9	0.058	7.236	311	80	239	21	191	1443	29
14	0.066	7.178	164	55	116	12	65	326	14
41	0.087	7.062	237	63	170	15	96	518	22
36	0.090	7.044	210	54	149	14	81	428	18
18	0.092	7.037	207	59	143	14	73	381	19
23	0.098	7.009	218	55	157	14	84	432	20
44	0.182	6.739	208	57	151	14	79	399	19
22	0.191	6.718	197	51	137	13	74	353	18
25	0.282	6.549	240	57	189	15	95	512	24
40	0.290	6.537	344	70	227	18	123	771	29

29	0.323	6.491	240	57	189	15	95	512	24
30	0.333	6.478	260	62	207	16	109	624	25
31	0.346	6.461	237	55	186	15	92	508	24
19	0.457	6.340	163	55	115	12	61	321	14
20	0.732	6.136	196	58	139	14	76	383	18
17	0.734	6.134	164	55	116	17	66	324	14
16	0.737	6.133	206	58	142	14	72	386	19

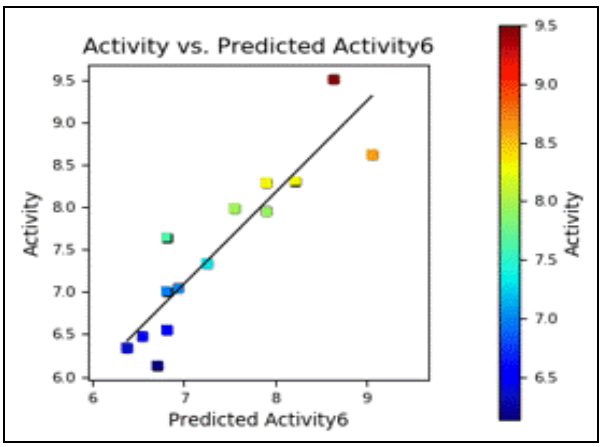
RESULT AND DISCUSSION:

Atom Based 3D QSAR analysis:

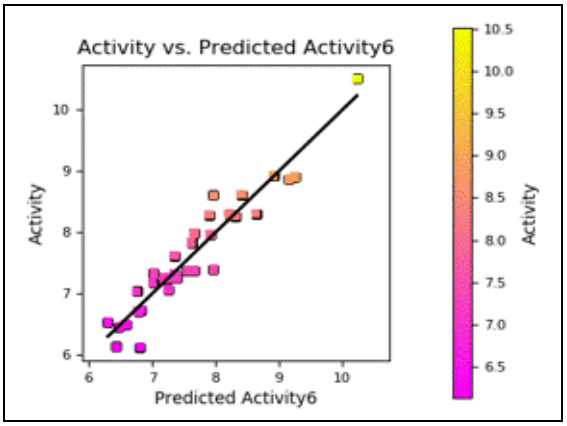
The atom based 3D QSAR models were developed on the basis of the flexible ligand alignment[19,20,21]. The predicted activity as well the predicted errors along with the test and training set selection of the dataset is displayed on **Table 1.** This model was chosen due to its high Q^2 (0.8070) and R^2 (0.9128) which proves it was credible and precise. The statistical parameters of the atom based QSAR constructed based on 6 PLS factors are presented at the **Table 3.** The F factor and the standard deviation were 47.1 and 0.3078 respectively. The scatter plots of the test and training set of the atom based 3D QSAR are shown in **Fig2.**

Table 3. Atom based 3D QSAR Statistics

Factors	SD	R^2	F	Q^2
1	0.7185	0.4369	24.8	0.1978
2	0.6280	0.5833	21.7	0.4807
3	0.5170	0.7267	26.6	0.6520
4	0.3920	0.8481	40.5	0.7925
5	0.3650	0.8728	38.4	0.7925
6	0.3078	0.9128	47.1	0.8070



Test data set plot



Training Dataset Plot

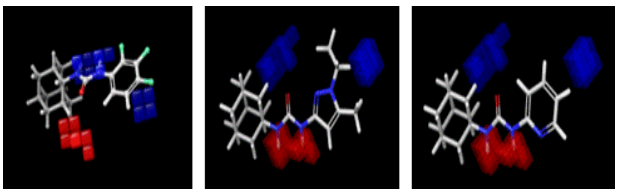
Figure 2. Scatter plot for Atom Based 3D QSAR showing relation between activity observed and the predicted activity 6

3D QSAR visualization:

The statistics Atom type fractions are seen in **Table 4.** The QSAR study was visualised in fields such as Hydrogen Bond Donor, negative ionic, hydrophobic, electron withdrawing and others factors which helped us to gain insight regarding the regions of the chemical framework of the adamantyl urea derivatives in the dataset and information regarding addition or removal of particular groups which causes either positive or negative effect on the anti-tubercular activity of the molecule. The visualisation of the QSAR model are shown in **Figure3.** The analysis was done using the ligand molecule 1 as the reference as it has the highest MmpL3 inhibitory activity among the chosen data set and 16 as molecule with the least inhibitory activity and 42 as the molecule showing the average MmpL3 inhibitory activity. The visualisation was focused on the Hydrogen Bond Donor, negative ionic, hydrophobic, electron withdrawing and others factors in which it is shown that the dark blue and red colour regions represent the positive and negative effects on the activity.

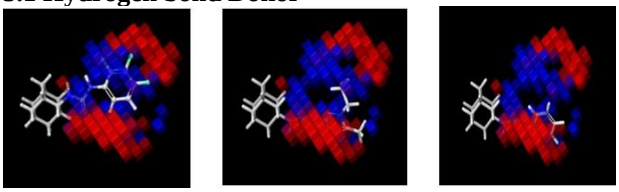
Table 4. Atom Type Fractions

Factors	H-bond donor	Hydrophobic/non-polar	Negative ionic	Electron-withdrawing	Other
1.	0.100787	0.455793	0.007573	0.396786	`0.039061
2.	0.054826	0.461581	0.016217	0.384467	0.082910
3.	0.022115	0.508655	0.022350	0.368735	0.078146
4.	0.013001	0.573151	0.019799	0.308392	0.085658
5.	0.012788	0.570752	0.004901	0.320661	0.090899
6.	0.009478	0.606266	0.010302	0.268104	0.105851



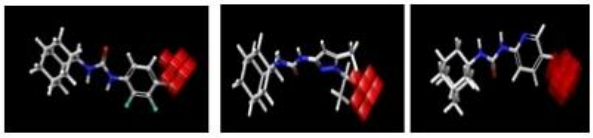
(1) (42) (16)

3.1 Hydrogen bond Donor



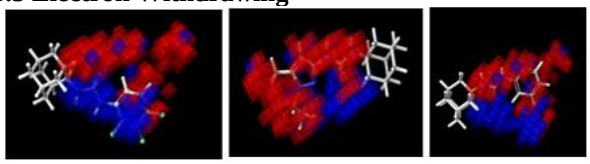
(1) (42) (16)

3.2 Hydrophobic



(1) (42) (16)

3.3 Electron Withdrawing



(1) (42) (16)

3.4 Negative ionic:

Figure 3. Atom based 3D QSAR visualisation in various fields as mentioned above.

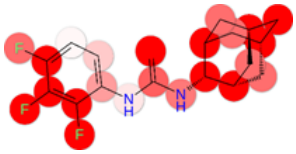
Fingerprint based 2D QSAR study and visualization:

The fingerprint based models in atom pair, atom triplet, dendritic, molprint, linear, radial and topological models were developed using the dataset in Table2[22, 23, 24,25]. The topological model was found out to be the most suitable model among all the fingerprints based 2D QSAR model which helps in predicting the activity more precisely with the validating factors of training set R^2 and standard deviation values as 0.9644 and 0.2005 respectively and test validating set Q^2 and RMSE values as 0.6758 and 0.5386 respectively with six KPLS factors. The statistical data of the Fingerprint based 2D QSAR model are presented at the Table 5. The 2D QSAR is visualised for all the fingerprint model with the ligand molecule 1 as the reference as it has the highest MmpL3 inhibitory activity among the chosen data set and 16 as the least inhibitory activity and 42 and the average. The 2D QSAR fingerprint visualisation of the topological model can be seen in figure 4. It is shown such that the red and blue colour regions represent the positive and negative effects on the activity.

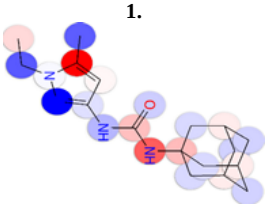
Table 5. Fingerprint based 2d qsar model statistics

Fingerprint based 2D QSAR (fp topological_7 model) Statistics

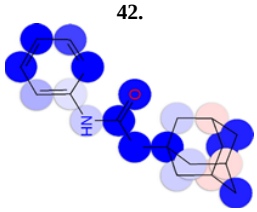
	Training Set	Test Set
Kpls Factors	SD	R^2
1	0.3896	0.8406
2	0.2869	0.9163
3	0.2304	0.9478
4	0.2159	0.9557
5	0.2075	0.9604
6	0.2005	0.9644
Kpls Factors	RMSE	Q^2
1	0.634	0.5506
2	0.566	0.6419
3	0.5036	0.7166
4	0.5093	0.7102
5	0.5288	0.6874
6	0.5386	0.6758



1.



42.



16.

Figure 4. Fingerprint based 2D QSAR topological based model visualisation

CONCLUSION:

The atom based 3D QSAR model and fingerprint based canvas 2D QSAR model were designed and the most precise and suitable model was generated to predict the MmpL3 inhibitory activity of 1-Adamantyl-3-Heteroaryl Urea derivatives. The given study indicates that the ligand molecule 1 has high anti-tubercular activity with various possibilities of structural alteration to develop potential molecule with significant MmpL3 inhibitory activity and also predict the activity of any unknown derivative and determine its pMIC values. The data reported by the above QSAR models provides guidance for the designing of structurally new adamantyl urea inhibitors with potential activity against MmpL3 of M. tuberculosis.

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RESEARCH ARTICLE

Logistic Regression and Artificial Neural Network: A Comparative Study in Diagnosing Breast Cancer

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ABSTRACT:
Breast tumor is a common problem in gynecology. A reliable test for preoperative discrimination between benign and malignant breast tumor is highly helpful for clinicians in culling the malignant cells through felicitous treatment for patients. This paper is carried out to generate and estimate both logistic regression technique and Artificial Neural Network (ANN) technique to predict the malignancy of breast tumor, utilizing Wisconsin Diagnosis Breast Cancer Database (WDBC). Our aim in this Paper is: (i) to compare the diagnostic performance of both methods in distinguishing between malignant and benign patterns, (ii) to truncate the number of benign cases sent for biopsy utilizing the best model as an auxiliary implement, and (iii) to authenticate the capability of each model to recognize incipient cases as an expert system.

KEYWORDS: Breast cancer (BC), Neural Network (NN), Logistic Regression (LR), learning vector quantization, maximum likelihood estimators.

INTRODUCTION:
Medical diagnosis is a major problem in medical care. This includes the limitations of human expertise in diagnosing the disease manually. Variety of methods have been studied in the past to attain the impeccable relegation of cancerous and non-cancerous cells by driving computers into the field. Arjun Patidar et al. [1] presented the comprehensive study on BC. Jean Tresa [2] researched lymphedema prevention and treatment in BC sufferers. Jayashree and Malarkodi [3] discussed BC and various prognostic biomarkers for disease diagnosis. Hussein and Ibrahim [4] investigated the role of miRNA-145 as a good indicator for women's BC detection and treatment. Muthu Lakshmi and Ramya [5] discussed BC knowledge and mammography awareness among age group women of 30 to 50 years. Geetha et al. [6] examined the practice of Breast Self-Examination (BSE) among women and awareness about BC risk factors. Sampoornam [7, 8] has explored the stress level and quality of life among patients with BC.

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Nayna and Joe [9] investigated BSE's social media paradigm of health belief. Sara et al. [10] addressed chromosomal damage contributing to the development of micro nucleated lymphocytes that is more common in BC patient following treatment with chemotherapy compared to control group. Recently, the developing technology of NN has largely exploited to implement a system towards relegation and clustering of cells [11]. The patients find most breast cancers as lumps in the breast. Many lumps to the breast are benign. And hence, diagnosing breast cancer is binding, that is, distinguishing benign lumps from malignant ones.

Mammography, fine needle aspirate (FNA) with visual interpretation and surgical biopsy are three obtainable methods for diagnosing breast cancer. The main objective of the diagnostic aspect of this paper is to improve a relatively objective system to diagnose FNA with an accuracy that is best attained visually. ANN, as a well-established computer aided in diagnostic (CAD) system, is a computer algorithm capable of learning significant relationship from a set of data and applying this knowledge to assess new cases. The outcome of FNA confirms the presence or absence of the malignancy and therefore is a binary outcome. A commonly used statistical logistic model can evaluate

this binary outcome, which is a useful method for discrimination. This paper is carried out to generate and assess both ANN model and LR model to prognosticate malignancy of breast tumor.

MATERIAL AND METHODS:

This paper uses the WDBC made publicly available at http://ftp.ics.uci.edu/pub/machine-learning-database/ breastcancer-wisconsin/. This data set is the outcome of efforts made at the University of Wisconsin Hospital for the diagnosis of breast tumor exclusively based on the FNA test. This test implicates fluid extraction from a breast mass utilizing a diminutive gauge needle, and then a visual inspection of the fluid under a microscope.

The dataset for WDBC is composed of 699 samples. The sample consists of visually measured FNA nuclear characteristics taken from the breast of a patient. Each sample has 11 attributes. Each attribute was assigned a 9-dimensional vector and has a value 1 corresponding to a normal state and 10 corresponding to a most abnormal state in the interval 1 to 10. Attribute 1 is sample number and attribute 11 indicates whether the sample is malignant or benign. The attributes 2 to 10 are as follows: clump thickness, uniformity of cell size, uniformity of cell shape, marginal adhesion, single epithelial cell size, bare nuclei, blend chromatin, normal nucleoli and mitosis. There are 16 samples having a single missing (i.e., unavailable) attribute value and deleted from the database, separating the remaining 683 samples from each other. Each sample has one of the two possible types, benign or malignant. Of 683 samples, 444 are benign and the remaining 239 are malignant, as the WDBC dataset provides. Multi-surface method of pattern separation for medical diagnosis applied to breast lump is studied by Wolberg and Mangasarian [12]. Carpenter and Markuzan [13] got nearly the same performance levels by the application of specialized back propagation (BP-ANN) algorithms. To determine the performance of the established models in practical usage, we have divided the database into two separate databases: (a) the training samples comprising 500 patients records (303 benign, 197 malignant) and (b) the validation samples comprising 183 patients records (141 benign, 42 malignant). Initially, the models trained by changing the weight values for the ANN's interconnection limits and estimating the parameters required to define the classification rules for the logistic regression model are used for the patient records in the training set. The patients reported in the validation sample (n = 183) was then used separately to test the generalizing capability of the developed models. Comparison was made of the best performance of established models in terms of accuracy, sensitivity, specificity, false positive and false negative.

Neural Network Structure:

The implementation of human intelligence in scientific equipment has long been the subject of scientific research and in the last decade of medical research. The biological neural network was first used in computer simulation in the 1950's. In 1951, McCullogh and Pitts stated the first artificial neuron definition [14]. Between 1982 and 1987, mathematical models which could be applied for realistic applications were developed based on the works by Hopfield, Kohohnen, McLelland, and Rummelhart [15, 16, 17]. Neural networks can be applied to medicine in four core fields: modeling, processing of bioelectrical signals, diagnosis and prognosis.

Self-Organization based classification is a powerful tool every time huge higher-dimensional data sets have to be divided into separate clusters [18]. The neural networks [19] are capable of automatically discovering (estimating) the underlying number of clusters and can learn incrementally, without checking the old case. Many ANN's for data clustering have been introduced in recent years, that is, ART, ART1, ART2, ART3, FUZZART, ART-2A and their variants [20, 21, 22, 23]. A similarity measure is integrated in most of these systems into an unsupervised learning algorithm, which allows for distinction between similar and dissimilar patterns. Those networks are based on the Grossberg [24] ART created.

Learning Vector Quantization (LVQ) was developed in 1986 by Kohonen [25] and summarizes three versions of the algorithm in 1990[26]. This is a supervised technique of learning that can classify vectors based on Vector Quantization. This paper was developed in the adoption of LVQ [27] as a breast cancer diagnostic technique. The LVQ version presented here is LVQ1, which was Kohonen's first version of the quantization of learning vectors. The LVQ1 training process proceeds with an input vector being randomly selected from the "labeled" training set (along with the correct class for that vector, hence the supervised learning). Given an input vector x_i to the network, the "output neuron" (i.e., the class or category) in LVQ1is deemed to be a "winner" according to, min $d(x_i, w_j) = \min \| x_i - w_j \|^2$, where w_j are the synaptic weight vectors and they are adjusted in the following manner: If the class associated with the weight vector and the class label of the input are the same,

that is, $Cw_j = Cx_i$, then

$w_j(k+1)=w_j(k)+\mu(k)[x_i-w_j(k)]$ (1)

where $0< \mu(k)<1$ (the learning rate parameter). But if, $Cw_j \neq Cx_i$, then

$w_j(k+1)=w_j(k)-\mu(k)[x_i-w_j(k)]$ (2)

and the other weight vectors are not adapted. The weight

vector w_j is moved in the direction of the weight x_i, according to the learning rule in (1), only when the class label of the input vector and the weight vector agree. If the class is not correct, however, the weight vector moves away from the input vector in the opposite direction, as per (2). The learning rate parameter μ(k) is monotonically decreased in accordance with the discrete time index k (e.g., linearly decreased in time, starting at 0.01 or 0.02; However, many times 0.1 is used as the initial value).

Logistic regression model:

Next, we used logistic regression model (LRM) as a classifier to predict the outcome of the same WDBC dataset. The training and validation samples were used, respectively, to build and validate the logistic regression model. Briefly, the study of logistic regression is a statistical technique by which to analyze the relationship between a dependent variable (FNA result) and a set of independent variables (visually evaluated nuclear characteristics). Then the independent variables will be selected which could give the best prediction. This approach is commonly used to predict membership by using a set of predictors in two groups. Suppose we have two populations with different top probabilities. The posterior probabilities for each category were calculated using the cases provided in the training samples as well as the top probability. Based on the obtained posterior probability associated with variables, the cases presented in the validation samples were separated then.

The simplest method of optimizing discrimination is to maximize the posterior probability of correct allocation. Using the Maximum Likelihood Estimation [28], the logistic coefficients could be estimated to obtain the posterior probability. Allocation of new cases can be accomplished using the logit function, which can be obtained using the natural logarithm of the calculated posterior probability ratio. If the outcome of the logit function is negative, the individual is allocated to class one (benign group). On the other hand, if the outcome is positive, the case is allocated to class two (malignant group).

Assume that we have observed a sequence of N outcomes {0, 1}. These indicate the absent/present of malignant for a patient. $y_i = 1$ implies that the patient i has malignant. We are interested in estimating the percentage of patients having malignant. Call this percentage as E and our estimate of E will be $\hat{E}$. The likelihood principle says that all inference about a parameter should utilize observed data only through how it affects the likelihood function, the probability of observing the observed data given E.

i.e., $L(E) = E^{\sum y_i} (1\text{-}E)^{N-\sum y_i}$

It is often more convenient to work with the log-likelihood function.

i.e., $\log L(E) = \ell(E) = (\sum y_i) \log E + (N - \sum y_i) \log (1 - E)$. If we replace E with $E(x_i)$, we obtain, $\ell(E) = \sum [\, y_i \log\{E(x_i) / (1 - E(x_i))\} + \log (1 - E(x_i))]$. Here $E(x_i)$ enters the likelihood through the function $\log [E(x_i) / (1 - E(x_i))]$, commonly known as the logit of E or the log–odds. This function is also known as the canonical link function for logistic regression. Conveniently, it takes values on the whole real line, making it a simplified modeling process. We will re-parameterize E(x) to be $\{1 / [1 + \exp (- f(x))]\}$. Then the log-likelihood for f(x) is $\ell(f) = \sum [y_i f(x_i) - \log (1 + \exp(f(x_i)))]$

This is the loss function because our goal is to obtain good estimates of E(x). We need to determine a functional form for f(x) and an optimization method. We assume $f(x) = \beta' x$. For optimization, Taylor's series tells us that any analytic function can be approximated as $\ell(\beta) \approx \ell(\beta_0) + (\beta - \beta_0)\, \ell'(\beta_0) + (1 / 2)\, (\beta - \beta_0)^2\, \ell''(\beta_0)$. To maximize $\ell(\beta)$ we can differentiate with respect to β and solve for β. That is, $\beta = \beta_0 - [\ell'(\beta_0) / \ell''(\beta_0)]$. β in the linear model is a vector, but Newton's method has a generalization (Newton-Raphson) using the multivariate Taylor's series, given by $\beta = \beta_0 - [\partial^2 \ell(\beta) / \partial\beta\, \partial\beta']^{-1} [\partial \ell(\beta) / \partial(\beta)]$, where $[\partial^2 \ell(\beta) / \partial\beta\, \partial\beta']$ is the matrix of second derivatives and $[\partial \ell(\beta) / \partial(\beta)]$ is the vector of first derivatives. Then the logistic log – likelihood linear modal becomes

$\ell(\beta) = \sum [\, y_i x_i' \beta - \log (1 + \exp(x_i' \beta))]$

$\partial \ell(\beta) / \partial \beta = X'(y - p)$, where $p = 1 / (1 + \exp(- x_i' \beta))$

$[\partial^2 \ell(\beta) / \partial\beta\, \partial\beta'] = - X' W X$,

where W is a diagonal matrix with element (i, i) equal to $p(x_i) (1 - p(x_i))$. Therefore,

$\beta = [\, X' W X]^{-1} [X' W Z]$,

where $Z = [X \beta_0 + W^{-1}(y - \beta)]$ (3)

For optimum least squares, we can estimate the coefficients as $[X' X]^{-1} [X' y]$. The expression in (3) is similar except that there is the weight matrix W and instead of y we have $[X \beta_0 + W^{-1}(y - \beta)]$. The expression in (3) is actually the solution to the weighted least square problem, namely, Minimize $\sum w_i [y_i - f(x_i)]^2$. To implement the solution, we use standard linear regression computer programs written in Matlab, by setting

$Z_i = x_i' \beta + [y_i - \beta(x_i)] / [\, p(x_i) (1 - p(x_i))]$ and $W_i = p(x_i) (1 - p(x_i))$

and we used weighted linear regression to predict z from x. To find the maximum likelihood estimator, (3) must be iterated in order to reach convergence. This process is called iteratively reweighed least square (IRLS).

Table-1: Comparative performance of the neural network and logistic regression

Data	Group		Predicted instances			
			Neural network		Logistic regression	
			Positive	Negative	Positive	Negative
Training Data (500)	Benign	303	09 (FP)	294 (TN)	46 (FP)	257 (TN)
	Malignant	197	182 (TP)	15 (FN)	171 (TP)	26 (FN)
Testing Data (183)	Benign	141	0 (FP)	141 (TN)	11 (FP)	130 (TN)
	Malignant	42	42 (TP)	0 (FN)	38 (TP)	4 (FN)
Total Data (683)	Benign	444	09 (FP)	435 (TN)	57 (FP)	387 (TN)
	Malignant	239	224 (TP)	15 (FN)	209 (TP)	30 (FN)

Table-2: Comparative results of the neural network and logistics regression

Data	Measurements	Predicted results in percentage	
		Neural network	Logistic Regression
Training Data (500)	Sensitivity	92.39	86.80
	Specificity	97.03	84.82
	Efficiency	95.20	85.60
	Predictive value (Positive)	95.29	78.80
	Predictive value (Negative)	95.15	90.81
Testing Data (183)	Sensitivity	100	90.48
	Specificity	100	92.20
	Efficiency	100	91.80
	Predictive value (Positive)	100	77.55
	Predictive value (Negative)	100	97.01
Total Data (683)	Sensitivity	93.72	87.45
	Specificity	97.97	87.16
	Efficiency	96.49	87.26
	Predictive value (Positive)	96.14	78.57
	Predictive value (Negative)	96.67	92.81

RESULTS:

Of the 683 samples of the WDBC data set, 500 were used to train the NN using the first version of the Kohonen's learning vector quantization technique and the remaining 183 samples were used to test the sample data. We initialize the learning rate parameter to $\mu(k=1) = \mu(1) = 0.1$ and decreasing it by k at every training epoch , for example $\mu(2) = \mu(1)/2$, $\mu(3) = \mu(2)/ 3$, etc. One method to initialize the codebook vectors is to use the first two samples, one from the benign and one from malignant. The associated classes for them are '1' and '2' respectively. The Matlab program is used to locate the codebook vectors, with the number of training epochs set at 35,000. After 35,000 training epochs, the final weight gives the codebook vectors.

If we measure the minimum distances between the remaining 183 samples and the codebook's computed weight vectors, the class to which each of the remaining samples belongs will be given. Table-1, presents the results of true positives (TP), false positives (FP), true negatives (TN) and false negatives (FN). Thus, the NN successfully identified 435 instances as negative and 224 instances as positive having an a priori known set of 444 benign and 239 malignant instances. Table-2 presents the sensitivity, specificity and efficiency of the LVQ1 technique, as well as the predicted values of a positive/negative test result, according to these observations,

Similarly, we applied the logistic regression model using the iterative reweighed least square (IRLS) method to these 683 samples of WDBC data set. The Matlab software is used to find the maximum Likelihood Estimators (MLE). Table -1, presents the results using this model. Out of 444 benign and 239 malignant instances, the logistic regression successfully identified 387 instances as negative and 209 instances as positive. In the same table-1, we also present the true positive (TP), false positive (FP), true negative (TN) and false negative (FN) results. According to these observations, we present the sensitivity, specificity and efficiency of the IRLS method using Logistic regression model in the Table-2. We also present in the table-2 the predicted values of a positive/negative test results.

CONCLUSION:

In this study, we presented an algorithmic model based on the analysis of logistic regression and a non-algorithmic model based on the ANN. We compared the ability of these models to distinguish malignant from benign tumor among a group of 683 patients. Our main objective is to investigate which model gets more reasonable specificity while maintaining high sensitivity. By doing so, we hope to decrease the number of cases sent to the biopsy; especially under the biopsy procedure for apparently benign lesions.

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RESEARCH ARTICLE

Stability indicating RP-HPLC method for the determination of Tenofovir in pharmaceutical formulation

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ABSTRACT:

Stability indicating high performance liquid chromatography (HPLC) method was developed for the assay of Tenofovir in bulk and solid dose formulation. The HPLC separation was achieved on kromasil C18 (100mm × 4.6mm, 5 µm) column using a mobile phase of Methanol: Potassium dihydrogen orthophosphate buffer (30:70,v/v) at a flow rate of 1 ml min^{-1} and UV detection at 260 nm. Peak elutes at 7.33 appropriate. The method was validated for linearity, repeatability, accuracy, precision, robustness, limit of detection and limit of quantification. The accuracy was between 99.14 - 99.97%. The highest R.S.D. amongst interday and Intraday precision was found 0.808 and 0.473 respectively.The assay was linear over the concentration range of 10-50 µg/ml (R≈0. 999). The method was robust as no significant change in chromatographic parameters. LOD and LOQ was found to be 0.90 and 2.71 respectively. The stress studies were performed per ICH guidelines to confirm its Stress testing was carried out in presence of acid, base, hydrogen peroxide, heat and light to demonstrate specificity of the method as per ICH guidelines. The developed method could separate the potential degradation products from the Tenofovir peak. It was concluded that highest degradation occurs in basic condition. This proposed method was suitable and practical for analysis the content of Tenofovir in pharmaceutical products and could be of benefit for the prediction shelf life of Tenofovir in marketed formulations.

KEYWORDS: Tenofovir, Liquid Chromatography, Assay, Development, Validation.

INTRODUCTION:

Tenofovir Alafenamide Fumarate (TAF) belongs to the class of nucleotide reverse transcriptase inhibitor (NRTI). It is a novel ester prodrug of the antiretroviral Tenofovir. It is chemically called as (2E)but-2-enedioicacid; bis(propan-2-yl(2S)-2-{[(S)-({[(2R)-1-(6-amino9H-purin-9-yl)propan-2yl]oxy}methyl)(phenoxy)phosphoryl]amino}propanoate).It has a molecular formula of$C_{23}H_{31}N_6OP$ and a molecular weight of 476.47 g/mol. It has the following structure (Figure 1) It is Slightly soluble in water, soluble in methanol, very slightly soluble in dichloromethane.[1,2]

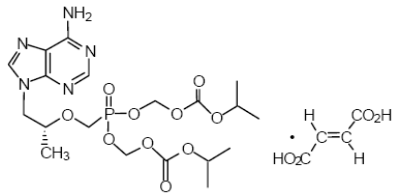

Fig. 1. Structure of Tenofovir

Several high-performance liquid chromatography (HPLC) methods have been published for direct analysis of Tenofovir in bulk materials and formulations in pharmaceutical product[3,16.] However, the purpose of this work was to develop and validate an economical, simple and stability-indicating HPLC method for Tenofovir bulk materials and pharmaceutical formulations using a C18 column for chromatographic separation followed by UV detection at 260 NM. Stress testing was carried out to demonstrate specificity of the method. [17] The developed method could be applied for prediction of shelf life of Tenofovir in related pharmaceutical products.

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MATERIALS AND METHODS:

Chemicals and Reagents:

Tenofovir bulk material was purchased from Mylan Pharmaceutical Ltd.Hyderabad. HPLC grade methanol was purchased from Merck Specialities Pvt. Ltd. with analytical reagent grade and water was obtained from Millipore.The marketed formulation was purchased from market and used for assay purpose.

HPLC Apparatus and conditions:

HPLC was selected as analytical technique for estimation of Tenofovir. The analysis of the drug was carried out on Agilent (S.K.) having Gradient System with UV Detector well Equipped with Reverse Phase (Cosmosil) C18 column (4.6 id x 250mm; 5µm), A SP930D pump and a 20µl injection loop with UV730D Absorbance detector and running Chemstation software. The elution was performed on isocratic solvent system using methanol: Potassium dihydrogen orthophosphate buffer (30:70v/v) at a flow rate of 1 ml min^{-1} , UV detection at 260 nm and the runtime was set to be 17 minutes with controlled temperature at 27ºC and injection volume was 20 µL for every samples and standard.

Stock and working solutions of standard compound:

Stock solution was prepared by accurately weighed standard Tenofovir 10 mg, dissolved in mobile phase, and adjusted to 100 mL with a volumetric flask. Working standard solutions were obtained by appropriate dilution of the stock solution with mobile phase.

Stress testing:

The stress conditions employed for the degradation study included base hydrolysis, acid hydrolysis, and oxidative condition. Stress testing was done by adding 50 µL of reagent to 1 mL of Tenofovir sample, 1N HCl, 1N sodium hydroxide, and hydrogen peroxide (3% v/v) were used as reagent for acid hydrolysis, base hydrolysis, and oxidative stress, respectively. Deionized water was used as solvent. Each sample was then analyzed with the proposed HPLC method after 1 and 2 hours.The peak purity of stressed samples was monitored by the diode array detector in the wavelength range of 200–400 nm[17]

Method validation:

Validation of the method was done according to the International Conference on Harmonization guideline (ICH, 1996/2005). The method was validated for linearity, precision, accuracy, limit of detection (LOD), and limit of quantification (LOQ).[18,19,20]

System precision/system suitability:

System precision was determined by measuring the peak area of standard solution containing 100% working concentration for six times and calculates the % RSD. The % RSD should be less than 2.0%. The relative standard deviation of six replicate measurement of standard solution found 0.193 % (limit NMT 2.0%), which indicates that the system is precise to analyze the sample.

Linearity:

From Tenofovir standard stock solution, different working standard solution (10-50µg/ml) were prepared in mobile phase. 20µl of sample solution was injected into the chromatographic system using mixed volume loop injector chromatogram were recorded.

The area for each concentration were recorded

Repeatability:

Repeatability is the result of the method operating over short time interval (within a day) under the same conditions. The peak area of 20µg/mldrug solution was analyzed six times on the same day. The %RSD was calculated for the resultant peak area and retention time.

Precision:

The intra-day precision was determined by analyzing the six-time injection within one day of Tenofovir, while the inter-day precision was examined for five consecutive days by the proposed method. The precision was expressed as percentage of relative standard deviation (%RSD).

Accuracy/Recovery:

Recovery studies were performed to validate the accuracy of developed method. Standard addition was performed with pre-analyzed standard solution. To pre-analyzed formulation, a definite concentration of standard drug (50%, 100%, and 150%) was added and then its recovery was analyzed. The recovery was calculated as follows: recovery (%) = 100 × (detected amount – original amount)/spiked amount. The % recovery was found to be within 99-100%

Robustness:

Robustness was performed by variation of flow rate, wavelength, mobile phase ratio and then the analysis was done.

Limit Detection and Limit Quantification:

Determination of signal-to-noise ratio was calculated under the proposed chromatographic condition. LOD was considered as 3:1 and LOQ as 10:1.

RESULTS AND DISCUSSION:

Selection of Wavelength:

From the figure 2, UV spectrum of Tenofovir, wavelength 260 nm was selected which showed the maximum absorbance in the wavelength range.

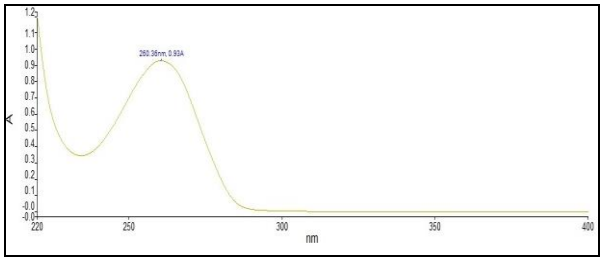

Figure-2: UV spectrum of Tenofovir

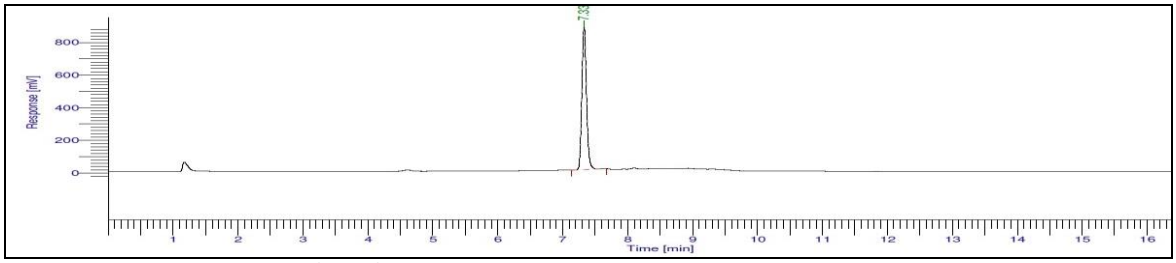

Figure-3: Optimised Chromatogram of Tenofovir

Development and optimisation of HPLC method:

A fast stability-indicating HPLC technique was developed for the analysis of Tenofovir in tablet formulation. From several trials, the mobile phase consisting of Methanol: Potassium dihydrogen orthophosphate buffer in the ratio 30:70 (v/v) was found to be an appropriate mobile phase allowing adequate separation of all the compounds using a symmetry C18 (100 mm×4.6 mm, 5 µm) column at a flow rate of 1.0 ml/min. It provided symmetrical peaks and has the most efficient separation and speed. The maximum absorbance 260 nm was used for wavelength detection. A typical chromatogram of separation of Tenofovir is shown in fig. 3.

Specificity:

There was no interferences observed at retention time of parent peak, so method was specific as shown in figure-4.

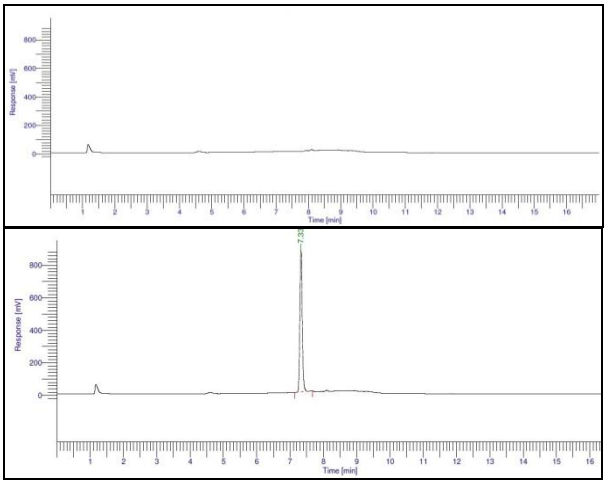

Figure (4a,b): Chromatogram of Blank Solution Chromatogram of optimized method

Validation Parameters:

The calibration curve yielded correlation coefficient (r^2) 0.9966 for Tenofovir respectively as shown in Table-1 which showed the method was linear. For repeatability studies, based on Peak area and retention time the %RSD was found to be 1.26 and 0.87 respectively as shown in Table-2. Based on recovery studies, the accuracy was between 99.14 - 99.97%. as shown in Table-3. The %RSD for intraday precision and interday precision was found to be between 0.29-0.34 and 0.45 and 0.80 respectively (table-5).By changing the mobile phase composition, flow rate and wavelength change, there was no significant change in chromatographic parameters. There was no effect of mobile phase composition on retention time (table 6).The LOD is the lowest limit that can be detected. Based on the S.D. deviation of the response and the slope the limit of detection (LOD) may be expressed as:

LOD = 3.3 (SD)/S = 3 = 0.90µg/ml Where, SD = Standard deviation of Y intercept S = Slope

The LOQ is the lowest concentration that can be quantitatively measured. Based on the S.D. deviation of the response and the slope, the quantitation limit (LOQ) may be expressed as LOQ = 10 (SD)/ S = 2.71µg/mlWhere, SD = Standard deviation Y intercept S = Slope

The LOD of Tenofovir was found to be 0.90 (µg/mL).The LOQ of Tenofovir was found to be 2.71 (µg/mL).

Table -1- Linearity data

Conc µg/ml	Peak area	Statistical analysis	
0	0	Slope	37363
20	887009.01		
40	1548374.76	Intercept	80923
60	2425678.67		
80	3114533.14	Correlation coefficient	0.9966
100	3718846.09		

Table-2 Repeatability study (n=6)

Concentration	% RSD[a]	% RSD[b]
20 µg ml^{-1}	1.26	0.87

[a]Based on peak area [b] Based on retention time

Table-3 Recovery studies for Tenofovir

Level of recovery	Drug	Mean % Recovery	Standard Deviation*	% RSD
50%	Tenofovir	99.14	0.47	0.48
100%	Tenofovir	97.65	2.76	2.82
150%	Tenofovir	99.97	0.66	0.66

Table-4 Precision Study (n=3)

Concentration	Mean Peak area	SD	%RSD
Interday			
20 µg ml^{-1}	1692255.11	1369.44	0.808
30 µg ml^{-1}	2171945.86	11621.64	0.535
40 µg ml^{-1}	3183764.76	14411.54	0.453
Intraday			
20 µg ml^{-1}	1711240.66	4980.75	0.291
30 µg ml^{-1}	2201504.68	10532.61	0.473
40 µg ml^{-1}	3110431.42	10778.43	0.346

Table. 5. Robustness study (n = 3)

Concentration	Conditions changed	% RSD	Mean RT
50 µg ml^{-1}	**Mobile phase composition**		
	80:20	1.13	2.17
	90:10	0.56	2.16
	Flow rate		
	0.8 ml min^{-1}	0.71	3.75
	1.2 ml min^{-1}	1.08	2.42

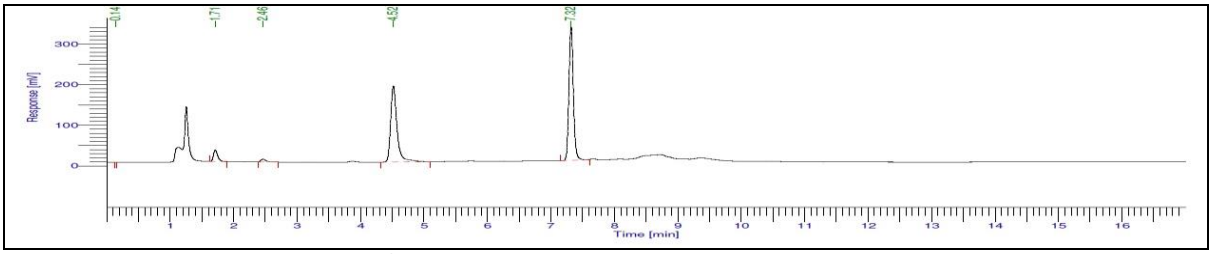

Figure-5ADegradation study with 1N HCl After 30 minute at room temperature

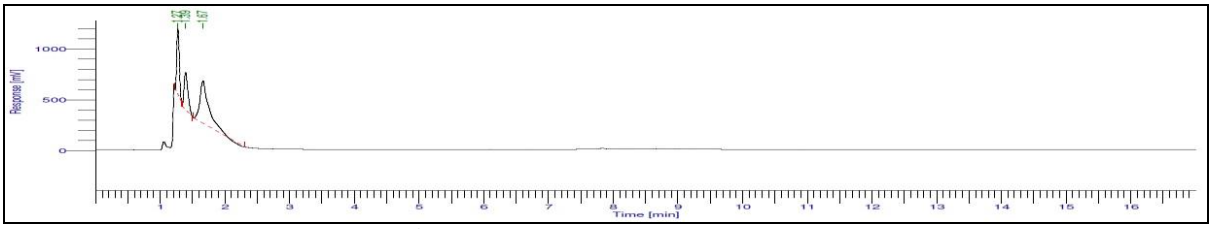

Figure-5B:Degradation study with 1N NaOH After 30 min at 80°C

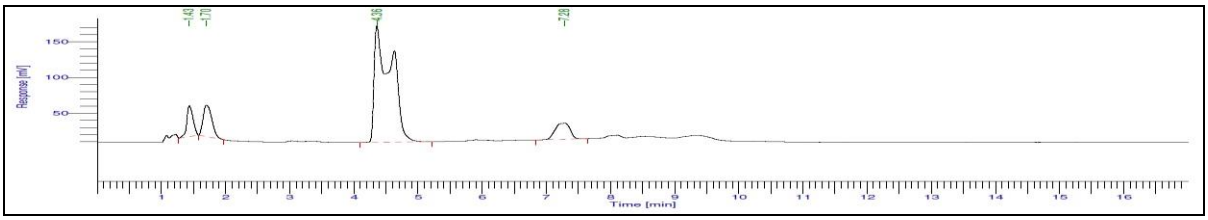

Figure-5CDegradation study with 0.01N NaOH initial at room temperature

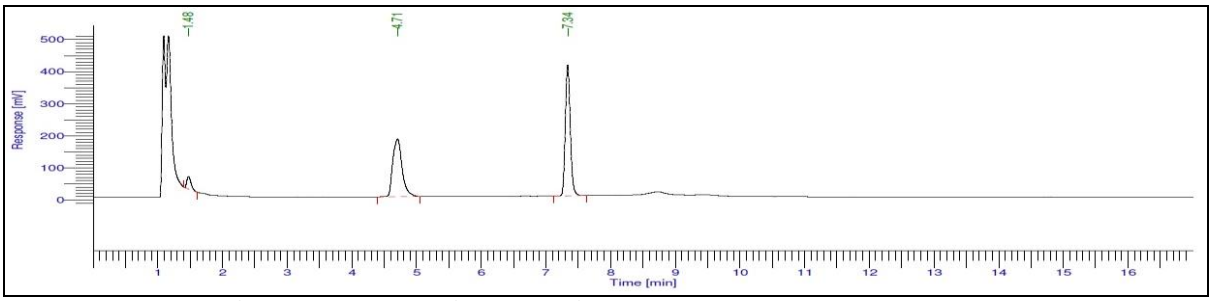

Figure-5DChromatogram of 3% H_2O_2 treated Tenofovir at 80°C for 30 min

Figure-5 A, B, C, D. Elution profile of Tenofovir obtained after different stress conditions: Figure 5A (1N HCl After 30 min at room temperature) Figure 5B (1N NaOH After 30 min at 80°C), Figure-5C (0.01N NaOH initial at room temperature) Figure-5D (3% H_2O_2 treated Tenofovir at 80°C for 30 min)

Forced degradation studies:

The experiment showed that the maximum degradation of Tenofovir occurred under basic conditions. The results of% degradation during forced degradation was shown in Table-6.

Figure 5(A, B, C,D) showed chromatograms of acidic, oxidative and basic stressed bulk drug after after 30 minutes by changing the temperature conditions. Baseline resolution between Tenofovir and degradation product peaks was achieved. The elevated peak at beginning of chromatogram showed degradation of Tenofovir under various stressed condition

Table-6 Degradation Data

Strength used	Temperature (°C)	Time (min)	% Degradation
Acid			
1 N HCl	80	30	48.04
0.1 N HCl	40	10	3.96
1 N HCl	40	60	41.96
Base			
1 N NaOH	80	30	100
1 N NaOH	80	5	100
0.1NaOH	40	5	100
0.01 N NaOH	40	5	91.1
Oxidative			
3 % H_2O_2	80	30	47.44

CONCLUSION:

A rapid and reliable isocratic RP-HPLC method for determination of Tenofovir has been developed and validated. This chromatographic assay fulfilled all the requirements to be identified as a reliable and feasible method, including accuracy, linearity, recovery, robustness and precision data. It is a highly specific and precise analytical procedure and having narrow range of chromatographic run time allows the analysis of a large number of samples in a short period of time. It was subjected to different stress conditions and the developed stability indicating method can able to detect and quantify the degraded productsTherefore, this economical and simple HPLC-UV method can be used as a routine sample analysis. The developed method was applied for the determination of Tenofovir content in marketed formulation. The result showed the method was suitable for stability-indicating analysis and assay for commercial products.

ACKNOWLEDGEMENTS:

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RESEARCH ARTICLE

Protective activity of *Ruta chalepensis* methanolic extract against nephrotoxicity and testicular damage induced by Carbon tetrachloride on albino male mice

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ABSTRACT:

Herbal medicinal products can contain whole or partially prepared plant components from plant leaves, bark, stems, flowers and seeds.They are administered orally, inhaled or directly applied in the skin. *Ruta chalepensis* is a wild herb of the Mediterranean region used by many countries in herbal medicine. The existence of bioactive molecules responsible for their pharmacological properties has been shown by phytochemical screening. Results of kidney protective activity of plant. Showed that: for total cholesterol, the effect was dose dependant (50 and 100 mg\kg) in which the plant decreased it in compared to positive and negative groups (162.1±1.83 and 154.6±1.11 mg\dl) compared to (202.1±1.13 and 167.5±2.96 mg\dl) respectively. For total protein, creatinin and albumin the plant also had the ability to keep it near control groups compared to CCL_4group.While the results of interaction groups indicated the ability of plant to provide protection against CCL_4 damage. the plant possessed the ability to keep testosterone, progesterone and estrogen hormones level near normal in compared to CCL_4 treated group (2.96±0.03, 1.93±0.01 and 3.63±0.04 ng\dl); (11.51±4.12, 9.85±2.18 and 11.78±3.42 ng\ml); (29.07±7.21, 30.11±9.11 and 30.67±8.98 ng\ml) for 50,100 mg\kg and negative control respectively. While for interaction group the results showed the ability of plant to counteract the damaged caused by CCL_4 (1.67±0.01, 2.54±0.02); (10.42±2.21, 13.65±4.37); (39.74±10.13, 35.45±9.91) for testosterone, progesterone and estrogen hormones in *Ruta chalepensis* +CCL_4 at dose (50 +0.02%) and (100+0.02%) respectively. All results of histo-architecture for kidney and testis showed the ability of plant to counteract any necrosis and abnormality caused by CCL_4.

KEYWORDS: *Ruta chalpensis*, Medicinal plant, CCL_4 , Nephrotoxicity, testes.

INTRODUCTION:

The vast majority of people on this planet still rely on their traditional medicinal plants and other materials. It is also a fact that one quarter of all medical prescriptions are formulations based on substances derived from plants or plant-derived synthetic analogs, and according to the World Health Organization (WHO), 80% of the world's population, primarily those of developing countries, rely on plant-derived medicines for their healthcare. In particular, a tremendous demand for knowledge on the uses and therapeutic properties of these plants has been found to be of worldwide significance in the use of medicinal plants and herbal plants[1]. All medicinal importance of different plant types

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attributed to its secondary metabolite and essentail oils rich in hydrocarbon compounds that have hydrogenated,oxygenated and dehydrogenated functional compounds are one of the major secondary metabolites of the plant[2]. *Ruta chalepensis.(Rutaceae)* is a medicinal plant that widely distributed in the Mediterranean area. It is an ancient medicinal plant unique presence used in the traditional medicine of many countries. Pharmacological investigations clearly indicated that the aerial part of *R. chalepensis* shares anti-inflammatory properties[3] Because of its high content of young stems and leaves with saponins ,amino acids, phenols, furocoumarins flavonoids and alkaloids. R. Chalepensis has pharmacological activities of pleiotropic origin[4]. and although these active components may be responsible for the plant's advantageous, the bioactivities of R. chalepensis extracts and its several preparations against tumor cells have involved only recently [5], accordingly, They are used primarily as anti-inflammatory, antispasmodic ,analgesics ,local anesthetic, anthelmintic, antiseptic and antipruritic, as well as many other therapeutic applications, as complementary herbal medicine and therapeutic agents [6]. Therefore, The objective of this study was to identify the activity of *R. chalepensis* methanolic extract of kidney and testes in CCL_4 damaged albino male mice.

MATERIAL AND METHOD:

Plant collection, identification and extraction:

The aerial parts of plant collected from Baghdad local market during march\ 2020\ which previously identified by National Herbarium of Iraq\ then these parts were washed with distill water and air dried (in shade) in addition to keep it in an oven for about 48 hours at 37°C and make it powder by glass mortar with the aid of a pestle. Plant powder have been extracted with 80% methanol (v/v) at 65°C using a soxhlet apparatus for about 3 hours for full extraction. Finally, the extract dried at 37 °C in incubator and dissolved to prepare require doses (50 and 100 mg\kg) [7].

Estimation of biochemical test.

This test was done by using enzymatic colorimetric method to evaluate of enzyme activity of kidney and testes function in mouse serum [8] For this purpose,use commercial kits (Randox Company) were used .

Tissue Preparation for Histological section:

By using 10% formalin samples remained stable for 24h and remove all water with series concertation of alcohol (30-to100%) for five min. next step embedding samples with paraffin wax to obtain blocks .the Cross sections were prepared of (5)µm thickness and stained with hematoxylin(Harison) and eosin as stated by standard method. Under light microscope histopathological changes stand achieved as compared to control group [9].

Experimental Design with Albino Male Mice Animals:

Albino male mice, 6-8 weeks old, weight of 23-25 gm were supplied from Al-Nahrain University, Biotechnology Research Center,Baghdad, Iraq. Four animals were housed per cage. They were divided into six groups: The first group (I) was mice treated with (50 mg\kg) of plant dose. The second group (II) was mice treated with (100 mg\kg) of plant dose. The third group (III) mice treated with 0.02% CCL4, the fourth group (IV) was control negative group (without any treatment), group five (V) and six (VI) were interaction between plant extract 50 and 100 mg/kg and 0.02% CCL4 respectively. Each investigated group was intraperitoneally (i.p.) injected with a single dose per day (0.1 mL) of the tested material for seven days while in interaction group, they received 0.02% CCL4 in day one and from day 2-7 received doses of plant extract (50 and 100 mg\kg) respectively. On day 8 of the experiment, the animals were sacrificed to carry out laboratory assessments [10].

Statistical Analysis:

The values of the investigated parameters were given in terms of mean ± standard deviation (SD), and differences between means were assessed by analysis of variance (ANOVA) followed by least significant difference (LSD) or Duncan test, using the computer programmer SPSS version 13.0.

RESULTS AND DISCUSSION:

Biochemical parameters function of *Ruta chalpensis* on kidney function test:

As shown in table 1, the biochemical parameters of plant extract activity different from one to other but all results indicated kidney protective activity of plant. For total cholesterol, the effect was dose dependent (50 and 100 mg\kg) in which the plant decreased it in compared to positive and negative groups (162.1±1.83 and 154.6±1.11 mg\dl) compared to (202.1±1.13 and 167.5±2.96 mg\dl) respectively. For total protein, the plant also had the ability to keep it near control groups compared to CCL4group (8.51±0.19, 9.48±0.20 and 8.15±0.23 g\dl) for 50,100 mg\kg and negative control group respectively in comparison with (4.22±0.25). Accordingly, the results of creatinin and albumin declared the ability of *Ruta chalepensis* plant to maintain the level within control group: for creatinin (1.35±0.02, 1.20±0.03 and 1.57±0.02mg\dl) compared to CCL4 (4.22±0.25mg\dl); for albumin (5.48±0.09, 6.23±0.06 and 5.56±0.09 g\dl) compared to ccl4 (3.67±0.04 g\dl) , for 50,100 mg\kg and negative control group respectively. While the results of interaction groups indicated the ability of plant to provide protection against CCL4 damage as shown in table below.

Table 1: Effect of *Ruta chalpensis* methanolic extract and CCL_4 on biochemical parameter function in albino male mice.

Mice groups	Doses (mg\kg)	Total cholesterol (mg\dL) Mean±S.E.	Total protein (g\dl) Mean±S.E.	Creatinin (mg\dL) Mean±S.E.	Albumin (g\dL) Mean±S.E.
***Ruta chalpensis* (I)**	50	162.1±1.83	8.51±0.19	1.35±0.02	5.48±0.09
***Ruta chalpensis* (II)**	100	154.6±1.11	9.48±0.20	1.20±0.03	6.23±0.06
CCL4 (III)	0.02%	202.1±1.13	4.22±0.25	1.78±0.03	3.67±0.04
Control group (IV)	----------	167.5±2.96	8.15±0.23	1.57±0.02	5.56±0.09
***Ruta chalpensis*+CCL4(V)**	50+0.02%	178.3±2.26	6.51±0.13	1.59±0.03	5.78±0.02
***Ruta chalpensis*+CCL4 (VI)**	100+0.02%	173± 2.81	7.32±0.56	1.58±0.01	5.17±0.09[b]

Table 2: Represented the effect of Ruta chalepensis on some testes hormones.

Mice groups	Doses	Testosterone Hormone ng/dl	Progesterone Hormone ng/ml	Estrogen hormone ng/ml
***Ruta chalpensis* (I)**	50	2.96±0.03	11.51±4.12	29.07±7.21
***Ruta chalpensis* (II)**	100	1.93±0.01	9.85±2.18	30.11±9.11
CCL4 (III)	0.02%	1.07±0.01	17.21±5.21	45.32±11.65
Control group (IV)	----------	3.63±0.04	11.78±3.42	30.67±8.98
***Ruta chalpensis*+CCL4(V)**	50+0.02%	1.67±0.01	10.42±2.21	39.74±10.13
***Ruta alpensis*+CCL4 (VI)**	100+0.02%	2.54±0.02	13.65±4.37	35.45±9.91

Histo-architecture activity of *Ruta chalpensis*:

All results of histological effect of plant explained in figure (2, and 3) which indicated the kidney protective activity of plant compared to CCL4 group which cause necrosis together with mild inflammatory cell infiltrate (mononuclear cells) and fatty changes in kidney tissue of mice treated with CCL4 in figure(1).

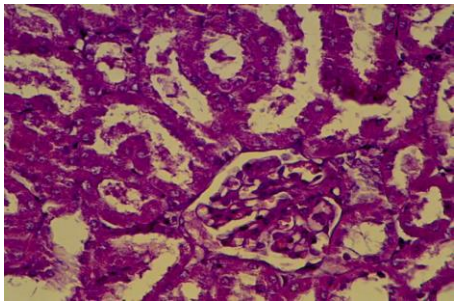

Fig 1:Necrosis in kidney tissue that present together with mild infiltration of mononuclear cells and fatty changes on mice treated with CCL4 (200×; H and E).

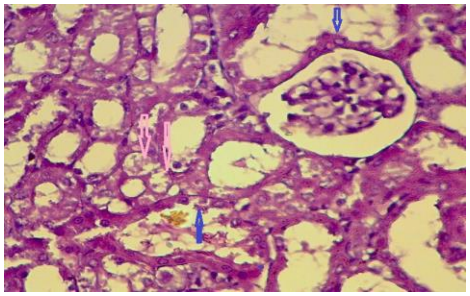

Fig 2: Tubules degenerative changes (blue arrow) and necrosis of lining epithelial cells (pink arrow) in mice treated with plant extract at dose of (100 mg\kg)

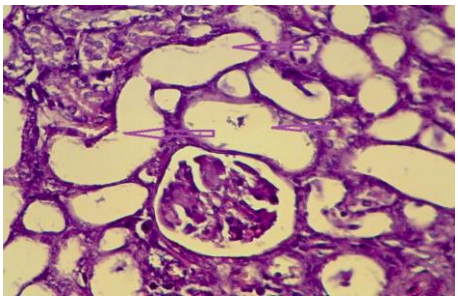

Fig 3: Cystic dilatation of renal tubules(pink arrow)due to degeneration of epithelial lining cells in interaction group (100 mg\kg +ccl4).

Biochemical parameters of *Ruta chalpensis* on some testes function test:

As explained below the plant possessed the ability to keep testosterone, progesterone and estrogen hormones level near normal in compared to CCL4 treated group (2.96±0.03, 1.93±0.01 and 3.63±0.04 ng\dl); (11.51±4.12, 9.85±2.18 and 11.78±3.42 ng\ml); (29.07±7.21, 30.11±9.11 and 30.67±8.98 ng\ml) for 50,100 mg\kg and negative control respectively. While for interaction group the results showed the ability of plant to counteract the damaged caused by CCL4 (1.67±0.01, 2.54±0.02); (10.42±2.21, 13.65±4.37); (39.74±10.13, 35.45±9.91) for testosterone, progesterone and estrogen hormones in Ruta chalepensis +CCL4 at dose (50 +0.02%) and (100+0.02%) respectively.

Histo-architecture activity of Ruta chalpensis on testes:

The results of histological activity of plant extract indicated ability of plant to counteract the damaged caused by CCL4(figure 4) which lead to widespread of apoptotic germinal cells in seminiferous tubule germinal epithelium and in the lumen (figure 5).

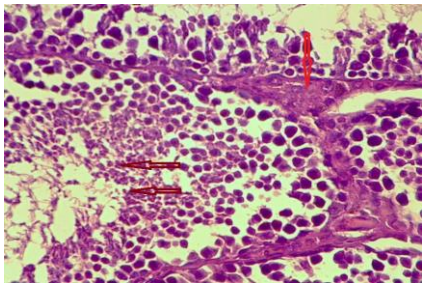

Fig 4: Normal development of spermatogonia cells and huge numbers of sperms((red arrow) inside the lumen and normal structure of leydig cells.

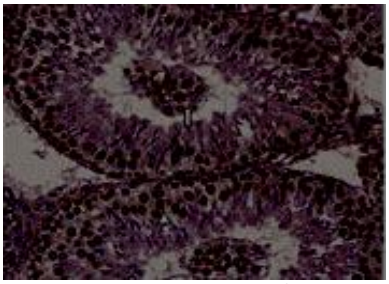

Fig 5:Apoptotic germinal cells in seminiferous tubule germinal epithelium and in the lumen testis tissue in mice treated with methotrexate drug (200×; H and E).

DISCUSSION:

In recent years there has been increasing attention in oxidative status and particularly in the pathologic role of free radicals that mediating peroxidation of membrane lipids and oxidative damage to DNA, are associated with a variety of chronic health disorders, such as testis and kidney diseases [11,12]. Additional, some drugs cause kidney disorder such as Gentamicin[13]. Also Increase level of Serum Creatinine and urea lead to kidney Deterioration[14].on another hand, some medical plant such as Gokshura Fruit and Varun Bark, before treated with nephrotoxicity induction, demonstrated kidney safety against the induction of Gentamicin[15]. Consequently, antioxidant inhibition of oxidative damage converted a good therapeutic strategy [16] to decrease the incidence of these diseases, inflammatory production has been identified as a risk factor for the human kidney, mainly and testes [17,18].

This study represent the protection and the effective of *Ruta chalepensis* extract against CCL4 toxicity on kidney and testis. Affected kidney is static a chief health problematic, because of the high quantity of patients affected long time. It is, consequently, significant to catch new, dependable signs enabling a primary diagnosis of this pathology [19,20].

The phenolic phytochemicals has antioxidant activity that, has been extensively examined in current years [21,22]. Many pharmaceutical compounds that isolated from plants may protect against contraction and vaculation of glomerular tuft through numerous mechanisms[23], Ruta chalepensis was recorded in previous revisions. Instead of treating the kidney bladder, prophylaxis presumably blocked the CCL4 role [24]. Many studies demonstrated that flavonoid have been activity related to antineoplastic and anti-inflammatory mechanisms in different plants [25-28]. *Ruta chalepensis* ethanolic extract and rutin preserve human kidney RBCs and testicles from oxidative hemolysis. This indicated that the defensive role of *Ruta chalepensis* and rutin ethanolic extract is due to its antioxidant and scavenger capability [29].

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RESEARCH ARTICLE

Effect of soft beverages on the *in vitro* dissolution of gastro-resistant tablets containing low dose Acetylsalicylic acid

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ABSTRACT:

The need for additional fluids for easy absorption is typical for elderly patients and those with dysphagia. Most often, these patients take their medication with a glass of orange juice or another liquid instead of a glass of water. We conducted a dissolution test with gastro-resistant tablets acetylsalicylic acid where different kind of orange juice or soft drink were added to the release medium. As a control, release medium - buffers 1.2, 4.5 and 6.8 were used. The released aspirin was determined after HPLC analysis. The obtained data were fitted to different kinetic models. The results of the dissolution test in medium buffers with added different beverage showed results similar to those obtained in pure buffer, where it is used an artificial sweetener and different, when sugar or glucose-fructose syrup was used to sweeten the beverage. The most significant change was observed in the release kinetics of the active substance.To exclude the possibility that the other beverage ingredients or excipients used to make the tablets affect the release profile of acetylsalicylic acid, we conducted a beverage-like dissolution test. Instead of a original beverage, we used water and sugar syrup, in a concentration that is declared on the label of the original beverages. The results obtained confirm that different sugar concentrations alter the release profile of acetylsalicylic acid from gastro resistant tablets when they are taken with a glass of sugar-containing beverage instead of a glass of water.

KEYWORDS: Acetylsalicylic acid, HPLC, orange juice, soft drinks, dissolution test.

INTRODUCTION:

For most of the people the type of the liquid they use to administer the drug is with no significance at all. In turn this presents the possibility for altering drug concentration due to various interactions between the drug or dosage form excipients with different ingredients in the liquid that differs from potable water. It is also very typical for patients that they rarely use sufficient amount of liquid for swallowing the tablets or capsules. This can without a doubt affect drug release and absorption[1]

Worldwide a lot of researchers investigate and evaluate the influence of food and drinks on pharmacokinetic behavior of drugs[2-5]. Drug absorption depends on many factors such as type and properties of the dosage form, physico-chemical properties of the drug itself and also the physiological conditions present in the gastro-intestinal tract[6].

The interaction between different components of the formulation and food or drinks can lead to inhibition of the active pharmaceutical ingredient (API) transport through the mucosa and the respective change in drug absorption[4].

It is also well known that different furanocoumarins, found in the grapefruit juice can affect the metabolism of some drugs for example Calcium-channel blockers[7,8].

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Drug-food or drug-beverage interactions can influence as well the dosage form disintegration as the API release[9]. Foodstuffs and drinks affect the gastro-intestinal conditions. Changes in the motility, volume and pH of the liquid are established in the presence of such substances. These changes can entail changes in the release characteristics, e.g. especially for dosage forms with pH-dependent modified release. The physiological gastric pH in fasted state is in the range of 1.0-2.5 and alongside the small intestine it varies reaching values of 7.0±0.5[10,11].

Postprandial pH is susceptible to variations in each area of the gastro-intestinal tract and depends as well on the type of administered food or liquid as on the intra-individual variations[12].

All those interactions and influences are a prerequisite for the need of study and evaluation of the potential effect foodstuffs can render to drug action. Hence it is not without concern whether the patient takes his medications with a glass of water or with a glass of orange juice or another liquid.

Furthermore, some statistical analyses show increase in the consumption of soft-beverages[13,14].

People drink more frequently such beverages and patients in particular sometimes use them for solid dosage form administration. According to our studies (not present here) this phenomenon is becoming more persistent. The need of additional liquids for the ease of swallowing is very typical for elderly patients and for the ones with dysphagia[15].

Investigation of the in vitro release profiles is an easily available method which can be very useful for probability estimation of *in vivo* interaction manifestation. The dissolution test is a physical method used for characterization of API release from different dosage forms in vitro. It is a basic pharmacopoeia method since 1970 for the purposes of dosage forms design and also quality control. Because the method is non-invasive and in the presence of appropriate operating conditions it can be ground for determining *in vivo-in vitro* correlation it is one of the most frequently used methods for biopharmaceutical characterization of medicinal products[16,17]. Due to its specificity and excellent sensitivity, HPLC is often used to separate and quantify different target compounds, both in dissolution testing procedures[18-20] and in various other studies[21-27].

According to World Health Organization (WHO) data for 2016 there are four major causes of death worldwide- cardio-vascular diseases, cancer, diabetes and chronic lung conditions[28].

Therefore, the cardio-vascular risk prophylaxis is of significant importance. Prophylaxis and prevention can be related to different means including changes in the patient's lifestyle and also pharmacological therapy. According to WHO the prophylaxis can be classified in two groups- primary (without previous manifestation) and secondary (in patients with cardio-vascular conditions). Acetylsalicylic acid is considered as suitable for all cases of secondary prophylaxis and for some cases of primary one when the benefits overweight the risks[29].

Acetylsalicylic acid (ASA) belongs to the group of Non-steroid anti-inflammatory drugs (NSAIDs), possess pronounced anti-inflammatory activity and can affect pain with different origin and in addition it is widely used in the symptomatic treatment of fever[30].

Its activity is due to the irreversible non-selective inhibition of the COX-1 and COX-2 (isoforms of the Cyclooxigenase family)[31,32]. The hindering of COX-1 activity in platelets with administration of low-dose ASA leads to inhibition of Thromboxane A2 production[33] which in turn leads to inhibition of platelet aggregation[34].

Due to this anti-aggregation potential low-dose ASA is indicated both for primary and secondary prophylaxis of acute coronary syndromes and ischemia[35].

According to different authors the dose of low-dose acetylsalicylic acid formulation which is sufficient for an adequate therapeutic efficacy lies between 30-325mg/day[36-40].

The low dose is recommended due to the fact that higher doses are more commonly related to manifestation of side effects such as hypersensitivity, gastro-intestinal discomfort and even bleeding[41].

For adequate cardio- vascular risk prophylaxis long duration of treatment is required. It depends on the type of the co-morbidity and the presence of previous manifestation and usually is between 18 months and 4 years or longer[42]. This systemic or chronic drug administration can be accompanied by patient's non-compliance and discontinuation of treatment. Patients are also very susceptible to spontaneous changes of therapy or they very often do not follow exactly the prescription and the instructions for drug administration[43]. The strict following of the therapy is a prerequisite for its success and respectively for increasing the quality of life (QALY)[44].

Regarding all the issues related to chronic drug therapy and the application of liquids other than water as means

to ease up swallowing in the present work the influence of different commercial soft beverages and orange juices on the *in vitro* release of low-dose acetylsalicylic acid from gastro-resistant tablets was investigated in order to evaluate possible interactions.

MATERIALS AND METHODS:

Materials:

Aspirin protect® (100mg acetylsalicylic acid (PubChem CID:10745), gastro-resistant tablets, Bayer Schering Pharma, batch number: BTADR11) (containing 100mg acetylsalicylic acid cellulose powder, maize starch with film coating containing: methacrylic acid-ethyl acrylate copolymer 1:10 dispersion 30% (Ph. Eur.) talc, triethyl citrate) was purchased from a local Pharmacy.

Soft beverages: Coca-Cola®, Coca-Cola Bulgaria (containing water, glucose-fructose syrup, carbon dioxide, coloring agent E150d, phosphoric acid, natural flavors including caffeine); Coca-Cola Zero®, Coca-Cola Bulgaria (containing water, carbon dioxide, coloring agent E150d, Sodium cyclamate, Acesulfam Potassium, Aspartame, phosphoric acid, natural flavors including caffeine, sodium citrate) were purchased from a local store.

Different commercial orange juices were purchased also from a local store. Orange juice Pfanner® (100% orange juice, 25mg/100ml ascorbic acid, 9% sugar, 0.7% proteins, 0.2g% fats, Sodium <0.01%), Orange juice Rauch® (100% orange juice, 32mg/100ml ascorbic acid, 9.4% sugar, 0.7g% proteins, 0.2% fats, salt 0.005%) and Orange juice Cappy® (51% orange juice from concentrate containing fructose- glucose syrup, citric acid and ascorbic acid with 22% sugars).

Disodium hydrogen phosphate (PubChem CID:24203) extra pure, Ph. Eur. grade and Sodium-dihydrogen phosphate- dodecahydrate (PubChem CID:21902471) extra pure, Ph. Eur. grade; Hydrochloric acid 37% (PubChem CID:313), pure for analysis; Sodium 1-heptanesulfonate (PubChem CID:23672332) were purchased from Merck, Germany.

Acetonitrile (PubChem CID:6342), Formic acid (PubChem CID:284) were purchased from Sigma Aldrich, Germany.

Purified water was prepared in house by distillation with Boeco Water Still WS 7500, Boeco, Germany.

Methods:

Determination of the pH of commercial non-alcoholic beverages:

For the pH measurement 713 pH Meter was used (Metrohm, Germany). The pH was evaluated as well for the pure soft drink as for the mixtures imitating the physiological conditions. The pH was measured at 22.0 ± 0.5^0C until a constant value is displayed.

Dissolution test:

The dissolution test was carried out with RC-8D Dissolution tester, Minhua Pharmaceutical Machinery Co, Limited, Shanghai, China. The Paddle apparatus (dissolution test 2) was used as described in the European Pharmacopoeia (Ph. Eur., 2010). The process parameters included temperature 37˚C ± 0.5°C; 100rpm for paddle rotation. The dissolution media consisted of 200ml soft beverage and 700 ml simulated gastric fluid (pH=1.2, 1M Hydrochloric acid), simulated duodenal fluid (pH=4.5 0.1M acetate buffer) or simulated intestinal fluid (pH=6.8 0.1M phosphate buffer, Ph. Eur).Samples were taken at predetermined time intervals. As a reference dissolution test was performed also in 900ml media containing only simulated gastric, duodenal or intestinal fluid.

Dissolution media containing phosphate buffer with pH 6.8 and different soft beverages for facilitation were coded as shown in Table 1.

Table 1: Composition of different dissolution media.

Medium composition	Coding
Phosphate buffer with pH 6.8, 900ml	Medium A
Phosphate buffer with pH 6.8, 700ml + orange juice Cappy, 200ml	Medium B
Phosphate buffer with pH 6.8, 700ml + orange juice Pfanner, 200ml	Medium C
Phosphate buffer with pH 6.8, 700ml + orange juice Rauch, 200ml	Medium D
Phosphate buffer with pH 6.8, 700ml + Coca Cola, 200ml	Medium E
Phosphate buffer with pH 6.8, 700ml + Cola zero, 200ml	Medium F

HPLC analysis:

Before being injected into the HPLC system the samples were initially filtered through a Glass 0.45 µm. Diluent of the samples was acetonitrile and formic acid in the ratio of 99:1. The samples were centrifuged and the supernatant was used for the HPLC assay.

For the analysis of the dissolution samples an HPLC system consisted of a Shimadzu DGU-$20A_5$ vacuum degasser, a Shimadzu LC-20AD quaternary pump, a Shimadzu SPD-20AUV/VIS detector and SIL-20A auto sampler was selected. A chromatography data system was used to record and evaluate the data collected during and following chromatographic analysis. The separation was achieved on a LiChrospher® RP-18 column packed with octadecylsilyl silica gel 10 µm, 250x4 mm. The mobile phase was acetonitrile – water, containing 1 g of sodium 1-heptanesulfonate, the pH

was adjusted to 3.4 with glacial acetic acid (85:15) and pumped at a constant flow rate 2.0 mL per minute. The eluent was monitored using UV/VIS detector at a wavelength of 280 nm. The column was maintained at room temperature and volume of 20 µl was injected. Under these conditions, the retention times (t_R) of Salicylic acid and Acetylsalicylic acid were approximately 5 and 8 min.

RESULTS AND DISCUSSION:

pH determination:

The pH of each of the commercial soft drinks was measured in order to evaluate its potential role in the acetylsalicylic acid dissolution from gastro-resistant tablets. From the data presented in Table 2 it can be seen that these liquids are acidic. This may influence the release rate of gastro-resistant tablets due to change in the pH.

Table 2: pH values of the different commercial beverages

Soft drink	pH
Coca-Cola	2.700
Coca-Cola Zero	2.710
Orange juice (Pfanner)	3.817
Orange juice (Rauch)	3.876
Orange juice (Cappy)	3.682

The pH values of dissolution media consisted of 200ml soft beverage and 700ml of pH=1.2, 1M Hydrochloric acid, pH=4.5 0.1M acetate buffer or pH=6.8 0.1M phosphate buffer, Ph. Eur. were also measured. The values are given in Table 3.

Table 3: pH values in dissolution media consisted of 200ml soft beverage and 700ml of buffers.

Soft drink 200 ml /Buffer 700 ml	pH (1.2)	pH (4.5)
Coca-Cola	1.781	4.568
Coca-Cola Zero	1.786	4.573
Orange juice (Pfanner)	3.555	4.462
Orange juice (Rauch)	3.515	4.416
Orange juice (Cappy)	3.565	4.496

As it can be seen in Table 3 the pH in simulated gastric fluid without enzymes is changed to higher pH values due to the fact that it is not a buffered medium. Slight but not significant decrease is noticed in the other media in the presence of a soft beverage (Table 3 and Table 4).

Table 4: pH values in dissolution media consisted of 200ml soft beverage and 700ml of buffers

Soft drink 200 ml /Buffer 700 ml	Medium	pH (6.8)
Coca-Cola	**B**	7.019
Coca-Cola Zero	**C**	7.025
Orange juice (Pfanner)	**D**	6.793
Orange juice (Rauch)	**E**	6.786
Orange juice (Cappy)	**F**	6.801

Release profiles from dissolution of ASA in different media:

In the release media with pH 1.2 1 M Hydrochloric acid and pH 4.5 0.1 M acetate buffer either without or with the addition of a soft beverage no release was detected. The represented profiles (Fig. 1) are for the samples in pH 6.8 0.1M phosphate buffer. A slight difference is observed in the dissolution profiles in the presence of additional liquid to the release medium.

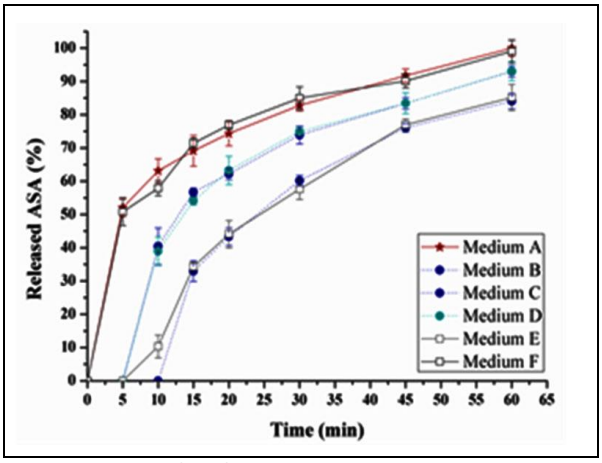

Fig. 1: Release profile of % Acetylsalicylic acid released with time (Zero order kinetics).

The release kinetics of the dissolution data were considered by various models such as Zero Order (Fig. 1), First Order (Fig. 2a), Higuchi (Fig. 2b), Hixon-Crowell (Fig. 2c), and Korsmeyer-Peppas (Fig. 2d).

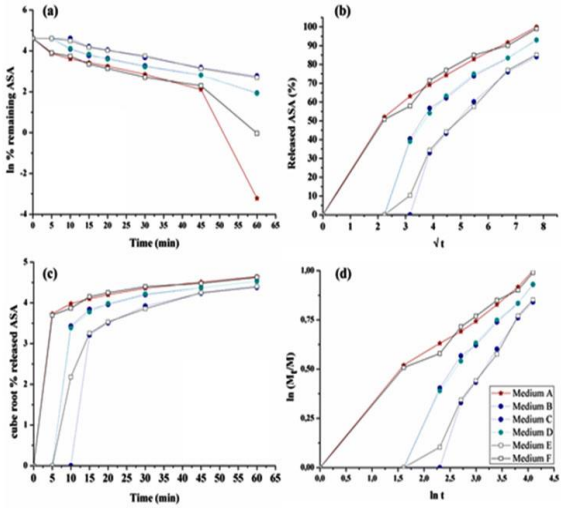

Fig. 2: Release kinetic plots for: (a) First order model; (b) Higuchi model; (c) Hixon-Crowell model; (d) Korsmeyer-Peppas model.

The data obtained from the *in vitro* drug release study in 700ml media with the presence of 200ml various soft beverages were fitted to different kinetic models. The resulting coefficients are presented in Table 5.

Table 5: Correlation coefficient (R^2) and dissolution constant (k) received in intestinal dissolution media A-F in presence of different soft beverages.

Medium	Zero order	First order	Higuchi	Hixon - Crowell	Korsmeyer-Peppas
A	R^2 = 0.62316 k = 1.23309	R^2 = 0.755 k = -0.10516	R^2 = 0.89651 k = 11.9414	R^2 = 0.26251 k = 0.04453	**R^2 = 0.99847** **n = 0.26086**
B	R^2 = 0.88448 k = 1.59292	**R^2 = 0.97648** **k = -0.03353**	R^2 = 0.84981 k = 13.034	R^2 = 0.63961 k = 0.08147	R^2 = 0.97162 n = 0.68712
C	R^2 = 0.75899 k = 1.5273	**R^2 = 0.97375** **k = -0.04351**	R^2 = 0.88414 k = 13.54015	R^2 = 0.46302 k = 0.06771	R^2 = 0.95323 n = 0.43003
D	R^2 = 0.76849 k = 1.54274	**R^2 = 0.97589** **k = -0.04395**	R^2 = 0.88879 k = 13.63745	R^2 = 0.47075 k = 0.06807	R^2 = 0.94868 n = 0.43961
E	R^2 = 0.91694 k = 1.55748	**R^2 = 0.98777** **k = -0.03394**	R^2 = 0.89772 k = 12.84395	R^2 = 0.63918 k = 0.07198	R^2 =0.81675 n = 1.05325
F	R^2 = 0.61579 k = 1.2365	R^2 = 0.91023 k = -0.06546	R^2 = 0.89429 k = 11.98061	R^2 = 0.26602 k = 0.04475	**R^2 = 0.97315** **n = 0.27695**

It was established that the ASA release in dissolution medium A and F shows Korsmeyer-Peppas model kinetics which represent ln Mt/M vs ln t, release exponent (*n*) value of 0.23125 and 0.23331 respectively were obtained, indicating Fickian diffusion. In dissolution medium B-E first order correlation coefficient was predominant in the dissolution release kinetics.

Looking at the graphs (Fig. 2) three groups release of ASA from different media can be clearly distinguished. The first group included the release of ASA from media A and F. The second group included the release of ASA in media C and D and third group - the release in media B and E.The release starts with about 50% released ASA after 5 minutes in A and F media (first group). Different orange juices added to the medium change the conditions thus the release starts later at 10min and at 15min and the percent of dissolved ASA increases in the following order media: B (15^{th} min), D and C (10^{th} min) respectively to 33 %, 39 % and 40 %. The release of ASA in medium E starts at 10^{th} min with 10% release, on 15^{th} min the concentration of released ASA is identical to the one in medium B or more precisely 34% (third group). Within 60 min the concentration of released ASA from media A and F was closer to 100%, from media C and D – 93%, and from media B and E – 85%.

The observed changes are not related to the established pH values of the media. Tablets with ASA were coated with EUDRAGIT L 30 D-55, anionic polymer with metacrylic acid as a functional group. The dissolution threshold of the enteric polymer was pH 5.5. The reason for release of ASA and the differences in % released active substance could be due to the composition the soft beverages, pKa of acetylsalicylic acid, pH of medium and the composition of the gastro-protective shell of Aspirin protect® tablets.

From the obtained results and the content of drinks we assume that presence of glucose-fructose syrup in dissolution medium decreased the dissolution rate of ASA. Dissolution media with content of higher % of glucose-fructose syrup showed lower dissolution rate of ASA.

We conducted dissolution test under the same conditions as have replaced the soft drink with the water and sucrose syrup. The concentration of sucrose corresponds to that declared on label of the soft drink. Test conducted with 200ml of water when sucrose quantity was 11.2% like in Coca Cola (www.coca-cola.bg), 9% like in orange juice "Pfanner", 9.4% like in orange juice "Rauch", 10.6% like in orange juice "Cappy",20%, 32% and 64%. Dissolution media containing phosphate buffer with pH 6.8 and solution with different sucrose concentration for facilitation were coded as shown in Fig. 3.

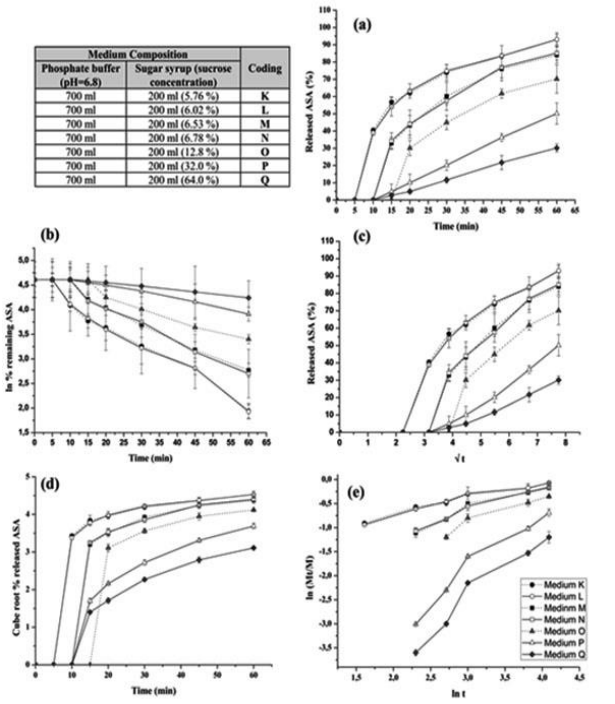

Fig. 3: Release kinetic plots for: (a) Zero order model (b) First order model; (c) Higuchi model; (d) Hixon-Crowell model; (e) Korsmeyer-Peppas model.

Table 6: pH values in different dissolution media

Medium	K	L	M	N	O	P	Q
pH	6.719	6.725	6.793	6.786	6.801	6.668	6.604

Table 7: Correlation coefficient (R^2) and dissolution constant (k) received in intestinal dissolution media K-Q in presence of different concentration of sucrose solution

Medium	Zero order	First order	Higuchi	Hixon - Crowell	Korsmeyer-Peppas
K	R^2 = 0.88464 k = 1.59278	R^2 = **0.97677** k = **- 0.03351**	R^2 = 0.84987 k = 13.03224	R^2 = 0.63961 k = 0.08147	R^2 = 0.91493 n = **0.50511**
L	R^2 = 0.75899 k = 1.5273	R^2 = **0.97374** k = **- 0.04351**	R^2 = 0.88414 k = 13.54015	R^2 = 0.46302 k = 0.06771	R^2 = 0.94871 n = 0.32008
M	R^2 = 0.76849 k = 1.60281	R^2 = **0.97589** k = **-0.04395**	R^2 = 0.88879 k = 13.63745	R^2 = 0.47075 k = 0.06807	R^2 = 0.94193 n = 0.33416
N	R^2 = 0.8895 k = 1.38553	R^2 = **0.97683** k = **- 0.03442**	R^2 = 0.85158 k = 13.09502	R^2 = 0.63929 k = 0.08152	R^2 = 0.95859 n = **0.49822**
O	R^2 = 0.89752 k = 1.38553	R^2= **0.94317** k = **- 0.02297**	R^2 = 0.76779 k = 10.80315	R^2 = 0.73145 k = 0.08445	R^2 = 0.89767 n = **0.55986**
P	R^2 = **0.96985** k = **0.9036**	R^2 = 0.9531 k = - 0.01215	R^2 = 0.76409 k = 6.80413	R^2 = 0.84209 k = 0.06894	R^2 = 0.9353 n = **1.22722**
Q	R^2 = **0.96306** k = **0.54397**	R^2 = 0.9583 k = - 0.00653	R^2 = 0.74779 k = 4.0722	R^2 = 0.85744 k = 0.05826	R^2 = 0.93709 n = **1.30159**

The release kinetics of the dissolution data were considered by various models such as Zero Order (Fig. 3a), First Order (Fig. 3b), Higuchi (Fig. 3c), Hixon-Crowell (Fig. 3d), and Korsmeyer-Peppas (Fig. 3e). The pH values of dissolution media consisted of 200ml imitatingsoft beverage and 700ml of pH=6.8 0.1M phosphate buffer, Ph. Eur. were also measured. The values are given in Table 6.

The data obtained from the *in vitro* drug release study in 700ml media with the presence of 200ml sucrose solution imitating soft beverages were fitted to different kinetic models. The resulting coefficients are presented in Table 7.

The obtain results correlate with the results of dissolution test with original beverages.The release begins at 10th minute in medium with added imitation beverage with the lowest sucrose concentration (9% and 9.4%).At a concentration of sucrose above 10%, the release of the active substance begins at the 15th minute, at 20% - on the 20th minute and the released active substanceis about 30% of the declared on the label. At a concentration of 32 and 64%of sucrose in the medium, release starting at 15th minute but the released ASA is respectively 4.92% and 2.74%. In a medium with added sucrose the release profile follows a first order kinetics, like in a medium with added original beverages. The obtained results give us reason to suppose that the concentration of sugars in soft drinks is one of the factors that influence the release of ASA from Aspirin protect®. Due to the direct relationship between sugar concentration and the viscosity of the medium, we intend to investigate how the change of viscosity affects the release of the active substance in our next study.

CONCLUSION:

This study was carried out to determine if there is an alteration of *in vitro* dissolution release of gastro-resistant tablets when co-administered with soft beverages. The study showed reduction of the release rate in case of glucose-fructose syrup presence. Release rate depended on the concentration of sugar content in soft beverage. When the soft beverage does not contain sugar or glucose-fructose syrup no change in rate of release was observed. The presence of sugar or glucose-fructose syrup in soft drinks, which are often used by patients to swallow tablets, changes the kinetics of release of acetylsalicylic acid from the gastro-resistant tablets, compared to beverages with sweetening agents *in vitro*. The most significant change that we observed *in vitro* for the release of ASA from gastro-resistant tablets was the change in release kinetics in the presence of soft drinks containing sugar or glucose-fructose syrup, compared to those with artificial sweeteners. The release kinetics of the active substance in the medium with added beverage with sweeteners followed Korsmeyer-Peppas model release, the same model of release like in simulated intestinal fluid, while the model that best described the release from the medium with added soft drink with sugar content is First order. The habit of swallowing the drug with liquids other than water must be done with caution although differences in the amount released aspirin was not significant, change in release kinetic is sufficient reason.

CONFLICT OF INTEREST:

The authors declare no conflict of interest.

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RESEARCH ARTICLE

The analysis of barriers to entry into the pharmaceutical market of Ukraine

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ABSTRACT:

The pharmaceutical market of Ukraine is characterized by a tendency to dominate imports over exports, which indicates its economic attractiveness for foreign companies that face various obstacles when entering the market. Entry barriers are understood as factors of an objective or subjective nature that prevent new firms from organizing profitable operations in the industry. The presence and impact of market barriers prove the need for their identification and comprehensive research. It has been established that when entering the pharmaceutical market of Ukraine, there are restrictive barriers related to state policy (for example, licensing, registration of medicines, examination, certification, etc.), barriers due to competition, and barriers of a non-legal nature. The results of the analysis of the competitive situation as a possible barrier characterize the pharmaceutical market of Ukraine as a market of free competition, which contributes to the relatively free entry of foreign manufacturers. Market entry barriers are also caused by anti-competitive behavior such as mergers and acquisitions, unfair competition, informal agreements, and so on. The results of the research can be used in making decisions about entering new markets or market segments for pharmaceutical companies, in forming competitive advantages and business strategies in order to develop potential in the long term.

KEYWORDS: Market Barriers, Pharmaceutical Market, Enterprise, Competition, Ukraine.

INTRODUCTION:

The study of market barriers is an important area of factors study that affect the state of market conditions and the enterprises activities. The opportunities assessment for new business entities to enter the market is based on the assumption of expected high profit with an acceptable risk level. The presence and height of barriers to entry into the market or its separate segment (or exit from it) determine the nature and timing of business decisions that affect the formation of the company's marketing strategy and tactics. Assessing the significance of barriers to entry of subjects to the pharmaceutical market is complicated by the lack of a universal formula for calculating their "height". Moreover, some barriers to entry do not lend themselves to quantitative measurement.

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MATERIAL AND METHODS:

The study uses data from the Ministry of Health of Ukraine, the State statistics service, and research companies that are publicly available. The analysis of regulatory system governing the pharmaceutical market of Ukraine, as well as monitoring of publications related to the research topic in scientific and practical publications was carried out. In the research methods of analogy and comparison (the study of scientific, professional and scientific literature on the problems of formation and development of the pharmaceutical market), methods of specification and systematization, methods of descriptive modeling (formulation of conclusions) were used, tabular and graphical means of visual representation of the results obtained.

The barriers to entry may be structural in nature, due to the characteristics of the industry (production technology, start- up, current costs, demand, market development, type of competition) or determined by the behavior of economic entities. A special place is occupied by obstacles resulting from the actions of the authorities (licensing, taxation, price fixing, government assistance to individual subjects).

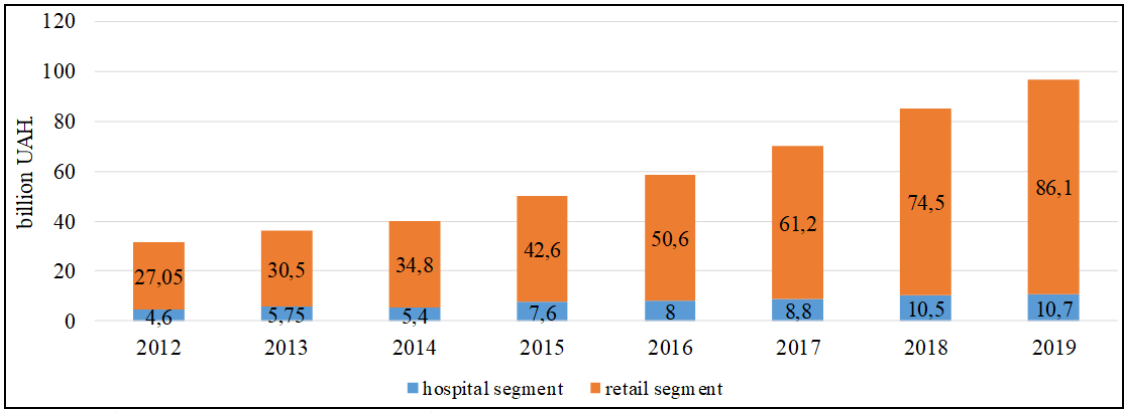

Fig. 1: Dynamics of the hospital and retail segments of the pharmaceutical market in Ukraine (2012-2019)

Distinguish between non-strategic and strategic barriers. Non-strategic barriers can be generated by objective characteristics of the industry market related to production technology, the nature of consumer preferences, demand dynamics, competition, etc. Another type of barriers are barriers caused by the strategic behavior of firms operating in the market (strategic pricing that restricts the entry of potential competitors into the industry, strategic policy in the field of research and innovation spending, patents, globalization, vertical integration and product differentiation, etc.) [1,2,3,4,5]

According to the nature of barriers impact on the activities of enterprises that are already on the market and entering it, the following types of entry barriers are distinguished: economic, regulatory, structural, administrative, organizational, technical (technological), financial, information, geographical, environmental, demographic, cultural, as well as non -legal barriers. [6-10]

When considering the subjects and factors that determine the emergence of entry barriers, we distinguish objective barriers (formed as a result of objective market trends and determined by its market conditions), as well as subjective barriers (established by the state and existing competitors).

Next, we consider the factors that determine the emergence of entry barriers for the pharmaceutical market in Ukraine.

RESULTS AND DISCUSSION:

The conjuncture of the pharmaceutical market of Ukraine and the main trends of its development:

The Ukrainian pharmaceutical market is promising and belongs to fast-growing ones: the average growth rate over the past 6 years is 17.8%. At the same time, most of the market is represented by retail sales, the hospital segment is located in the range of 12.35–15.86% (Fig. 1). [11,12,13]

The structure of the pharmaceutical market is dominated by imported drugs in monetary terms (62.5%), and 71.5% in physical terms belong to the products of Ukrainian manufacturers. Experts note that a significant increase in the role of domestic producers in the Ukrainian pharmaceutical market, which began in 2010, was the result of a significant devaluation of the hryvnia due to the global financial crisis of 2008-2009, but now price advantage factor of Ukrainian pharmaceutical companies is gradually losing its importance in their competition with foreign manufacturers in the Ukrainian market.[12]

A specific feature of the pharmaceutical market in Ukraine is the significant predominance of imports over exports (Fig. 2), for example, in 2018, one US dollar received from the export of Ukrainian pharmaceutical products accounted for nine dollars spent on the import of such products to Ukraine.[12]

In Ukraine, GDP growth in recent years is in the range of 3.3-3.5%, the forecast for 2020 is 3.7%. Both the minimum and average wages continue to grow, and the inflation index is assumed to be at the level of 5±1%.[13,14]

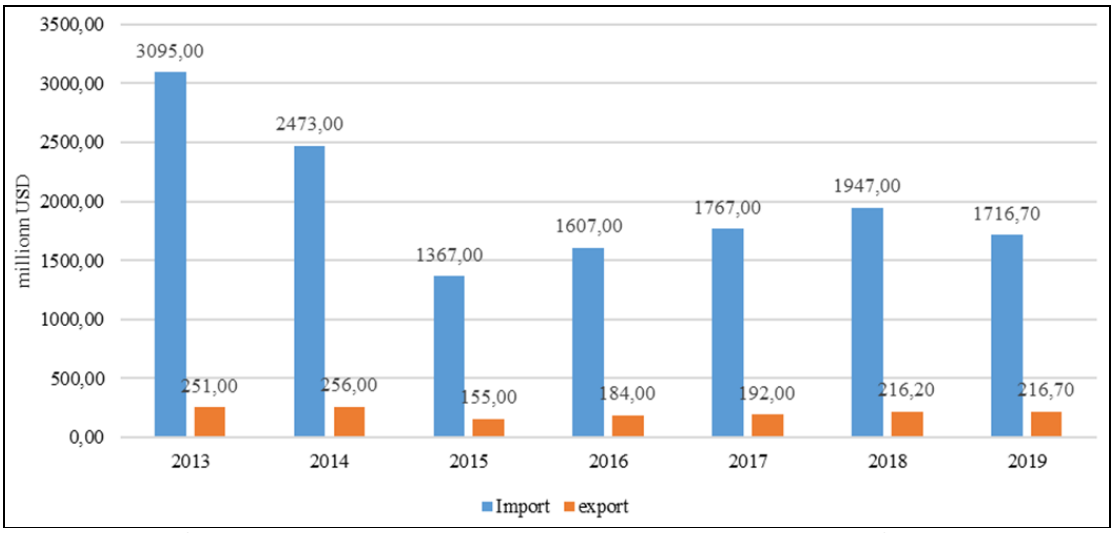

Fig. 2: Import and export of medicines in Ukraine (2013-2019) according to the State Statistics Service of Ukraine

All these factors can have a positive impact on the purchasing power of consumers in the pharmaceutical market, which is quite low. Drug consumption in Ukraine is significantly lower than in the EU. If one Ukrainian spends an average of $73 per year on medicines, then, for example, an Estonian – $258, and a Swede - $509[15].

This is due to the fact that almost 90% of medicines are paid by Ukrainian citizens on their own, while in Europe most of the drug costs are covered by insurance and special state programs. The launch of such programs in Ukraine can give a boost to the market. Thus, the action of the "Affordable Medicines" program and other existing drug reimbursement programs have already changed the proportion of Rx and OTC drug consumption. According to Proxima Research, over the past ten years, the ratio of Rx and OTC drugs in the consumer basket of Ukrainian consumers has changed significantly: if in 2008 it was 33% and 67% in physical terms, in 2018 it was 60% and 40%, respectively. [15]

According to Proxima Research forecasts, the market will continue to show double digit growth in monetary terms in the coming years. Optimistic forecasts for the development of the pharmaceutical market allow us to expect that by the end of 2020, Ukraine will be able to re-enter the group of Pharmerging Markets-promising emerging markets that are an attractive segment for international investors. [16]

Barriers set by the state:

In order to enter the pharmaceutical market, it is necessary to overcome the restrictive barriers associated with the state policy in this product market: licensing, product registration, examination, certification, etc.

In accordance with the legislation of Ukraine, an enterprise must obtain a license for the production of drugs, while the enterprise or entrepreneur must have the appropriate material and technical base and qualified personnel. In addition, since 2009, mandatory licensing requirements have included certification for compliance with the requirements of good manufacturing practice (GMP).

Only after state registration, which is carried out by the Ministry of Health of Ukraine through an examination of the quality, safety and effectiveness of medicines, medicinal preparations can be launched into the drug market. After registration, a certificate is issued for a period of 5 years, and after re-registration, the certificate is issued without expiration (except for cases when the Ministry of Health decides to conduct additional re-registration after 5 years for justified reasons related to pharmacovigilance).

For registration and re-registration of drugs, a certificate of compliance for the conditions of drugs production with the requirements of the GMP or a conclusion on the recognition of a PIC/S GMP certificate is also required. The conclusion and Certificate are issued by the State Service of Ukraine for Medicines and Drug Control. The recognition of certificates issued in other countries has been possible since 2011, when Ukraine became a member country of the PIC/S, an international tool for interaction between countries and competent authorities in the field of medicines quality control, which ensure cooperation in compliance with GMP requirements, inspecting and licensing production.[17] Since 2016 an updated version of the guidelines "Medicinal Products. Good Manufacturing Practice" entered into force in Ukraine, which corresponds to the document "The Rules Governing Medicinal Products in the European Union.

Volume 4 EU Guidelines to Good Manufacturing Practice Medicinal Products for Human and Veterinary Use".

Since 2005, Ukrainian legislation on the registration and circulation of drugs has been harmonized with EU legislation. As part of the harmonization in 2016, a provision was added to Article 9 of the Law of Ukraine "On Medicines", providing for simplified state registration of medicines registered in the United States, Switzerland, Japan, Australia, Canada, and medicines registered by the competent EU authority under a centralized procedure for applications in the territories of these countries or EU Member States, respectively. The procedure for registering such drugs involves submitting a smaller set of documents, reducing the registration period (17 working days), and eliminating the need for examination of registration materials.[18]

Such model does not correspond to the European approach, but it is transitional for Ukraine, which will allow filling the state market with drugs, the effectiveness of which has been verified by the competent authorities of countries with strict regulatory policies, according to a shortened procedure.[11] In the EU, the mutual recognition procedure applies to most so-called conventional medicines. This procedure is based on the principle of existing national marketing permits recognition (registration) in some EU countries. In Ukraine this procedure applies only to public procurement of medicines.[18]

In 2008, Ukraine joined the WTO. In order to bring the legislation in line with the obligations assumed in the framework of joining this organization, a number of laws were adopted to limit barriers to entry into the Ukrainian markets for foreign manufacturers. Among other things, Ukraine has set customs tariffs zero rates for a number of goods, including pharmaceuticals.

Another decision related to joining the WTO was that since January 2017, amendments to the TRIPS Agreement (the WTO agreement on trade — related aspects of intellectual property rights) have come into effect, according to which a permanent mechanism for compulsory licensing of patented pharmaceutical products has been introduced, which makes it possible to ensure that the necessary generic drugs produced in other States are available to WTO member countries that need it.[19]

The adoption of a law that fixes the provision on compulsory licensing in Ukraine has caused concern among manufacturers of original drugs, who believe that the introduction of rules on compulsory licensing can lead to abuse and violations of patent rights. According to experts, this may lead to the withdrawal of innovative companies from the market that do not want to give generic companies the products of their innovations.

In recent years, discussions have continued in Ukraine regarding the possibility of allowing parallel imports. Its supporters believe that this method of import and sale of goods helps to reduce its cost, and for manufacturers and official distributors, parallel import creates a negative competitive situation. At the same time, especially in the pharmaceutical market, there is a question of compliance with the conditions of storage and transportation of the corresponding drug or medical device, which directly affects the quality and safety of the product.[20]

Price regulation in Ukraine is carried out by setting border retail and wholesale allowances for medicines included in the List of Vital Medicines, but it does not affect manufacturers' pricing. An exception is the pricing for medicines that participate in the "Affordable medicines" program and other cost-compensated programs for which reference pricing is applied. That is, the sale price of manufactured pharmaceutical products cannot exceed the state-established borders. However, the inclusion of drugs in the list of reimbursable drugs allows manufacturers to increase their sales, so participation in such programs is usually beneficial for pharmaceutical companies. First of all, we are talking about generic manufacturers.

Barriers related to the competitive situation:

A significant barrier to entering the market is the level of competition.[21,22,23] To analyze the competitive situation, we analyzed the market shares of pharmaceutical manufacturers that were in the TOP 20 in terms of pharmacy sales of "pharmacy basket" products in monetary terms for 2017-2019, according to Proxima Research.[11]

There are 8 domestic companies in the TOP 20 manufacturers. We calculated the values of market concentration indicators for three, ten and twenty producers. The results obtained allow us to characterize the market as weakly concentrated and conclude that there is a state of free competition in the market. The Herfindahl-Hirschman index calculated for the TOP 20 confirms these conclusions (Table 1). The number of manufacturers present in the pharmaceutical market of Ukraine is significant, but since the companies holding the last positions of the TOP 20 have a market share of 1.23-1.25%, adding the remaining manufacturers to the analysis for calculating the Herfindahl-Hirschman index will not have a significant impact on the result.

Table 1: Calculated values of standard indicators of market concentration and the Herfindahl-Hirschman index for the TOP 20 manufacturers of medicines in terms of pharmacy sales of "pharmacy basket" products in monetary terms for 2017-2020.

Indicators	2017	2018	2019	2020
CR_3	12,35	12,14	12,03	12,73
CR_{10}	31,22	30,36	30,17	33,59
CR_{20}	47,56	46,63	46,44	50,16
HHI	132,72	127,66	126,95	148,82

Comparison of the results of competition analysis in the pharmaceutical market with studies conducted in 2010 shows that the level of competition has not changed significantly, at that time the CR_{10} was about 30%.[24]

The fact that Ukrainian pharmaceutical companies, lacking sufficient resources to search for fundamentally new drugs, have focused their efforts on the production of generics, which makes it possible for manufacturers of original drugs to enter the market relatively freely. But, at the same time, the low purchasing power of drug users does not allow them to get the maximum profit by setting high prices.

To ensure the success of the business, manufacturers have to bear significant costs for the promotion of the product, the main place in which is occupied by advertising. As domestic practice shows, those drugs that are actively advertised occupy the first lines in the sales ratings.

For the organization of a new production enterprise in the pharmaceutical industry, significant investment and competent management decisions are expected.

Barriers of a non-legal nature. Anticompetitive behavior of pharmaceutical market entities:

Anticompetitive behavior of its subjects can create or strengthen barriers to entry into the market, such as mergers and acquisitions, informal agreements, abuse of dominant position, unfair competition, abuse of authority, etc. In order to prevent monopolization of commodity markets, the Antimonopoly Committee of Ukraine monitors the concentration of business entities. Procedures and rules for merging companies were significantly simplified in 2016 as part of the harmonization of Ukrainian legislation with the European one.

Among the negative trends in the pharmaceutical market, the spread of counterfeit medicines and corruption are of concern to business entities. Moreover, counterfeiting is a big problem for the retail segment of the pharmaceutical market, and corruption actions – for the hospital.[12]

CONCLUSION:

The development trends of the Ukrainian pharmaceutical market allow us to predict that by the end of 2020, Ukraine will be able to enter the Pharmerging Markets group, which indicates its attractiveness to foreign companies. When entering the pharmaceutical market of a country, companies need to overcome barriers caused by state influence, such as licensing, drugs registration, examination, certification, etc. Drug manufacturers must obtain a license and have a certificate of compliance with the GMP requirements for the production of medicines, all medicines must be registered by the Ministry of Health of Ukraine after examination of their quality, safety and effectiveness. It should be noted that Ukraine has a simplified state registration of medicines registered in the United States, Switzerland, Japan, Australia, Canada, and medicines registered by the EU competent authority under a centralized procedure for use in the territory of these countries or EU member States. Zero rates of customs tariffs for pharmaceuticals also contribute to entering the Ukrainian market. Features of state regulation of drug pricing allow manufacturers of generic drugs to increase sales volumes by participating in the "Affordable medicines" program and other cost-compensated programs. To assess the barriers associated with the competitive situation, the market shares of pharmaceutical manufacturers for 2017-2019 were analyzed, which led to the conclusion that there is a state of free competition in the market. At the same time, the orientation of national producers to the production of generics creates an opportunity for producers of original drugs to enter the market relatively freely. When making a decision to enter the Ukrainian market, foreign manufacturers need to take into account the purchasing power of drug users and the cost of promoting the product. It has been established that the source of barriers is the anticompetitive behavior of pharmaceutical market entities, the negative impact of which is minimized by state control bodies. At present, Ukraine has simplified the procedures and rules for merging companies within the framework of harmonization of national legislation with European legislation, and introduced methods to counter the spread of counterfeit medicines and corruption in the hospital segment. It should be noted the stimulating nature of barriers to the development of market competition and the creation of obstacles to the entry of unscrupulous manufacturers into the market. At the same time, a high level of entry barriers can negatively affect consumers' access to quality goods and services, whose producers do not have sufficient financial resources to overcome competition, for example, in the field of marketing. In general, the results of our research allow us to assess the prospects of foreign companies entering the Ukrainian pharmaceutical market, taking

into account existing market barriers.

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RESEARCH ARTICLE

Ameliorative potential of Crinum asiaticum Linn leaf extracts on Elevated blood glucose in Alloxan-induced Diabetic Rats

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ABSTRACT:
In the present investigation the glucose lowering potential of the leaf extracts of Crinum asiaticum were prepared using cold maceration technique in solvents of varying polarity. The extracts exhibited the presence of flavonoids, alkaloids, phenolics and tannins. The oral toxicity of the aqueous and ethanolic extracts was determined and these two extracts were used of evaluating the antidiabetic activity. Oral glucose tolerance test was performed and diabetes was induced using alloxan (150 mg/kg) in rats. Ethanolic and aqueous extracts at two dose levels (200 mg/kg and 400mg/kg) were used for evaluating glucose lowering capability. Both the aqueous and the ethanolic extracts were found to significantly reduce glucose levels with the aqueous extract at dose level 200 mg/kg being the most effective (50% reduction) whereas the ethanolic extract was able to reduce the blood glucose by around 35% at the same dose level.

KEYWORDS: Antidiabetic, Alloxan, glucose, extract, crinum asiaticum.

INTRODUCTION:
Medicinal plants have always been a fundamental segment of healthcare system since times of antiquity. The medicinal property of any plant is contributed through some potential chemical constituents that have the capability to produce some sort of effects on the physiological processes of the body. Diabetes mellitus is a graving health condition that affects people with all socio-economic status and is characterized by continual accumulation of glucose in blood leading to excessive urination, weight loss, fatigue and stupor.[1]

It is the most common endocrine disorder and it is estimated that by the end of 2025 over 300 million people worldwide would be affected by diabetes mellitus.[2] The use of ethnobotanicals has been known to have a long standing record in the treatment of abnormalities of blood glucose.[3-13] India has been known to have a rich source of ethnomedicine available in the various systems of alternative medicine practiced in the country.

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The leaves of *Crinum asiaticum* have been used to treat inflammation and skin diseases. It has also been reported to have antibacterial activity and is used in treatment of ulcers. The seeds of *Crinum asiaticum* are used as purgative and the stem is used in the treatment of Gonorrhoea.[14]

The aim of the present study was to investigate the glucose lowering potential of *C. asiaticum* by inducing diabetes in rats by alloxan.

MATERIAL AND METHODS:
Plant material:
C. asiaticum leaves were procured from the local area of Prayagraj, Uttar Pradesh, India during the month of May and the plant was taxonomically authenticated form Botanical Survey of India. The shade dried leaves were ground to powder prior to extraction with solvents of varying polarity.

Extraction of leaves of C. asiaticum:
The freshly collected leaves of *C. asiaticum* were shade dried, powdered and extracted successively using petroleum ether, chloroform, ethyl acetate, ethanol and water by cold maceration technique. The progressive dissolvable extracts were concentrated by rotary evaporator under vacuum and at low temperatures. The

yield of the extracts was recorded as percentage of the dried leaf powder.

Preliminary screening of the leaf powder and extracts[15]:
The dried leaf powder was subjected to determination of total-ash value, acid-insoluble ash, water soluble debris, alcohol soluble extractive, water soluble extractives and loss on drying. The extracts obtained from various solvents were screened for the presence of phtyochemicals like carbohydrates, proteins, gums, mucilage, aminoacids, steroids, glycosides, alkaloids, tannins and phenolics.

Detection of carbohydrates:
To 2ml of the extract, 5 to 8 drops of Fehling's solution (boiling) was added. Formation of a birck-red colored) precipitate was an indicator for the existence of reducing sugar.

Detection of proteins:
0.25% w/v ninhydrin reagent was mixed dropwise to the extracts and the mixture was boiled for some duration. Development of a blue-color in the solution was indicative of the presence of amino acids.

Detections of terpenoids (Salkowski's test):
5ml extract was dissolved in chloroform (2ml) and followed by the addition of 3ml conc. sulphuric acid. Development of a reddish-brown hue at the joint of the tow liquids indicated the existence of terpenoids in the extract.

Testing of steroids (Liebermann-Burchard test):
1ml plant extract was taken in a test tube and solubilized using 10ml chloroform; 1ml of acetic anhydride was added to the solution. 2 drops of conc. sulphuric acid was flown down the sides of the test tube. If the color of the solution turned red to blue and finally greenish, it exhibited the presence of steroids.

Testing for Cardiac glycosides (Keller Killiani test):
1ml of leaf extract was solubilized in 1ml glacial acetic acid by gentle heat and the solution was allowed to cool followed by the addition of 2 to 3 drops of ferric chloride. To this solution, 2ml of conc. H_2SO_4 was carefully added along the sides of the test tube. Development of reddish-brown colored ring at the junction of two layers denotes the existence of glycosides.

Testing for flavonoids:
To 2ml of the extract, 5ml ethanol (95%), few drops of concentrated HCl and 0.5g magnesium turnings were added. Appearance of orange, red to purple or pink color indicated the presence of flavonols.

Testing for alkaloids:
The extract as dissolved in dilute HCl, filtered and the following tests were carried out to confirm alkaloids.

Mayers test:
To 2ml of the filtrate, Mayer's reagent (1-2 drops) was added by the side of the test-tube. Occurrence of white or creamy precipitate denotes positive test for alkaloids.

Wagners test:
To 1ml of the filtrate, few drops of Wagner's reagent were mixed alongside the walls of the test tube. If a reddish-brown precipitate occurred, the test was considered as positive for alkaloids.

Testing for phenolics (Ferric Chloride Test):
1ml extract was solubilized in 2ml of distilled water followed by the addition of few drops of 10% ferric chloride solution. Formation of dark green color denotes the existence of Phenolic compounds.

Detection of tannins (Ferric Chloride Test):
1ml of extract was solubilized in 10ml distilled water and filtered. Aqueous Iron (III) chloride (1%w/v) solution was added to the filtrate. The occurence of intense green, purple, black or blue color in the solution denotes for the presence of tannins.

Animals:
Grown-up albino wistar rats (180-200g) of either sex were housed in polypropylene cages under standard conditions (12h light; 12 h dark cycle; 23±2°C, 50±5% relative humidity). The animals were fed with standard pellet diet and access to water *ad libitum*. All the animal experiments were planned and performed in accordance with the guidelines approved by CPCSEA, and Institutional Animal Ethics Committee (SIP-IAEC-01-15-01).

Acute toxicity Study:
The animals were fasted overnight and water *ad libitum*, followed by the oral administration of the extracts at dose of 5, 50, 300 and 2000mg/kg and observation for mortality over 14 days. The same dose was re-administered to confirm the toxic dose.

Antidiabetic activity:
Induction of experimental diabetes:
For induction of diabetes, animals were subjected to overnight fast (free access to water) for 12 hours to make them additionally susceptible to developing diabetes[16]. Diabetes was induced in the test animals by intraperitoneally administrating alloxan monohydrate (150mg/kg body weight) solubilized in normal saline. After 72 h mice with blood glucose range of 200 to 350 mg/dl were used for study[17].

Table 1: Screening of phytochemicals in various extracts of *C. asiaticum*

Phytoconstituents/ Extracts	Petroleum ether	Chloroform	Ethyl acetate	Ethanolic	Aqueous
Alkaloids	-	+	-	+	+
Glycosides	-	+	+	+	+
Carbohydrates	-	-	-	-	-
Tannins and Phenolic	-	-	-	+	+
Flavonoids	-	+	+	+	+
Sterols	-	-	-	+	-
Proteins	-	-	-	-	+

Experimental Setup:

Animals were categorized into seven groups, each consisting of six rats. Standard pellet diet and water *ad libitum* was provided to the animals.

Group I: Normal healthy rats administered only vehicle (0.5% Tween 80)

Group II: Diabetic control (Alloxan 150 mg/kg)

Group III: Diabetic rats of this group were administered with glibenclamide (10 mg/kg) from 6th day after first administration of alloxan

Group IV: Diabetic rats of this group were administered with *C. asiaticum* ethanolic extract (CAEE 200 mg/kg) from 6th day after first administration of alloxan

Group V: Diabetic rats of this group were administered with *C. asiaticum* ethanolic extract (CAEE 400 mg/kg) from 6th day after first administration of alloxan

Group VI: Diabetic rats of this group were administered with *C. asiaticum* aqueous extract (CAAE 200 mg/kg) from 6th day after first administration of alloxan

Group VII: Diabetic rats of this group were administered with *C. asiaticum* aqueous extract (CAAE 400mg/kg) from 6th day after first administration of alloxan

Oral glucose tolerance test[18]:

Prior to initiation of the experimental procedure, the rats were fed with a bolus of 2g/kg dose of glucose and the level of glucose in blood was estimated at 0, 30, 60 and 120 seconds after administration of glucose using glucometer.

Evaluation of antidiabetic activity:

The antidiabetic activity of the extracts was determined by measuring the blood glucose levels on 1st, 10th and 15th day of administering the extract to the diabetic rats. The decline in glucose level was taken as the indicator for glucose ameliorating potential of the leaf extracts. Histopathological analysis of the pancreatic cells for degeneration and regeneration of islets of Langerhans was also performed by sacrificing the animal as per approved procedure and dissecting for isolation of the pancreas.

Statistical analysis:

The results of the animal experimentation are reported as mean ± standard error of the mean. Statistical difference between the means was determined by ANOVA followed by Dunnett's test and $p< 0.05$ was accepted as significant.

RESULT AND DISCUSSION:

Cold maceration technique was employed to successively extract out the phyto-constituents of varying polarities from the leaves of *C. asiaticum*. The extraction yield followed the order aqueous > ethanolic > chloroform > petroleum ether > ethylacetate. The qualitative assessment of the phyto-constituents in the extracts of indicated the existence of flavonoids, sterols, glycosides, alkaloids, tannins and phenolics, proteins and saponins in various extracts (Table 1).

The ash values, extractives and loss on drying of the powdered material were calculated as per the procedures and the results of the same are reported in Table 2.

Table 2 Physicochemical values of the leaf extracts of *C. asiaticum*

Characteristic	Percentage (%w/v)
Total ash	25
Acid insoluble ash	9.0
Water soluble ash	18
Alcohol soluble extractives	23
Water soluble extractives	32
Loss on drying	14.62

Diabetes was induced in experimental animal using 150 mg/kg dose of alloxan administered intra-peritoneal over a period of 5 days. Ethanolic and aqueous extracts were used for the study of oral glucose tolerance as well as for evaluating the antidiabetic efficacy against alloxan-induced diabetes in rats. The presence of phenolics, tannins, flavonols and phytosterols has been widely responsible for various pharmacological actions of plant extracts and hence only ethanolic and aqueous extracts out of all the extracts of *C. asiaticum* were considered for pharmacological evaluation. The effect of *C. asiaticum* extracts on tolerating the bolus of glucose (OGTT) is presented in Table 3 and the changes in levels of blood glucose in normal, diabetic and extract treated diabetic animals is presented in Table 4.

Table 3 Effect of ethanol and aqueous extract of leaves of *C. asiaticum* on OGTT

Groups	Treatment / dose	Blood glucose (mg/dl)			
		0 h	0.5 h	1.0 h	2 h
I	Normal control	89 ± 1.3	124 ± 1.2 (↑40.0%)	115 ± 1.4 (↑30.0%)	91 ± 0.9 (↑3.33%)
II	Glibenclamide, 10 mg/kg	232 ± 1.9	293 ± 2.26 (↑26.06%)	289 ± 1.5 (↑ 24.35%)	285.4 ± 2.6 (↑22.64%)
III	Control, 0.5% Tween 80	177.2± 0.8	206.9 ± 1.6 (↑16.57%)	220 ± 1.02 (↑23.88%)	184.1 ± 0.8 (↑3.85%)
IV	CAEE 200 mg/kg	164.3±0.9	210.6 ± 1.09 (↑27.84%)a	237.9 ± 0.9 (↑44.25%)	194.1 ± 0.78(↑17.91%)
V	CAEE 400 mg/kg	181.3±0.6	210.6 ± 1.05 (↑15.98%)a	246.3 ± 0.76 (↑35.46%)	187.9 ± 1.5 (↓3.6%)a
VI	CAAE 200 mg/kg	207.1±1.4	239.5 ± 0.6 (↑15.49%)	260.6 ± 0.56 (↑25.10%)	190 ± 0.96 (↓8.18%)
VII	CAAE 400 mg/kg	193.5± 2.1	^{s}225.7 ± 0.76 (↑6.47%)a	234 ± 0.11 (↑20.71%)	178.1 ± 0.7 (↓12.77%)

Values are represented as mean ± SEM for (n=6); **p<0.01, *p<0.05 and ^{ns} p>0.05 compared to control

Table 4 Effect of ethanolic and aqueous extract of leaves of *C. asiaticum* on blood glucose in alloxan-induced diabetic rats

Groups	Level of Blood sugar in Group (15 days) mg/dL (mean ± SD)				
	Initial	Day 1	Day 5	Day 10	Day 15
Normal Control	68.76 ± 6.02	63.03±9.31	64.68±9.83	65.00 ±7.39	63.46 ±5.86
Glibenclamide,10 mg/kg	247.74±8.83	260.26±14.73	283.83±4.76	307.18±8.07	311.26 ±4.71
Control, 0.5% Tween 80	248.83±8.38	250.47 ± 5.55	237.21±8.40	202.36±5.82	190.01±5.78
CAEE 200 mg/kg	246.02±3.87	247.63 ± 7.83	219.22±5.39	187.8 ± 8.20	176.12 ±9.28
CAEE 400 mg/kg	247.68±8.83	254.06 ± 4.96	237.86±8.82	212.21±3.31	180.83 ±4.55
CAAE 200mg/kg	249.82±4.88	254.55 ± 5.55	231.43±6.28	190.75±4.87	152.83±10.22
CAAE 400mg/kg	246.36±3.48	249.15 ± 8.12	215.95±4.50	188.08±7.89	178.19 ±8.66

Values are represented as mean ± SD for (n=6); **p<0.01, and ^{ns} p>0.05 compared to control.

The level of blood glucose was found to decrease significantly in the diabetic rats when compared to control at the end of the 15th day of study. In the OGTT, the ethanolic extract was found to be not significant in reducing the glucose level at dose of 200 mg/kg while the aqueous extract was found to be significantly reducing the increased blood glucose level that occurred due to the administration of the glucose bolus. Both the aqueous and the ethanolic extracts were found to significantly reduce glucose levels with the aqueous extract at dose level 200 mg/kg being the most effective (50% reduction) whereas the ethanolic extract was able to reduce the blood glucose by around 35% at the same dose level.

The demand for natural products that exhibit antidiabetic potential has risen over years owing to the side effects associated with long term use of the synthetic oral hypoglycemic agents. Several plants are known to be used for the treatment of diabetes and a few of them have actually scientifically proven to be highly effective. The presence of the phytochemicals like tannins, flavonoids, and sterols has been identified to be responsible for the glucose lowering potential exhibited by plants[19]. Alloxan is considered to be the most common chemical substance to induce diabetes in experimental animals. It has been proven that alloxan can lead to rapid depletion or degeneration of the β cells of the islets of Langerhans thereby causing diabetes[20].

The histopathological analysis of the pancreatic cells revealed that the extracts of *C. asiaticum* were able to enhance the regeneration of the islet of langerhans that was caused due to the induction of diabetes in the experimental animals (Figure 1). The aqueous extract was able to produced higher regeneration of the damaged cells of the islets of Langerhans compared to the ethanolic extract.

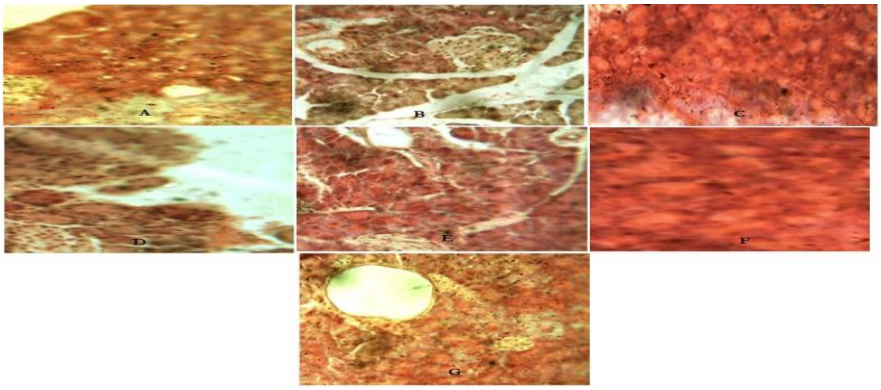

Figure 1 Histopathological study of the pancreatic cell.

A. Normal pancreatic cell; B. Damaged islets of Langerhans; C. Regenerated islets due to standard drug; D. Moderate islet regeneration due to ethanolic extract (200 mg/kg) of *C. asiaticum*; E. Significant islet regeneration caused by ethanolic extract (400 mg/kg); F. Moderate islet regeneration due to aqueous extract (200 mg/kg); G. No noticeable degeneration of the islets on administration of aqueous extract (400 mg/kg).

A previous study also reveals that the ethanolic extract of *C. asiaticum* has the potential to reduce the oxidative stress and blood glucose levels in alloxan induced diabetic mice[4]. Our study reinstates the findings and provides evidence that both ethanolic and aqueous extracts of *C. asiaticum* have the capability to regenerate the β cells and thereby enhance the insulin levels causing a decrease in serum glucose concentration.

CONCLUSION:

The present investigation provides proof that the ethanolic as well as aqueous cold extracts of the leaves of *Crinum asiaticum* possess the potential to ameliorate the elevated serum glucose levels that are caused due to the depletion of the β cells by alloxan. Furthermore, a comprehensive study to investigate the possible mechanism of action of the extracts as well as standardization of the extracts to develop into a potent natural medication has to be undertaken.

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CONFLICT OF INTEREST:

The authors declare no conflict of interest.

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RESEARCH ARTICLE

Evaluation of Antiepileptic activity of Mosapride in Albino wistar rats

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ABSTRACT:

Serotonin causes a significant shift in the excitability of neurons and endogenous serotonin and drugs acting on serotonergic receptors play a role in pathogenesis of epilepsy. This study was done to study the effect of Mosapride, a serotonin receptor $5HT_4$ agonist, in animal models of epilepsy. Albino Wistar rats were divided into 5 groups with six animals in each group. Group 1 was control group, group 2 was standard group and group 3, 4 and 5 received test drug mosapride in low dose (3mg/kg), high dose (6mg/kg) and mosapride plus standard antiepileptic drug respectively. The antiepileptic efficacy was evaluated using Maximal Electroshock Seizure model (MES) and Pentylenetetrazole (PTZ) induced convulsions. Data was analysed using ANOVA followed by post hoc Tukeys test. Mosapride treated animals showed statistically significant decrease ($p<0.001$) in the duration of flexion, hind limb extension and post ictal depression in MES model which was comparable to phenytoin group. In PTZ model, mosapride alone did not show any significant difference as compared to control group in terms of latency and duration of seizures ($p>0.05$). The antiepileptic efficacy of mosapride is similar to phenytoin in MES model. However, in PTZ model mosapride did not show any beneficial antiepileptic effect

KEYWORDS: Antiepileptic drugs, Mosapride, $5HT_4$ receptors, Serotonin, Epilepsy.

INTRODUCTION:

Epilepsy, a chronic neurologic disorder, classically presents as repeated episodes of seizure which are secondary to paroxysmal rampant discharges of neurons in the central nervous system.[1] It is the second most common neurological condition that burdens individuals, families, and the health care system.[2] Not only medical, epilepsy also poses social burden on different communities due to fear, unpredictability and associated social stigma leading to discrimination.[3]

Even patients with epilepsy have poor quality of life and this can further lead to mild to moderate depression.[4] Imbalance between neurotransmitter Glutamine (excitatory) and GABA (inhibitoty) is known to be a cause of seizure genesis. Changes in volatage, release and uptake of neurotransmitters have also been reported as mechanism of seizure development along with neurodegenrative and congenital developmental etiologies.[5] Antiepileptic drugs serve as a mainstay of treatment in epilepsy by reversing the seizure genesis. The majority of the epileptic seizures are controlled by available antiepileptic drugs but about one-third of patients remain uncontrolled despite the drug therapy.[6] Seizure frequency is also a significant predictor of psychological distress.[7] Adverse effects with currently available antiepileptics lead to poor patient compliance. Thus a need for a new class of drugs with different mechanisms is always felt.

Seizure threshold can be modulated using serotonin neurotransmitters in both focal and generalized seizures. Many studies have been conducted to test the efficiency of selective serotonin reuptake inhibitors (SSRI's) on seizure threshold and have shown significant results.[8-10] Evidence shows that the pathogenesis of epilepsy is significantly affected by endogenous serotonin and

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drugs acting as agonists and antagonists on serotonin receptors.[11] 5HT4 receptors have a facilitatory control over the release of serotonin.[12] Audiogenic seizures in animal models of epilepsy have known to be controlled by 5-HT7 receptors antagonists.[13]

Mosapride, a prokinetic drug, is a 5HT4 agonist and is used for gastroesophageal reflux disorders[14], functional dyspepsia, and irritable bowel syndrome. This study was designed to carry out the evaluation of the effect of mosapride in maximal electroshock seizures and pentylenetetrazole induced convulsion in animal models of epilepsy on the basis of the hypothesized linkage between serotonin and epilepsy.

MATERIAL AND METHODS:

Albino Wistar rats aged 6 months weighing between 150-200g were used in this study. The animals were kept in a standard environmental conditions (temperature of 22-24^{0}C with a 12 hour light/dark cycle) in an animal house with access to food and water. Institutional animal ethics committee approval was taken before conducting any experiments and CDSCO guidelines were followed.

The test drug used was mosapride in low and high doses of 3mg/kg and 6mg/kg respectively. Human therapeutic dose was converted into animal dose using Paget and Barnes's table.[15] Sodium valproate 108mg/kg[16] and phenytoin 100mg/kg[17] were used as the standard drugs for epilepsy. The animals were dosed for three days as previous studies report serotonin release and effect to be maximum on the third day of dosing.[18] Carboxymethyl cellulose (CMC) 0.5% was administered to control group and Pentylenetetrazole 60mg/kg[19] was used to induce seizures in the pentylenetetrazole model of epilepsy.

Experimental Design:

a) Maximal Electroshock Seizure (MES) Models:
In this model, 30 animals were used and divided into 5 groups as shown in table 1.

Table 1: Groups of MES model.

Group	Drug	Dose
Group 1 (control)	CMC 0.5%	2ml
Group 2	Phenytoin	100mg/kg
Group 3	Mosapride	3mg/kg
Group 4	Mosapride	6mg/kg
Group 5	Mosapride+Phenytoin	3mg/kg+ 100mg/kg

Group 1 received CMC 0.5% as a vehicle for 3 days. Group 2, 3, 4, 5 received respective drugs intraperitoneally for 3 days. On the third day after 30 minutes of receiving the drugs, electroshock at the intensity of 150mA, 50Hz for 0.2 sec was given. Later on, each animal was observed for the onset of seizures, duration of hind limb extension, the total duration of seizures, recovery/death in individual polypropylene cages. The scoring of the seizures was done according to the following scale: 0- No seizure, 1- Forelimb extension without hindlimb extension, 2- Complete forelimb extension with partial hindlimb extension, 3- Complete tonic hind limb extension (hind limb parallel to tail), 4- Postictal depression.

Percentage protection calculation = Number of animals with Total hind limb extension absent ×100

Total number of animals:

The endpoint of the experiment was considered as the absence/presence of tonic hind limb extension following drug treatment. This is a position during the generalized tonic-clonic seizures in rodents when the tail and both hind limbs are parallel to each other.
b) Pentylenetetrazole (PTZ) induced convulsions:

In this model 30 rats were taken and divided into 5 groups as shown in table 2.

Table 2: Groups of PTZ model.

Group	Drug	Dose
Group 1 (control)	Distilled water + Pentylenetetrazole	2ml
Group 2	Valproate + Pentylenetetrazole	108mg/kg + 60mg/kg
Group 3	Mosapride + Pentylenetetrazole	3mg/kg + 60mg/kg
Group 4	Mosapride + Pentylenetetrazole	6mg/kg + 60mg/kg
Group	Mosapride+valproate+ Pentylenetetrazole	3mg/kg + 208mg/kg + 60mg/kg

Group I received distilled water and Group 2, 3, 4, and 5 received drugs as shown respectively intraperitoneally. On the third day, after 30 minutes of receiving the drugs, 60mg/kg pentylenetetrazole was injected intraperitoneally to induce seizures. Afterward each animal was observed for a period of one hour in individual polypropylene cages. Seizures and tonic-clonic convulsions were recorded. The interval between administration of pentylenetetrazole and the occurrence of seizures was observed.

STATISTICAL ANALYSIS:

The analysis was done using SPSS 23.0. Intergroup analysis was done using one-way analysis of variance followed by post hoc Tukey's test. Standard and control groups were compared with the test groups. *P* value of <0.05 was considered statistically significant and results were expressed in mean ± standard deviation.
The results are reported in (Table 3 and 4).

a) Maximal Electroshock Seizure Models:

The duration of flexion, hind limb extension and postictal depression is shown in Table 3.

Table 3. Duration of flexion, extension and postictal depression in MES model

Groups	Duration of flexion (sec)	Duration of hind limb extension (sec)	Postictal depression (sec)
GROUP 1	13.33 ± 1.20	11.33 ± 0.49	162.67 ± 9.04
GROUP 2	1.83 ± 0.31[a]	1.67 ± 0.33[a]	1.67 ± 0.33[a]
GROUP 3	4.17 ± 0.31[a]	6.83 ± 0.40[a e]	53.50 ± 1.23[a]
GROUP 4	4.17 ± 0.17[a]	4.00 ± 0.37[a b d]	60.33 ± 1.48[a]
GROUP 5	3.50 ± 0.4[a]	3.67 ± 0.67[a d c]	54.67 ± 4.74[a]

(Values = Mean ± SD)
SD = Stanadrd Deviation
[a] p value < 0.001 as compared to control
[b]p value < 0.05 as compared to mosapride low dose
[c]p value <0.001 as compared to mosapride low dose
[d]p value < 0.05 as compared to phenytoin
[e]p value< 0.001 as compared to phenytoin

PERCENTAGE PROTECTION:

Figure 1 shows the percentage protection in different groups. Percentage protection is calculated by dividing the no. of animals without hind limb extension by total no. of animals in a group. Mosapride low dose produced 83.3 % protection whereas standard and other test groups gave 100% protection.

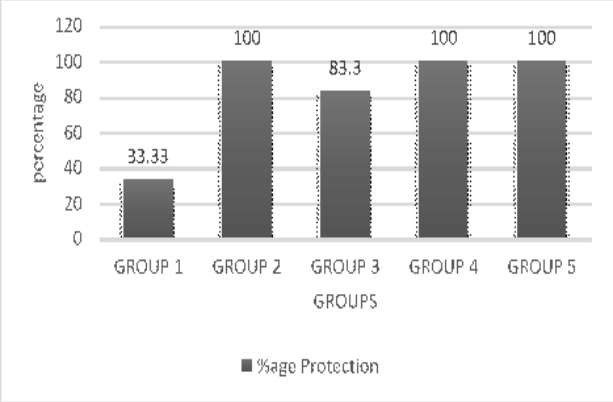

Figure 1. Graph showing percentage protection in different MES groups.

b) Pentylenetetrazole induced convulsions:

The latency and duration of seizures in PTZ model in different groups is shown in Table 4. Only phenytoin and combination group showed a statistically significant increase in latency (p<0.001) as compared to control.

Table 4. Latency and duration of seizures in PTZ model

Groups	LATENCY (sec)	DURATION (sec)
GROUP 1	1.25 ± 0.25[b d]	3.67 ± 0.33[c]
GROUP 2	6.33 ± 0.67[a]	1.25 ± 0.25[a]
GROUP 3	1.62 ± 0.36[b d]	4.17 ± 0.48[b]
GROUP 4	2.50 ± 0.56 [b d]	4.83 ± 0.48[b]
GROUP 5	5.50 ± 0.43[a]	2.83 ± 0.40

Values (Mean ± SD)
SD = Standard Deviation
[a] p value<0.001 as compared to control
[b]p value <0.001 as compared to valproate
[c] p value<0.01 as compared to valproate
[d]p value < 0.01 as compared to mosapride valproate combination

DISCUSSION:

Serotonin plays a significant role in the mechanism of action of psychiatric drugs like antidepressents, mood stabilisers, and sleep disorders and also as antiemetic.[20] which works by modulating the levels of serotonin in the central nervous system. The increased serotonin levels has even been found in metabolic disorders like diabetes.[21] The role of serotonin in the generation of seizures has been documented. Studies have shown that chronic administration of phenytoin modifies the levels of serotonin and produces antiepileptic action by modulating the proportions of monoamine neurotransmitters in various areas in the brain in animal studies.[22]

Maximum Electric Shock (MES) model is a gold standard in the screening of antiepileptic drugs used in Grand Mal seizures because of its simple setup and high predictive value for efficacy of the drug used.[23] This test has been used as a major initial screen for new drugs with a mechanism of action involving prolonged inactivation of the voltage-gated Na+ channels like phenytoin, carbamazepine, etc. In the present study, mosapride treated animals in the MES model showed a decrease in the duration of flexion, duration of hind limb extension and duration of postictal depression (p<0.001) in comparison to control which was statistically significant. (Table 1) The protective effect of mosapride treatment was comparable to the standard drug (phenytoin). No difference of statistical significance was shown between the two doses of mosapride and mosapride + phenytoin on different parameters in the MES model. However 100% protection was seen in phenytoin, Mosapride 6mg/kg and mosapride + phenytoin as compared to the lower dose of mosapride in which only 83% protection was seen (Figure 1.) In this study mosapride, a 5HT4 agonist has shown a protective effect in MES induced seizure model. Peripheral serum levels of serotonin play a role in seizures and post-seizure recovery.[24] An animal study by Buchchan et al put forth that serotonin regulates seizure threshold and regulates the severity of seizures and augmentation of serotonin can help in the prevention of seizures. Serotonin related neurons raise seizure threshold and decrease mortality related to seizures.[25] Phenytoin and carbamazepine increase extracellular serotonin and dopamine, using microdialysis in the hippocampus of freely moving rats.[26] In comparison with the control rats, serotonin depleted rats showed spontaneous seizures with kainic acid administration, and the authors concluded that low serotonin is an important risk factor in epilepsy.[27] On the contrary, stimulation of 5HT1A, 5HT2A, C, and 5HT3 receptors by fluoxetine did not alter the threshold and severity of seizures in male Wistar rats that was induced by electrical stimulation.[28] In the present study, 5HT4

receptor agonist, mosapride has shown a favorable effect comparable to that of phenytoin on the duration of flexion and postictal depression in the MES model. This can be hypothesized due to increasing activity at 5HT4 receptors mediated by Mosapride.

Drugs acting selectively on absence seizures can be identified by PTZ induced seizure models[23]. In this model latency and duration of the seizure-induced after giving the drug are noted. Latency is the time taken to induce seizures in an animal. In the sodium Valproate group, significant increase in latency in comparison to control group was noted (Table 2). Mosapride low dose (p= 0.98) and mosapride high dose (p = 0.37) did not show any significant difference as compared to the control group. The effect of Mosapride and sodium valproate was similar to valproate alone showing that the addition of mosapride has no benefit in PTZ induced seizures. Similarly, the duration of convulsions also did not show any improvement in the mosapride group. On recording the duration of convulsions, Valproate and mosapride combination was comparable to valproate alone. Overall mosapride did not show any protective effect on PTZ induced seizures, the effect being comparable to that of control. Thalamus and cortex play a role in the generation of absence seizures in experimental models.[29] There are monosynaptic connections between the thalamus and the hippocampus.[30] Studies show a protective effect of serotonin underactivity in absence seizures. Serotonin depletion produced by administering parachlorophenylalanine in rats blocked generation and propagation of absence seizures in the hippocampus and thus protected against experimental absence seizures.[31] However, in other studies, serotonin has shown a protective effect in absence seizures. In Groggy rats, serotonergic agonists acting on 5HT1A and 5HT2receptors decreased the incidence of absence like seizures. Also, SSRIs reduced the generation of seizures.[32] 5HT4 receptor knock out mice are more susceptible to PTZ related seizures. Authors further suggest that inhibitory influences on the excitability of neurons are mediated by 5HT4 receptors.[33]

To conclude, Mosapride has shown antiepileptic efficacy similar to phenytoin in the MES model, largely independent of dose. Owing to the burden of the disease and prevalence of current treatment failure, further experiments can be carried out to evaluate its efficacy in generalized tonic-clonic seizures. On the other hand, in the PTZ model of epilepsy, Mosapride did not show any promising effect.

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CONFLICT OF INTEREST:

The authors declare no conflict of interest.

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RESEARCH ARTICLE

Synthesis and Molecular Modeling Studies of 2-[2-(3-nitrophenyl)-1h-benzimidazol-1-yl]-acetamide Derivatives as Anthelmintic

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ABSTRACT:
Benzimidazole derivatives of substituted 2 [2-(3-nitrophenyl)-1H-benzimidazole-1-yl] acetamide analogues were synthesized and studied for antihelminthic activity. Compounds **3a–o** were obtained in three steps, starting with the Oxidative Condensation of the appropriate 3-nitrobenzaldeyde, o-phenylenediamine and sodium hydrogen sulfite to form 2-(3-nitrophenyl)-1H-benzimidazole **(1a)**. In second step Nucleophilic substitution, Chlorine atom of ethylchloroacetate will attach on nitrogen of benzimidazole by replacing hydrogen with elimination of hydrochloric acid to form ethyl [2-(3-nitrophenyl)-1H-benzimidazole-1-Yl acetate **(2a)**.In third step amide formation from ester takes place by substitution of electrophilic with loss of ethanol to form substituted 2 [2-(3-nitrophenyl)-1H-benzimidazole-1-yl] acetamide **3a–o** The antihelminthic activity showed that compounds **3f, 3h, 3i, 3j** and **3k** good activity against Indian earthworms (*Pheretima posthuma*) in comparison to albendazole.

KEYWORDS: Antihelminthic Activity, Benzimidazole, Docking, Molecular Modeling, Synthesis.

INTRODUCTION:
The benzimidazole nucleus is a useful structural moiety for the development of molecules for various pharmacological activities. Appropriately substituted benzimidazole derivatives have found diverse therapeutic applications such as in antiulcers, antihypertensive, antivirals, antifungals, anticancers, antihistaminics., antimicrobial, anti-inflammatory, anticonvulsant, antidepressant, antioxidant, radioprotective and anti-leishmanial. The benzimidazoles are broad–spectrum group of drug discovered in the 1960 with activity against GI helminthes[1].

The benzimidazoles are an important group of microtubule inhibitors which have found widespread use as broad-spectrum anthelmintics showing high efficacy against a range of nematode, trematode and cestode parasites. It is well established that benzimidazoles bind directly to the β-tubulin of nematodes, interfering with microtubule dynamics and consequently disturbing microtubule-based processes in these helminthes[2,3]. Benzimidazole derivatives have been found antidepressant and analgesic activity[4], inhibitor of Biotin Carboxylase[5] anti-inflammatory activity[6], antimicrobial activity[7,8,9], antidepressant activity[10]. On that reason the synthesis of new benzimidazoles and the investigation of their antihelminthic activity in order to compare it to the efficacy of Albendazole and to the activity of the most important commercial of pharmacological interest.

MATERIALS AND METHODS:
Melting points were determined by using VEEGO electronic (VMP-D) melting point apparatus and are uncorrected. The IR spectra of the compounds were recorded on a JASCO FT-IR 4100, Japan spectrometerin KBr. The ^{1}H NMR spectra were obtained on a VARIAN MERCURY YH-300 MHz NMR spectrometer in DMSO-d_6 as solvent and TMS as internal standard.

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Procedure:

2-(3-nitrophenyl)-1H-benzimidazole (1a):

Equimolar amounts (0.5 mmol) of *o*-phenylenediamine and 0.5mmol of 3-nitrobenzaldehyde were thoroughly mixed in 2 ml of N,N-dimethylformamide, 0.15 mmol of sodium hydrogen sulfite was added, and the mixture was stirred at 80°C for 40 min until the reaction was complete according to the TLC data. The mixture was cooled to room temperature and added dropwise to 20 ml of water under vigorous stirring. The product separated as a free flowing solid, it was collected by filtration, washed with water, and dried[11].

IR [cm^{-1}, KBr]: 3083 (NH), 2915 (CH), 1588 (C=N), 1518, 1339 (Ar NO_2), 1267 (C-N)

Ethyl [2-(3-nitrophenyl)-1H-benzimidazole-1-Yl] acetate (2a):

A mixture of equimolar alkaline solution (0.5 ml, 4 N NaOH) of 2-(3-nitrophenyl)-1H-benzimidazole (0.01 mol) in 50 ml of methanol and 0.01 mol of ethylchloroacetate in methanol (30 ml) was heated gently on boiling water bath for 0.5 hr. The solid thus obtained on cooling was recrystallized from chloroform to give product.

IR [cm^{-1}, KBr]: 3062 (CH), 1737 (C=O), 1521, 1348 (Ar NO_2), 1437 (C=N), 1128 (C-N), 1102 (C-O)

Substituted 2-[2-(3-nitrophenyl)-1H-benzimidazole-1-yl] acetamide analogues:

To a solution of *ethyl* [2-(3-nitrophenyl)-1H-benzimidazole-*1-Yl]acetate* (0.01 mol) dissolved in dry methanol (50 ml) 99 % primary amines, substituted hydrazine (1 ml) was added and the mixture was refluxed for 4–5 hr. The reaction mixture was cooled and the solid obtained was filtered, washed with small quantity of cold methanol to give product[5].

1. N-ethyl-2-[2-(3-nitrophenyl)-1H-benzimidazol-1-yl] acetamide (3a)

IR [cm^{-1}, KBr]: 3044 (NH), 2913 (CH), 1605 (C=O), 1515, 1341 (Ar-NO_2) , 1452 (C=N) 1128 (C-N)

^{1}H NMR: 11.166 (s, 1H, NH), 7.242-9.010 (m, 8H, Ar), 4.512 (s, 2H, CH_2), 3.385-3.407 (q, 2H, CH_2), 2.495-2.504 (t, 3H, CH_3).

2. N-benzyl-2-[2-(3-nitrophenyl)-1H-benzimidazol-1-yl] acetamide (3b)

IR [cm^{-1}, KBr]: 3049 (NH), 2905 (CH), 1605 (C=O), 1568, 1348 (Ar-NO_2) , 1428 (C=N) 1090 (C-N)

^{1}H NMR: 10.025 (s, 1H, NH), 6.902-7.853 (m, 10H, Ar), 3.027 (s, 2H, CH_2), 4.012 (s, 2H, CH_2).

3. N-(naphthalen-2-yl)-2-[2-3-nitrophenyl)-1H-benzimidazol-1-yl] acetamide (3c)

IR [cm^{-1}, KBr]: 3154 (NH), 2958 (CH), 1665 (C=O), 1535, 1362 (Ar-NO_2) , 1582 (C=N) 1178 (C-N)

^{1}H NMR: 10.906 (s, 1H, NH), 7.258-9.286 (m, 16H, Ar), 3.128 (s, 2H, CH_2)

4. 2-[2-(3-nitrophenyl)-1H-benzimidazol-1-yl]-N-phenylacetamide (3d)

IR [cm^{-1}, KBr]: 3055 (NH), 2926 (CH), 1611 (C=O), 1509, 1273 (Ar-NO_2) , 1428 (C=N) 1013 (C-N)

^{1}H NMR: 11.013 (s, 1H, NH), 7.250-9.010 (m, 13H, Aromatic), 2.800 (s, 2H, CH_2).

5. N-(2-nitrophenyl)-2-[2-(3-nitrophenyl)-1H-benzimidazol-1-yl] acetamide (3e)

IR [cm^{-1}, KBr]: 3122 (NH), 2958 (CH), 1681 (C=O), 1528, 1333 (Ar-NO_2) , 1542 (C=N) 1180 (C-N)

^{1}H NMR: 10.227 (s, 1H, NH), 7.320-8.017 (m, 12H, Ar), 3.242 (s, 2H, CH_2).

6. N-(3-chlorophenyl)-2-[2-(3-nitrophenyl)-1H-benzimidazol-1-yl] acetamide (3f)

IR [cm^{-1}, KBr]: 3068 (NH), 2932 (CH), 1655 (C=O), 1518, 1339 (Ar-NO_2) , 1433 (C=N) 1128 (C-N), 747 (C-Cl)

^{1}H NMR: 10.436 (s, 1H, NH), 7.427-8.148 (m, 12H, Ar), 3.019 (s, 2H, CH_2).

7. N-(3-nitrophenyl)-2-[2-(3-nitrophenyl)-1H-benzimidazol-1-yl] acetamide (3g)

IR [cm^{-1}, KBr]: 3156 (NH), 2980 (CH), 1668 (C=O), 1554, 1345 (Ar-NO_2) , 1584 (C=N) 1154 (C-N)

^{1}H NMR: 10.572 (s, 1H, NH), 7.182-8.923 (m, 12H, Ar), 3.012 (s, 2H, CH_2).

8. N-(4-bromophenyl)-2-[2-(3-nitrophenyl)-1H-benzimidazol-1-yl] acetamide (3h)

IR [cm^{-1}, KBr]: 3178 (NH), 2967 (CH), 1678 (C=O), 1557, 1334 (Ar-NO_2) , 1575 (C=N) 1170 (C-N), 667 (C-Br)

^{1}H NMR: 10.238 (s, 1H, NH), 7.580-8.207 (m, 12H, Ar), 3.549 (s, 2H, CH_2).

9. N-(4-nitrophenyl)-2-[2-(3-nitrophenyl)-1H-benzimidazol-1-yl] acetamide (3i)

IR [cm^{-1}, KBr]: 3062 (NH), 2918 (CH), 1771 (C=O), 1588, 1321 (Ar-NO_2) , 1428 (C=N) 1090 (C-N)

^{1}H NMR: 10.338 (s, 1H, NH), 7.028-8.003 (m, 12H, Ar), 3.129 (s, 2H, CH_2).

10. N-(4-hydroxyphenyl)-2-[2-(3-nitrophenyl)-1H-benzimidazol-1-yl] acetamide (3j)

IR [cm^{-1}, KBr]: 3318 (OH), 3196 (NH), 2985 (CH), 1676 (C=O), 1516, 1378 (Ar-NO_2) , 1539 (C=N), 1126 (C-N)

^{1}H NMR: 10.586 (s, 1H, NH), 7.238-8.213 (m, 12H, Ar), 3.028 (s, 1H, OH), 2.728 (s, 2H, CH_2).

11. 2-[2-(3-nitrophenyl)-1H-benzimidazol-1-yl]-N'-acetohydrazide (3k)

IR [cm^{-1}, KBr]: 3452 (NH_2), 3145 (NH), 2962 (CH),

1659 (C=O), 1539, 1343 (Ar-NO_2) , 1552 (C=N), 1183 (C-N)
^{1}H NMR: 11.203 (s, 1H, NH), 7.210-8.431 (m, 8H, Ar), 4.862 (s, 2H, NH_2) 3.827 (s, 2H, CH_2).

12. 2-[2-(3-nitrophenyl)-1H-benzimidazol-1-yl]-N'-phenylacetohydrazide (3l)
IR [cm^{-1}, KBr]: 3312 (NH), 3168 (NH), 2935 (CH), 1665 (C=O), 1548, 1352 (Ar-NO_2) , 1568 (C=N), 1162 (C-N)
^{1}H NMR: 11.238 (s, 1H, NH amide), 7.103-8.521 (m, 13H, Ar), 4.147 (s, 1H, NH) 3.802 (s, 2H, CH_2).

13. N'-(4-nitrophenyl)-2-[2-(3-nitrophenyl)-1H-benzimidazol-1-yl]- acetohydrazide (3m)
IR [cm^{-1}, KBr]: 3285 (NH), 3215 (NH), 2987 (CH), 1696 (C=O), 1527, 1346 (Ar-NO_2) , 1558 (C=N), 1142 (C-N)
^{1}H NMR: 11.272 (s, 1H, NH), 7.012-8.128 (m, 12H, Ar), 4.338 (s, 1H, NH) 3.923 (s, 2H, CH_2).

14. N'-(2,4-dinitrophenyl)-2-[2-(3-nitrophenyl)-1H-benzimidazol-1-yl]-acetohydrazide (3n)
IR [cm^{-1}, KBr]: 3236 (NH), 3285 (NH), 2994 (CH), 1678 (C=O), 1533, 1324 (Ar-NO_2) , 1548 (C=N), 1135 (C-N)
^{1}H NMR: 11.427 (s, 1H, NH), 7.578-9.025 (m, 11H, Ar), 4.402 (s, 1H, NH) 3.902 (s, 2H, CH_2).

15. 4-({[2-(3-nitrophenyl)-1H-benzimidazol-1-yl] acetyl} amino) benzoic acid (3o)
IR [cm^{-1}, KBr]: 3412 (OH), 3215 (NH), 2965 (CH), 1679, 1756 (C=O), 1545, 1334 (Ar-NO_2) , 1532 (C=N), 1245 (C-O), 1183 (C-N)
^{1}H NMR: 11.282 (s, 1H, NH), 7.624-9.123 (m, 12H, Ar), 9.032 (s, 1H, COOH) 3.435 (s, 2H, CH_2)

Procedure for Antihelminthic activity:
Three groups, of six earthworms each were released in 20 ml of desired formulations prepared in distilled water are as follows: saline, 20 mg/ml of Albendazole (Bandy, Mankind) and compounds **3a-3o** (20 mg/ml). Observations were made for the time taken to paralysis and death of individual worms. Paralysis was said to occur when the worm do not revive in normal saline. And death was concluded when the worms lost their motility followed with fading away of their body color[12-17].

RESULTS AND DISCUSSION:

Antihelminthic activity, Benzimidazole derivatives of substituted 2 [2-(3-nitrophenyl)-1H-benzimidazole-1-yl] acetamide **3a-3o** were evaluated for their activity against *Pheretima posthuma*. The antihelminthic activity showed that compounds **3f, 3h, 3i, 3j** and **3k** good activity against Indian earthworms (*Pheretima posthuma*) in comparison to albendazole. The docking studies yielded fitness score ranging from -5.380668 to -4.588958. The docking study indicate that compounds bind with *β*-tubulin by forming hydrophobic interaction with amino acid residues GLU 200B, THR 201B, TYR 202B, LEU 255B, MET 259B, van-der waal interaction with amino acid residues MET 166, THR 168, THR 198, ASP 199, GLU 200, THR 201, LEU 265, PHE 268. From the docking simulation it is found that title analogues have good interaction with *β*-tubulin.

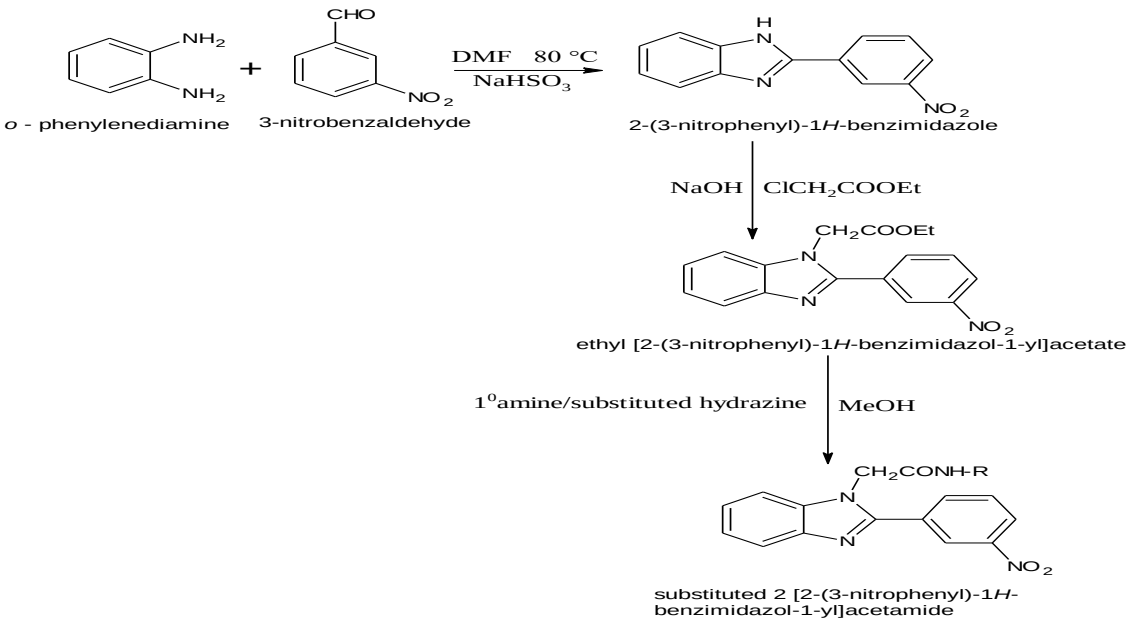

Fig. 1: Scheme for synthesis of 2-[2-(3-nitrophenyl)-1h-benzimidazol-1-yl]-acetamide derivatives

Table 1: Anthelmintic Activity of Compounds.

Compound	Paralysis Time (min) ±SEM	Death Time (min) ±SEM
Albendazole	17.56 ± 1.73	55.24 ± 1.40
3a	21.43 ± 1.34	63.36 ± 1.54
3b	31.17 ± 1.78	77.21 ± 1.53
3c	34.07 ± 1.10	86.50 ± 1.67
3d	24.31 ± 0.69	63.19 ±1.44
3e	26.08 ± 1.06	63.21 ± 1.50
3f	18.24 ± 1.35	58.56± 1.27
3g	29.04 ± 1.07	72.15 ± 1.44
3h	19.44 ± 1.32	56.59± 1.32
3i	18.58 ± 1.51	55.43 ± 1.33
3j	20.26 ± 1.04	58.43 ± 1.39
3k	19.08 ± 1.43	57.26 ± 0.96
3l	29.16 ± 0.77	63.04 ± 1.37
3m	32.37 ± 0.72	65.49 ± 1.25
3n	38.12 ± 1.55	72.16 ± 1.46
3o	27.24 ± 0.63	68.14 ± 1.08

Control worms were alive up to 24 hours of the experiment.

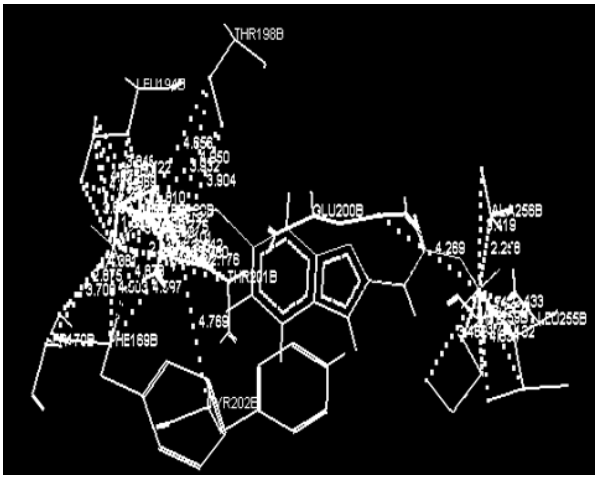

Figure 2: Hydrophobic interaction of Albendazole with *β*-tubulin

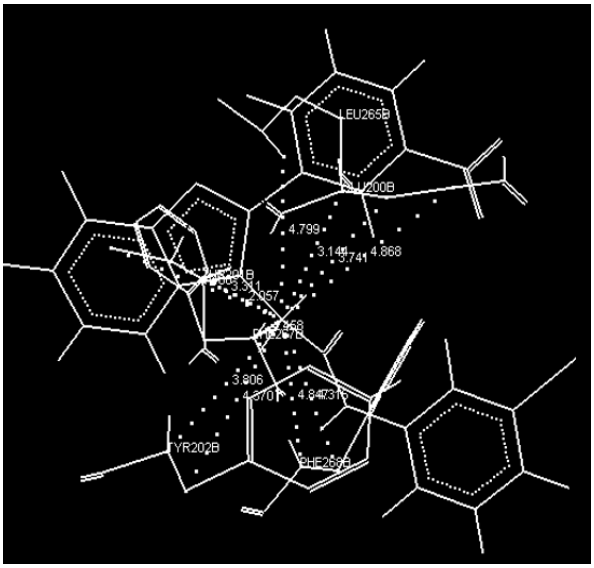

Figure 3: Hydrophobic interaction of compound 3f with *β*-tubulin.

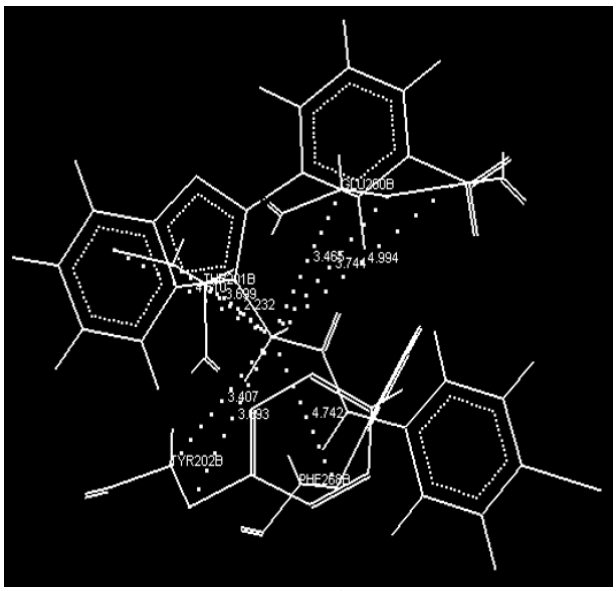

Figure 4: Hydrophobic interaction of compound 3h with *β*-tubulin.

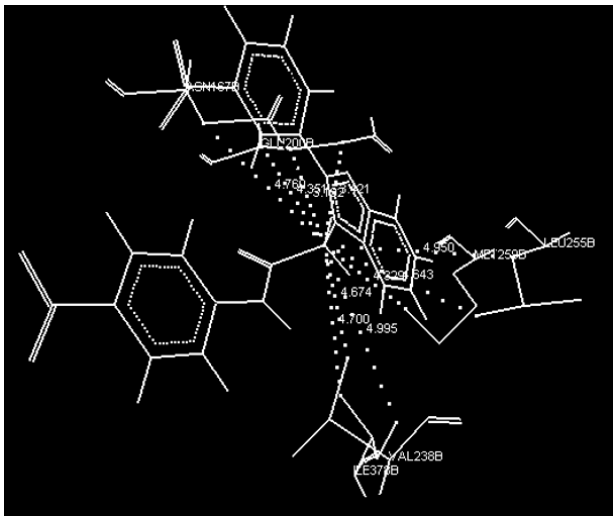

Figure 5: Hydrophobic interaction of compound 3i with *β*-tubulin.

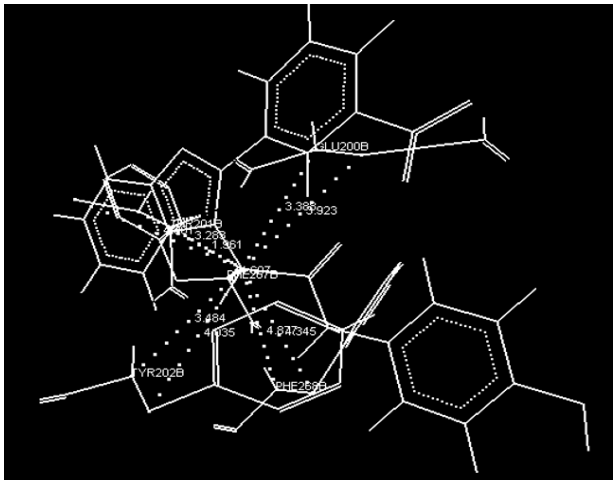

Figure 6: Hydrophobic interaction of compound 3j with *β*-tubulin.

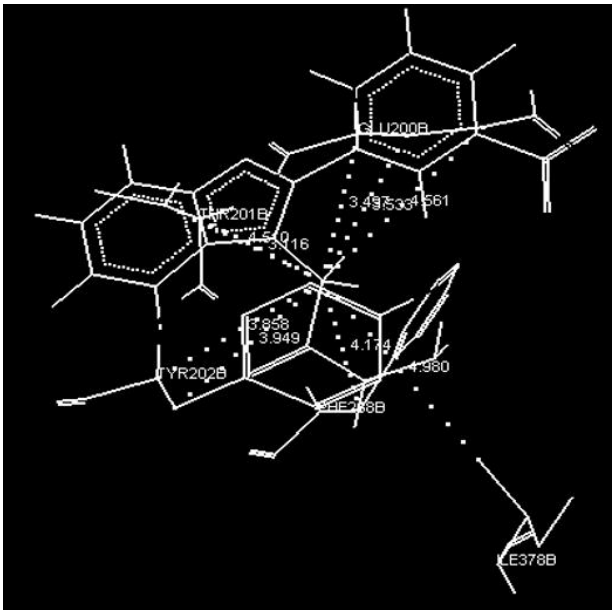

Figure 7: Hydrophobic interaction of compound 3k with *β*-tubulin.
Values are expressed as mean ± SEM, *n*=6

DOCKING RESULTS:

Table 2: Docking Score of Title Compounds

Sr.no.	Compound	Docking score
1	3a	-5.074641
2	3b	-4.588958
3	3c	-5.045692
4	3d	-4.978864
5	3e	-4.924625
6	**3f**	**-5.380668**
7	3g	-4.882526
8	**3h**	**-5.330436**
9	3i	-5.263447
10	3j	-5.110342
11	3k	-5.246499
12	3l	-5.080505
13	3m	-4.960719
14	3n	-5.081316
15	3o	-5.088659
16	Albendazole	-5.125293

CONCLUSION:

1. The evaluation of anthelmintic activity reveals that, **3f, 3h, 3i, 3j and 3k**, shows good anthelmintic activity and most of these compounds contain electronegative group at first and second position of Benzimidazole moiety.
2. Molecular docking study indicates that the observed anthelmintic activity may be due to inhibition of *β*-tubulin of the worms.
3. The title compounds are the good anthelmintic agents as per anthelmintic activity and molecular modeling studies.

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RESEARCH ARTICLE

Effect of Ethyl Acetate Fraction of *Marsilea crenata* Presl. Leaf Extract on Major Histocompatibility Complex Class II Expression in Microglial HMC3 Cell Lines

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ABSTRACT:

Phytoestrogens are plant-derived chemical substances that have estrogen-like structures or estrogenic functions. Deficiency of estrogen in human brain causes neuroinflammation characterized by increase of major histocompatibility complex class II (MHC II) expression as a marker of M1 phenotype in microglia. Recent research found phytoestrogen compounds in *Marsilea crenata* Presl. The aim of this study was to investigate the effect of ethyl acetate fraction of *Marsilea crenata* Presl. leaf extract in MHC II expression of microglial HMC3 cell lines, for resolution of inflammation and tissue repair. The fractions were given at concentrations of 62.5, 125, and 250 ppm to microglia, that had been previously induced by IFNγ 10 ng for 24 hours to stimulate the cells into M1 phenotype. Genistein as phytoestrogen was given at a concentration of 50 μM as positive control. Expression of MHC II was analyzed using immunocytochemistry method. Result showed reduction in MHC II expression of microglial cells, which indicated the activity of all extracts and, showed that 250 ppm of the fraction showed the strongest effect with MHC II value expression of 148.632 AU, and ED50 of 1,590 ppm. It was concluded from the study, that ethyl acetate fraction of *Marsilea crenata* Presl. leaves has antineuroinflammation effect.

KEYWORDS: MHC II, Neuroinflammation, Phytoestrogen, *Marsilea crenata* Presl., Microglia.

INTRODUCTION:

Neuroinflammation is an inflammatory response of the brain mediated by major histocompatibility complex class II (MHC II), which is expressed by antigen presenting cells (APCs) of microglia[1]. They are molecules that have main function in presenting processed antigens from exogenous sources, and are expressed during the activation of microglia classical pathway to M1 polarity state. Polarity state is an active form of microglia cell which has proinflammation characteristics[2].

Estrogen deficiency among menopausal women is one of the causes of inflammation[3,4]. Treatment for estrogen deficiency-induced neuroinflammation include administering estrogen as hormone replacement therapy (HRT)[5]. However, long-term administration of HRT may lead to potential side effect[6], and therefore other alternatives which are safe and have minimum side effects, such as phytoestrogens, are needed[7,8,9].

Phytoestrogens are plant-derived chemical substances

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that have estrogen-like structures or estrogenic functions, and can replace endogenous estrogen for the functioning of all body organs to retain homeostasis[10,11,12]. From previous studies, it was known that *Marsilea crenata* Presl., one of popular plants from East Java Province of Indonesia, contain phytoestrogens. Some research showed that the leaves contain a high concentration of estrogen-like substances[13], and therefore could exert activities similar to endogenous estrogen, such as induction of bone formation, either in vitro in MC3T3-E1 cells, or in vivo in dexamethasone-induced mice[13,14,15,16]. Other studies showed, that *Marsilea crenata* Presl. contains secondary metabolites, such as flavonoids, polyphenols, steroids, alkaloids, and saponins[17,18,19].

The aim of the study was to understand the effect of ethyl acetate fraction of *Marsilea crenata* leaf extract on the inhibition of inflammation occured during activated M1 microglial state. Activation of the microglia was conducted by induction of IFNγ in microglial HMC3 cell lines for 24 h[2]. MHC II expression as a marker of the M2 alternative state resulted from the shift of M1 state of microglial HMC3 cell lines would be identified by immunocytochemistry method using confocal laser scanning microscope (CLSM).

MATERIAL AND METHODS:

Plant Material:

Marsilea crenata Presl. leaves were collected from Benowo District, East Java Province, Indonesia, and determination was conducted by Center for Plant Determination, UPT Materia Medica, Batu, Indonesia. The leaves were sun dried and powdered.

Chemical:

Ethanol 96%, n-hexane, and ethyl acetate purchased from Merck, fetal bovine serum (FBS), paraformaldehyde (PFA), phosphate buffered saline (PBS), penicillin-streptomycin, genistein as positive control, Tween 80, anti-rabbit MHC II were purchased from Abcam, anti-rabbit fluorescein isothiocyanate (FITC), dimethyl sulfoxide (DMSO), eagle's minimum essential medium (EMEM) were purchased from Sigma-Aldrich.

Apparatus:

Confocal laser scanning microscopy (CLSM) Olympus Fluoview Ver.4.2a. software.

Extraction and Fractionation:

1,5 kg of *Marsilea crenata* Presl. powdered leaves were extracted with 96% ethanol, and fractionated with a mixture of 96% ethanol and 700 ml of water. Further fractionation was conducted using mixtures of n-hexane and ethyl acetate in a ratio of 1:1. Ethyl acetate fraction was then separated and evaporated by rotary evaporator Heidolph Hei-VAP ML/G3. Ethyl acetate dissolved flavonoids contents of *Marsilea crenata* Presl. leaves.

Cell Culture:

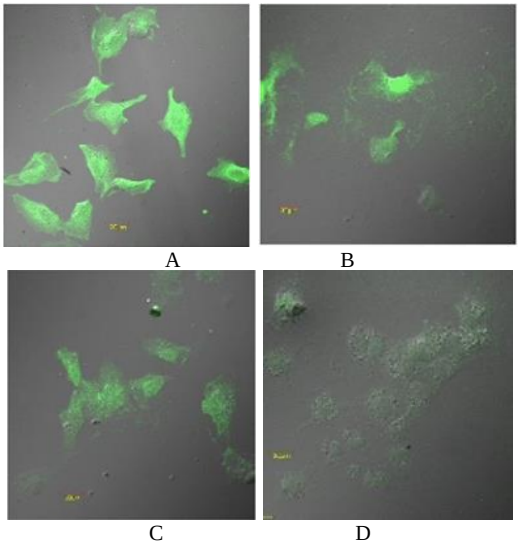
Microglial HMC3 cell line (ATCC CRL-3304) were purchased from American Type Culture Collection, USA. Cells were cultured in 24-well microplates in EMEM mixed with 10% FBS and 1% penicillin-streptomycin in a 5% CO2 incubator at 37°C for 6 days.

MHC II Measurement:

50 mg of ethyl acetate fractions were mixed with a 0.5% DMSO, and 0.5% of Tween 80, to prepare a suspension, and mixed with EMEM. The suspension was further diluted to obtain the desired concentration of 62.5, 125, and 250 ppm. Induction of IFNγ for 24 hours was conducted to cells that have been cultured on a 24-well microplate and reached 80% confluence. After induction, cells were rinsed with PBS, and mixed with the ethyl acetate suspension for another 48h incubation. After incubation, mixtures were fixated with 4% PFA. BSA and anti-rabbit MHC II were added and kept at 4°C for 24 h, and finally anti-rabbit FITC was added. The MHC II expression was analyzed using CLSM Olympus Fluoview Ver.4.2a. software at 488 nm. Data were analyzed by One Way Anova and Post-hoc LSD. Differences were considered significant at a significance level of $p<0.05$.

RESULT AND DISCUSSION:

This study was done using immunocytochemistry method to understand the antineuroinflammation activity through direct fluorescence antibody test[20]. Results were shown in Figure 1and 2.



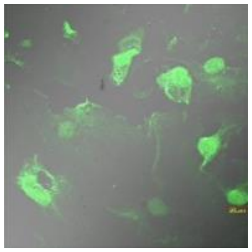


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Fig. 1. Immunofluorescence of HMC3 microglial cell, (A) Negative control group, (B) 62.5 ppm group, (C) 125 ppm group, (D) 250 ppm group (E) Positive control (Genistein) group.

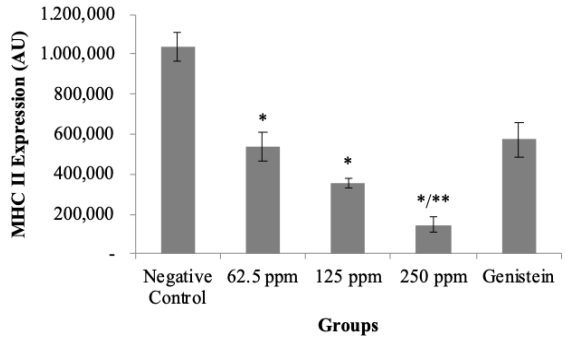

Fig. 2: MHC II Expression in microglial HMC3 cell lines after the administration of ethyl acetate fraction of *Marsilea crenata* Presl. leaves at concentrations of 62.5 ppm, 125 ppm, and 250 ppm. All fractions showed activities in lowering MHC II expressions which had significant differences compared to the negative control and positive control groups at $p < 0.05$. Strongest activity was found in 250 ppm fraction sample

Fig. 1 showed the immunofluorescence of HMC 3 cell lines in all groups. The strong intensity was found in negative control group, whereas the weak ones were shown in the genistein and treatment groups. This indicated, that genistein and treatment groups could reduce the expression of MHC II in microglial HMC3 cell lines. Microglial HMC3 cell lines of negative control group remained in M1 polarity, which was shown by its amoeboid-like morphology. Ethyl acetate fractions of treatment groups showed expressions that were significantly different compared to the negative control group. Treatment group of 250 ppm gave the lowest MHC II expression.

Fig. 2 described the quantitative results of MHC II expression of all groups. The ethyl acetate fractions of *Marsilea crenata* Presl. leaves of concentrations showed lower MHC II expressions compared to the negative control group. Interpretation of one-way ANOVA showed significant differences between the means of all treatment groups and the negative control at $p < 0.05$. ANOVA showed no significant difference between fraction of 62.5 ppm groups with positive control group. However, there is a significant difference between fractions of 125 and 250 ppm with genistein, in which both fractions were more active in reducing the MHC II expression than genistein. The strongest concentration was obtained by fraction of 250 ppm with the value of MHC II expression 148.632 AU. ED50 value of ethyl acetate fraction of *Marsilea crenata* Presl. leaf was 1.590 ppm. The value was calculated by statistical probit analysis of MHC II expressions of negative control and treatment groups.

Phytoestrogens are compounds produced in plants that can modulate the action of endogenous estrogens by binding to estrogen receptors. The compounds are known to have the ability to bind the estrogen receptors which is required for the ligand-receptor association[12]. Originally, the richest expression of ERβ is in the central nervous system, cardiovascular system, lungs, kidneys, urogenital tract, mammary glands, colon, and immune system. Genistein is also a phytoestrogen that showed a strong affinity for ERβ[21-28].

Lower blood estrogen level among menopausal women can cause various health problems, including inflammatory diseases, such as osteoporosis and neurodegenerative. The main cause of inflammation to occur is increasing production of cytokines, including TNF-α. As estrogen is known to have anti-inflammatory properties, hormone replacement therapy seems able to help in preventing inflammatory diseases associated with menopause[4,8,9].

Estrogen works by inhibiting NF-κB binding to the IL-6 promoter. Since NF-κB transcriptional factors activated inflammatory genes, it is supposed that estrogen blocks the inflammatory genes activation mediated by NF-κB. But, research found out, that actually estrogens exert anti-inflammatory effects through various mechanism. This can be explained through the fact, that estrogen did not repress the *TNF*-α gene by inhibiting the binding of NF-κB, but by recruiting steroid receptor coactivator 2 (SRC-2), which acts as a transcriptional repressor. This helps to understand that estrogenic drugs can have many targets responsible in the regulation of inflammatory genes. From such viewpoint, effort to find natural source of phytoestrogen will be helpful as alternative in replacing synthetic estrogens for hormone replacement therapy[27,29].

Previous research indicates the phytoestrogen contents of *Marsilea crenata* leaves. Phytoestrogens are bioactive compounds as secondary metabolites of plants that have therapeutic benefits[19,20,30]. They can modulate the action of endogenous estrogens by binding to estrogen receptors. The compounds are known to have the ability to bind to estrogen receptors which is required for ligand-receptor association. It is known that phytoestrogens could reduce the expression of MHC II

of microglia through estrogen receptor (ER) dependent pathway[10,11,12,31]. The binding between estrogen and ER can affects transcriptional gene through activated estrogen receptor (ER*). Transcriptional genes that are affected primarily was nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), which is a transcriptional factor responsible for immune system and inflammatory response[30]. Activation of NF-κB in microglial HMC3 cell lines will increase MHC II expression, and such activation occurred due to estrogen deficiency. Upon activation, NF-κB approached the nucleus and change the isomers of protein from monomers to oligomers which affect cell protein synthesis[32], such as MHC II. Therefore, an increase of ER* after administration of phytoestrogens could inhibit the activation of NF-κB, resulting in inhibition of MHC II expression and activation of microglia classical pathway to M1 polarity[10].

Microglia works through initiating inflammatory response to protect the body from any harmful stimuli, by producing various inflammatory cytokines such as TNFα, IL-6, IL-1β, and interferon-γ (IFNγ). Cytokines play a very pivotal role in the polarization process that will turn microglia into M1 activated state. In case of wound in the brain, inflammatory response has to be shifted into anti-inflammatory state for wound repairmen, and therefor proper transition from M1 to M2 phenotype is needed[2,33]

MHC II is a lipoprotein molecule that is important to regulate the immune response from extracellular pathogens. Increase expression of MHC II is caused by inflammatory inducing factors, such as IFNγ. As a result of IFNγ signaling, CD4+ T lymphocyte cells will be activated as a cellular specific immune system. CD4+ T lymphocyte cells will activate T helper 1 (Th1) cells, which in turn mediate MHC II expression as a proinflammatory marker in microglial cells. Th1 cells are responsible in immune response to intracellular pathogens, mainly through IFNγ secretion[33,34]. In this study inflammation occurred due to the induction of pro-inflammatory cytokine IFNγ in order to mimic the inflammation caused by estrogen deficiency. It aimed at activating microglial cells into M1 state[3,35].

It becomes clear from this study, that environment is critical in controlling the shift of M1 to M2 state, since prolonged inflammation could cause further neurodegeneration. Therefore, understanding M1/M2 dynamics is necessary, especially in diseases with chronic neuroinflammation[2,35].

CONCLUSION:

Ethyl acetate fraction of *Marsilea crenata* Presl. leaf extracts reduced MHC II expression in microglial HMC3 cell lines, due its phytoestrogen contents. This indicated the antineuroinflammation property of the phytoestrogens of the fraction. Further research is needed to investigate the specific types of phytoestrogen compounds.

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CONFLICT OF INTEREST:

The author states that there is no conflict of interest regarding the publication of this article.

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RESEARCH ARTICLE

Phytochemical and antimicrobial analyses of *Plectranthus amboinicus* leaf extracts

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ABSTRACT:
The present study is to evaluate the phytochemical constituents and antimicrobial activity of *Plectranthus amboinicus* leaf extract using petroleum ether, methanol and water as solvents. The antimicrobial activity of *P. amboinicus* leaf extracts were examined against the selected bacterial and fungal isolates namely *Escherichia coli, Staphylococcus aureus* and *Aspergillus niger, Candida albicans* using agar well diffusion method. FT –IR analysis was performed to identify the presence of functional groups in the methanol extract. The results of the study revealed the presence of distinctive active secondary metabolites in the tested leaf extract. The results revealed that the methanol extract exhibited maximum antimicrobial activity against the tested bacterial and fungal isolates when compared with aqueous and petroleum ether extracts. The FT-IR analysis reveals the presence of functional groups such as hydroxyl, amines, alkynes, ketones and carboxylic acid in the methanolic extract of *P. amboinicus*. Thus, *P. amboinicus* leaves are effective against the selected microbes and recommends that the plant derived phytochemicals are comparatively safer than synthetic alternative, thereby contributing insightful remedial benefits for the treatment of diseases.

KEYWORDS: *Plectranthus amboinicus*, Phytochemicals, FT – IR analysis, Antimicrobial activity.

INTRODUCTION:

Antibiotics are one of the most important weapon in combating bacterial infections and have significantly benefited the health associated quality of human life since their introduction. However, uncertainty of controlling the infections is the main problem commonly referred as "antibiotic resistance" which is found in several allopathic therapies. Therefore, it is essential to investigate newer drugs with lesser resistance. The multiple drug resistance has developed due to the indiscriminate use of antimicrobials and reemergence of diseases[1]. Adverse drug reactions and the high cost of antimicrobials have been key contributors to ineffective management of infectious diseases in many developing countries[2].

Systematic studies among various pharmacological compounds have revealed that any drug may have the possibility of possessing diverse functions and thus may have useful activity in completely different spheres of medicine[3]. In the recent past, about 61% of new drugs were developed based on natural products and they have been very successful especially in the areas of infectious disease and cancer[4]. On contrary, 2020 is more into the integration of machine learning networks like Artificial Intelligence which is time consuming and accurate in the identification of diseases. Hence it is clear that research towards discovery rate of active novel chemical entities is declining[5]. Certainly there is a need to bioprospect new sources and if possible from less explored regions and habitats to maximize the discovery of novel bioactive metabolites.

Plectranthus amboinicus (Lour.) known as Indian Borage belongs to the Lamiaceae family, and it is a commonly available aromatic pubescent medicinal herb with essential oil producing capability. It is extensively used in folk medicine to cure conditions such as respiratory ailments, constipation, headache, fever and

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skin diseases. The leaves are frequently consumed and used as flavoring agents, or integrated as a constituent in the preparation of traditional food[6]. Hence, the present study is attempted to evaluate the phytochemical constituents and antimicrobial activity of the leaf extracts of *P. amboinicus*.

MATERIAL AND METHODS:

Collection and identification of the plant material:

Fresh leaves of *Plectranthus amboinicus* was collected from Malappuram, Kerala, India. The sample was authentically identified from Kerala Forest Research Institute, Peechi, Thrissur, Kerala. The leaves were thoroughly rinsed with tap water and ensured that it was devoid of contaminants. The leaves were air dried at room temperature and pulverized to fine powder and stored in air tight container at room temperature for further analysis.

Preparation of leaf extracts:

The extract was prepared using three solvents of increasing order of polarity viz., petroleum ether, methanol and water (aqueous). For the preparation of aqueous extract, about 10g of the leaves were homogenised with 10ml of hot water using mortar and pestle, 90ml of hot water was mixed with the residue and stirred for 30min. The finely pooled extract was centrifuged at 10,000rpm for 15 min at 4°C. The collected supernatant was concentrated using rotary evaporator and used for further analysis. While, for the methanol and petroleum ether extraction, 10g of the dried leaf powder was added to 100ml of the respective solvent and extraction was performed by cold maceration method for 72h. After extraction, it was filtered using Whatman filter paper and the solvent was evaporated to dryness under vacuum using a rotary evaporator. The crude extract was weighed and dissolved in a known volume of dimethyl sulphoxide (DMSO). The extraction yield was expressed as

$$\text{Extraction yield (\%)} = \frac{\text{Weight of the dry extract (g)}}{\text{Weight of the sample used for the extraction (g)}} \times 100$$

Phytochemical analysis:

The petroleum ether, methanol and aqueous extracts of *P. amboinicus* leaves were subjected to preliminary phytochemical tests namely carbohydrates, proteins and aminoacids, phenols, sterols, glycosides, quinones/anthroquinones, alkaloids, tannins, anthocyanin, flavonoids, terpenoids, saponins and leucoanthocyanin as per the standard methods[7].

FT- IR analysis:

In FT-IR study, methanolic extract (10mg) of *P. amboinicus* leaf powder was taken in a mortar and pestle, grounded with 2.5mg of dry potassium bromide (KBr), filled in a 2mm internal diameter micro-cup and loaded onto FT-IR set at 26ºC±1ºC. The sample was scanned using infrared in the range of 4000-750cm^{-1} using Fourier Transform Spectrometer (Shimadzu, IR Affinity 1, Japan). The spectral data obtained was compared with the reference chart to identify the functional groups present in the sample[8].

Antimicrobial analysis:

Agar well diffusion method was employed to determine the antimicrobial activity of the leaf extract of *P. amboinicus*. The bacterial strains viz., *Escherichia coli*, and *Staphylococcus aureus* were selected for evaluation. These bacteria were cultured on the nutrient broth (Himedia, Mumbai, India) at 37±1°C. While, the fungal strains *Aspergillus niger* and *Candida albicans* were chosen to evaluate the antifungal property of leaf extracts of *P. amboinicus*. The fungi were cultured in Sabouraud dextrose broth (Himedia, Mumbai, India) at 28 ± 1°C. Five wells were bored on each Petri plate and the well grown bacterial and fungal cultures were spread plated or swabbed on sterile Muller Hinton and Rose Bengal chloramphenicol agar medium separately. To each well 20µl of the leaf extracts were added and incubated at 37°C for 24hrs (bacteria) and at room temperature for 5 days (fungi) respectively. In addition, standard antibiotic discs namely chloramphenicol (Bacteria) and flucanozole (Fungi) were used as positive control and DMSO served as negative control. After incubation period the diameter of the inhibition zone was measured and expressed in millimetre[9,10].

RESULTS AND DISCUSSION:

Plant-based medicines are widely employed in various public health practices throughout the globe as they are safe and cost-effective, and efficiently combat various deadly diseases and help in maintaining good health. Amongst the wide number of existing medicinal plants, *Plectranthus amboinicus* (Loureiro) Sprengel is one of the most documented species in the family Lamiaceae[11].World Health Organisation stated that about three-fourth of the world population is still dependent on traditional herb-based medications due to their low cost, easy accessibility and likely negligible side effects in comparison to allopathic medicines[12]. Undoubtedly, many allopathic medicines have their integral active metabolites from the plants, which itself explains the importance of the practice of traditional medicine.

Phytochemical analysis:

The yield of the extracts varied according to the character of solvent used for extraction. Accordingly, the quantification of the dried extracts was 3.86g, 5.42g and 4.37g for petroleum ether, methanol and distilled

water respectively. Several methods of extraction have been employed and the net yield varies. For instance it was reported that hexane extraction method produced the highest oil yield (6.52%) when compared to steam distillation (0.55%) and supercritical CO_2 extraction methods (1.40%)[13]. It was also noteworthy to mention that that there was a difference in aroma of the extracts which was attributed to qualitative and quantitative dissimilarity in chemical composition. Significantly, the phytochemical composition varies according to the geographic variation which proportionally reflects on the pharmacological properties of the plant.

The phytochemical analysis of leaf extracts of *P. amboinicus* confirmed the presence of various phytochemicals. Significantly, the methanol extract indicated the presence of carbohydrates, proteins, amino acids, phenols, saponins, flavonoids, alkaloids, sterols, glycosides and the absence of tannins, terpenoids, quinones/ anthroquinones, anthocyanin and leucoanthocyanin. The major constituent in the leaf of *P. amboinicus* are the phenolic compounds which is mainly due to the natural production of essential oil with high amount of bioactive compounds such as carvacrol, thymol, β-caryophyllene, α-humulene, γ-terpinene, p-cymene, α-terpineol and β-selinene[14,15].

On contrary, in the aqueous extract alkaloids, phenols, flavonoids and sterols were present and the rest of the secondary metabolites were absent. The non-polar solvent, petroleum ether indicated the presence of phenols, terpenoids and sterols whereas all the other phytoconstituents were absent (Table 1). A research work reported the bench mark composition that *P. amboinicus* oil is rich in oxygenated monoterpenes, monoterpene hydrocarbons, sesquiterpene hydrocarbons and oxygenated sesquiterpenes[16]. In addition several research findings pointed that essential oil of leaf of *P. amboinicus* was particularly rich in phenolic monoterpenes, which are speculated to exert various pharmacological properties[17,18,19]. The results of phytochemical from *Begonia versicolar* Irmsch also indicated the presence of alkaloids, flavonoids, phenols, terpenoids, steroids, carbohydrates, saponins and reducing sugar which supported the findings of the present study[20]. The phytochemical screening of ethanol and aqueous extracts of *Barleria cristata* revealed the presence of alkaloids, flavonoids, glycosides, saponins, phenols and tannins[21]. More recently, interest among phytochemists and biologists has focused on the isolation of specific bioactive compounds of *Plectranthus amboinicus* and comprehending their pharmacological importance.

Table 1. Phytochemical screening of leaf extracts of *Plectranthus amboinicus*

S. No.	Phytochemicals	Petroleum ether extract	Methanol extract	Aqueous extract
1	Carbohydrates			
	Molisch test	-	+	-
	Benedict test	-	+	-
2	Proteins and Aminoacids			
	Ninhydrin test	-	+	-
	Biuret test	-	+	-
3	Saponins	-	+	-
4	Alkaloids			
	Mayer's test	-	+	+
	Dragendroff's test	-	+	+
	Wagner's test	-	+	+
5	Tannins	-	-	-
6	Phenols			
	Ferric chloride test	+	+	+
	Lead acetate test	+	+	+
7	Flavonoids	+	+	+
8	Glycosides			
	Legal's test	-	+	-
	Keller's-killiani test	-	+	-
9	Terpenoids	+	-	+
10	Sterols	+	+	+
11	Quinones/ Anthroquinones			
	Borntrager's test	-	-	-
12	Anthocyanin	-	-	-
13	Leucoanthocyanin	-	-	-

FT –IR analysis:

The FT-IR spectrum of *P. amboinicus* was studied to identify the nature of functional groups present. The peaks and functional groups of the spectrum are depicted in Figure 1. The spectrum revealed a strong and broad peak at 3346cm^{-1} which is attributed to –OH and –NH stretching vibrations in hydroxyl and phenol groups respectively. The peaks at 2349cm^{-1} and 2308cm^{-1} revealed the strong –COO stretching vibrations of carboxylic acids. The band at 2137 cm^{-1} attributed to –C≡C– stretching vibrations of alkyne groups. The peaks at 1641 cm^{-1} and 1544 cm^{-1} are indicative of strong C=O and N=O stretching. The peaks at 1228 cm^{-1} and 1217 cm^{-1} represents the strong C-N stretching vibrations of amine groups. Thus the FT-IR analysis suggests the presence of different functional groups such as hydroxyl, amines, alkynes, ketones and carboxylic acid in the methanolic extract of *P. amboinicus*.

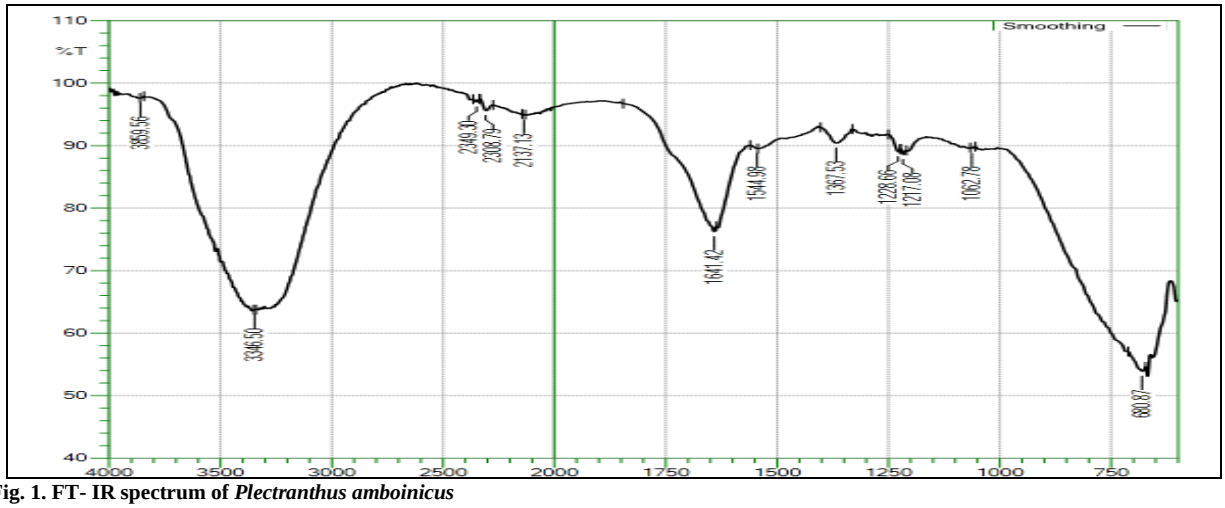

Fig. 1. FT- IR spectrum of *Plectranthus amboinicus*

Antimicrobial activity:

P. amboinicus has appreciable pharmacognostic properties such as antioxidant, antibacterial, antimicrobial, anti-inflammatory and fungitoxic activities due to the presence of volatile essential oils in its leaves[22]. In last two decades the medicinal plants have regained a wide recognition due to an escalating faith in herbal medicine in view of its lesser side effects compared to allopathic medicine[23].

The leaf extract of *P. amboinicus* with the selected solvents was evaluated for antimicrobial activity against selected bacterial and fungal isolates and the zone of inhibition was depicted in Table 2. Their antimicrobial property was evaluated by comparing with the positive controls. Comparative study analysis amongst the different solvents limelight the efficacy of the different phytoconstituents extracted based on the nature of solvents. It is significant to mention that there is a definite correlation between the nature of solvents used for extraction and their respective expression of antimicrobial properties.

Table 2. Antimicrobial activity of leaf extracts of *Plectranthus amboinicus*

Microorganisms	Zone of inhibition (mm)				
	Methanol extract	Petroleum Ether extract	Aqueous extract	Positive control	Negative control
Bacterial isolates					
E. coli	17.3±0.98	10.1±1.3	17.7±1.5	20±2	-
S.aureus	11.3±1.2	5±1	9.9±0.85	14.1±1.4	-
Fungal isolates					
A. niger	22.2±0.68	13.1±1.02	19±1.25	24.1±0.8	-
C. albicans	19±2	9.1±0.9	12.3±2	21.1±1.15	-

* The values are represented as Mean±SD
Positive control: Chloramphenicol (Bacteria) / Flucanozole (Fungi), Negative control: DMSO

In the present study the leaf extracted using methanol invariably exerted a good zone of inhibition against *E. coli* (17.3±0.98mm), *S. aureus* (11.3±1.2mm) and *A. niger* (22.2±0.68mm), *C. albicans* (19±2mm) respectively. This can be justified by the presence of phenolic and flavonoid compounds that natively has a trait to control the growth of microorganisms. While the aqueous extract inhibited the microbial strains in a better manner than the petroleum ether leaf extracts. When compared with the standard antibiotics chloramphenical (bacteria) and flucanozole (fungi), the methanol extract had a remarkable antibacterial and antifungal activity (Table 2). *Coleus aromaticus* proved to be a fairly good antibiotic and a very effective antifungal herb. It had inhibitory action against, *Candida krusei* and *Candida albicans* with a marginal difference. Whereas, the antibacterial activity was distinctive against the series of bacterial strains and the least susceptibility for *C. aromaticus* was recorded against *Proteus mirabilis, Escherichia coli, Staphylococcus aureus, Enterococcus faecalis, Klebsiella pneumoniae* and *Neisseria gonorrhoea*[24,25]. Also, *Jatropha curcas, Passiflora foetida* and *Cassia tora* leaves possess potent bactericidal activity against *Escherichia coli, Staphylococcus aureus, Bacillus spp.* and *Pseudomonas aeruginosa*[26,27]. The ethanolic leaves extract of *Convolvulus arvensis* and *Thymus capitatus* exhibited considerable antibacterial activity against the tested Gram positive bacterial (*Staphylococcus epidermidis, Staphylococcus saprophyticus*) and Gram negative bacterial (*Proteus vulgaris, Escherichia coli, Citrobactor freundii*) strains respectively[28].

The *in vitro* antimicrobial activity of the essential oils of *C. aromaticus* and *C. zeylanicus* against bacteria (*Bacillus megaterium, B. subtilis, Escherichia coli, Staphylococcus aureus, Proteus vulgaris, Pseudomonas aeruginosa* and *Xanthomonas campestris*) and fungi (*Aspergillus niger, A. parasiticus, Rhizopus oryzae, Rhizoctonia oryzae-sativae, Colletotrichum musae, Fusarium solani, Candida albicans,* and *Alternaria brassicicola*) revealed that the essential oil of *C. zeylanicus* had slightly higher inhibitory activity against a wide spectrum of bacteria and fungi [29]. *Muntingia calabura* showed higher inhibitory effect against the pathogens like *Staphylococcus aureus, Bacillus subtilis, Escherichia coli. Basella rubra* showed mild and good inhibitory activity against *Staphylococcus aureus* and *Aspergillus niger*[30]. Thus the phytochemicals present in the plants are acknowledged for their priceless effects and also gained increasing attention among consumers and scientific community as epidemiological studies has indicated that habitual consumption of plants rich in diverse phytoconstituents is related to a lower risk of non-communicable diseases in human[31,32].

CONCLUSION:

The present study vividly exhibited that methanolic extract of *Plectranthus amboinicus* exhibited potential antibacterial and antifungal activity due to the presence of active bioactive compounds. It is more necessary to understand that a variety of species of *P. amboinicus* exist and their phytocomposition varies according to different geographic regions. More research needs to be done to unveil the inhibitory effect of this plant against virus and other novel antigens. The utilization of this plant has always been a tradition and mostly a community based medicine that is passed on from one generation to another. Hence through this study we insist that the traditional medicine has to be streamlined and properly documented and should be available as evidence based therapies for the benefit of humankind against various infectious diseases.

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CONFLICT OF INTEREST:

The authors declare no conflict of interest.

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RESEARCH ARTICLE

Comparative Pharmacokinetic Studies of Marketed and Microsponges Gel Loaded with Diclofenac Diethylamine in Rabbits

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ABSTRACT:
Oral administration of the non-steroidal anti-inflammatory drug, Diclofenac Diethylamine (DDEA) is often associated with gastrointestinal ulcers, bleeding and extensive first-pass hepatic metabolism. As an alternative to oral administration, formulated microsponges-based gel of DDEA was developed for topical administration, to quantify diclofenac diethylamine in plasma of rabbits for this a sensitive Reverse Phase High-Performance Liquid Chromatography (RP-HPLC) method was developed using carbamazepine as Internal standard (IS) and DDEA (pure drug) was provoked on Hypersil RP C18 column (250mm × 4.6mm 5µm) using a mobile phase mixture of potassium dihydrogen buffer pH 2.5 and acetonitrile in the ratio of 30:70 v/v at an isocratic flow rate of 1mL/min. The drug peak area was detection and found at 276nm. The retention time of DDEA was found to be 5.3 min. The calibration curve was linear over the concentration range of 50-750ng/ml of DDEA. This method was accurate for quantitative estimation of the drug from the marketed gel and optimized microsponge gel. The main of investigation is to compare pharmacokinetic profile of diclofenac diethylamine in pharmaceutical dosage forms (marketed gel and microsponges gel) using WinNonlin software version 8.1.

KEYWORDS: Pharmacokinetic Parameters, RP-HPLC, DDEA, Formulated Microspongial gel, Marketed gel, Phoenix WinNonlin.

INTRODUCTION:
Non-Steroidal Anti-Inflammatory drugs (NSAIDs) have been extensively used in the treatment of rheumatoid arthritis and other related conditions. However, they carry the risk of adverse systemic side effects and gastrointestinal irritation at the usual dose of oral administration[1]. Topical and transdermal products are vital classes of drug delivery systems, and their use in therapy is becoming more common as they offer many increased patient acceptability (non-invasiveness), avoidance of gastrointestinal disturbances, and first pass metabolism of the drug[2].

Diclofenac is a nonsteroidal anti-inflammatory agent, widely used in musculoskeletal disorders, arthritis, for symptomatic relief of pain and inflammation. Diclofenac diethylamine (DDEA) is a novel NSAID of the aryl-acetic acid class. It is chemically 2- {2- [(2, 6-dichloro phenyl) amino] phenyl} acetic acid is a white crystalline powder, amphiphilic in nature, sparingly soluble in water and acetone, freely soluble in ethanol and methanol. Diclofenac diethylammonium inhibits cyclooxygenase (COX-1 and -2), which is responsible for production of prostaglandin and is used in the treatment of local pain, inflammation, soft tissue rheumatism, and degeneration of inflammatory lesions of the tendons, ligaments, and joints[3].

Due to oral administration of DDEA, it has increased risk of serious to fatal cardiovascular events, heart attack or stroke, ulcers and perforation in the stomach and intestine[4]. It has analgesic and anti-inflammatory properties and also effective in relieving symptoms of muscle aches, back aches, dysmenorrhea, dental pain, menstrual cramps and sport injuries[5].

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A few types of research are reported determination of DDEA concentration in rabbit study using HPLC method. The present investigation by the author explains pharmacokinetic profile of diclofenac diethylamine in microsponges gel in rabbit compared with marketed gel, this type of dosage form is suitable for diclofenac diethylamine for its the unique property of penetration into skin barriers to reach joints muscles and synovial fluid due to the presence of dimethylammonium salts of diclofenac owing to its lipophilic nature as shown in figure 1, and topical application of gel is more suitable for geriatric patients with increased patients acceptability[6].

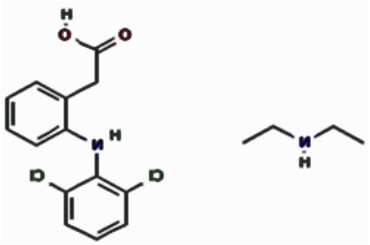

Figure. 1: structure of Diclofenac Diethylamine

MATERIAL AND METHOD:

Diclofenac Diethylamine was a gift sample from Santa Cruz, Mumbai. India and Carbamazepine were obtained as a gift sample from Vasudha Pharma, Chem. Ltd., India. KH_2PO_4 (Potassium dihydrogen phosphate), Orthophosphoric acid (OPA) and Acetonitrile was procured from Merck, Mumbai. Water for HPLC was procured from Sigma-Aldrich Chem Pvt. Ltd., Bangalore. Animal Ethical Committee has approved the study, and the studies were piloted in accordance with standard institutional guidelines.

Surgical blade, cotton adsorbent, gauze needle, syringe, Gloves, 1.5 mL Eppendorf tubes, Whatman filter paper, Buffers pH 2.5, 5.0, C18 column, Triethanolamine (TEA) from Qualikems Fine Chem Pvt. Ltd., Safe labs K3EDTA tubes, CML Biotech, Pvt. Ltd., Cyclomixer (Remi Model cm101. LC solution software 2.1 version. Voltarol (Marketed Gel). Centrifuge (Model R-8C, Remi Elektrotechnik, Vasai, India).

Preparation of Optimized Formulation of DDEA:

The DDEA microsponges were prepared by emulsion solvent diffusion technique using an internal phase consisted of Ethylcellulose as a polymer and Triethyl citrate (1% w/v) as a plasticizer dissolved in 10mL of dichloromethane alone (Internal Phase). This was followed by the addition of Drug: polymer ratio of 1:2. The mixture was then poured into an aqueous solution of Poly Vinyl alcohol which acts as the external phase with continuous stirring at 1000rpm for 180 min. Then the microsponges were formed due to the removal of dichloromethane from the system by evaporation[7]. Prepared microsponges were then filtered, washed with distilled water for thrice, and are left for drying under desiccator.

Preparation of Buffer:

10mM Potassium dihydrogen orthophosphate was prepared by taking 0.272mg of KH_2PO_4 in 200mL of HPLC water, sonication is required for complete dissolution of KH_2PO_4, still, particles are removed by filtration through filter paper with a pore size of 0.45µm (Spectrum laboratories)[8]. Further buffer is adjusted to pH 2.5 with orthophosphoric acid and triethanolamine.

Preparation of Mobile Phase:

Mobile phase was prepared by mixing acetonitrile and freshly prepared 10mM KH_2PO_4 by using distilled water in the ratio of 70:30 v/v, the mobile phase was filtered through 0.45µm membrane filter and was degassed with the help of a bath sonicator (Ultrasonic).[9]

Preparation of Internal Standard:

Accurately weighed 10mg of carbamazepine as Internal standard (IS) was transferred into 100mL the volumetric flask containing 10mL of acetonitrile (HPLC grade) and sonicate for about 10 mins. The volume was made up to the mark with acetonitrile. The stock solution was further diluted with mobile phase to give the final concentration of 1000ng/mL of each[10].

Detection of Peak:

From the various trails of Internal standard and drug concentration, the optimized concentrations of Internal standard (carbamazepine) 1000ng/mL and pure drug (DDEA) 100ng/mL was dissolved in a similar solvent and injected into HPLC to elute the peak, for identification of individual peaks of ACN, Carbamazepine (Internal standard), and DDEA (pure drug).

Chromatographic Conditions:

The chromatographic system consists of a Shimadzu LC (Liquid chromatography)-20AD Toyoko Japan, solvent delivery pump equipped with a 20µL loop and rheodyne sample injector, Hypersil RP-C18 column (250mm x 4.6 mm) Purosphur Star, encapped (5µm particle size) Darmstadt, Germany. Thermo scientific-analytical column was used. The detector equipped with SPD (Shimadzu Prominence Detector) 20-A, Toyoko Japan, dual-wavelength UV-Visible detector, and the eluate was measured at 276nm, the sensitivity was set at 0.0001 AUFS, the isocratic flow rate was kept at 1 mL/min, the data were recorded using LC solution software version 2.1 (Liquid chromatography).

A 20µL Hamilton injection syringe was used for sample injection. The flow rate of mobile was increased gradually with maintained constant pressure Kilogram-force (Kgf) and finally flow rate was stabilized constant at 1mL/min, the column temperature was maintained at 25±0.5°C, and the detection of the drug was carried out at 276nm. HPLC grade Acetonitrile, water and analytical grade potassium dihydrogen phosphate of Merck Limited, Mumbai. India.

Construction of Calibration Curve:

The column was equilibrating with the mobile phase for at least 30 min to the injection of serial concentrations of the drug. The linearity of the peak area response was determined by taking measurement at nine concentrations point including zero concentration, each concentration is measured in triplicate (n=3)

Calibration curve was constructed with rabbit plasma, these involves various concentrations of DDEA (0, 50, 100, 200, 300, 400, 500, 600, 750ng/mL as given in table 1) and a fixed concentration of carbamazepine (internal standard) were prepared by taking suitable samples of the working standard solution in different 2 mL Eppendorf tubes and dilution was made up to the mark with the mobile phase. 15µL of the dilution sample was taken and injected into the column at an isocratic flow rate of 1 mL/min, each dilution was injected three times into the column. The drug eluates are monitored at 276 nm and the corresponding chromatograms were obtained. From these chromatograms mean peak area was calculated and a plot of concentration over the peak area was constructed. A linear relationship in the range was found to be 50-750 ng/mL. the correlation coefficient R^2 = 0.9976 and y= 201.74x+1381.9 from the above equation concentration of drug was estimated and tabulated. The slope of the plot was determined by the method of least square regression analysis and was used to calculate the DDEA concentration in the unknown samples as shown in figure 2.

Table. 1: Calibration Curve of Diclofenac Diethylamine at 276nm wavelength

S. No	Concentration of DDEA (ng/mL)	Mean Peak Area (n=3)
1	0	0
2	50	10495.3
3	100	20990.6
4	200	41981.2
5	300	62450.4
6	400	83962.3
7	500	104321.9
8	600	125943.2
9	750	147350.0

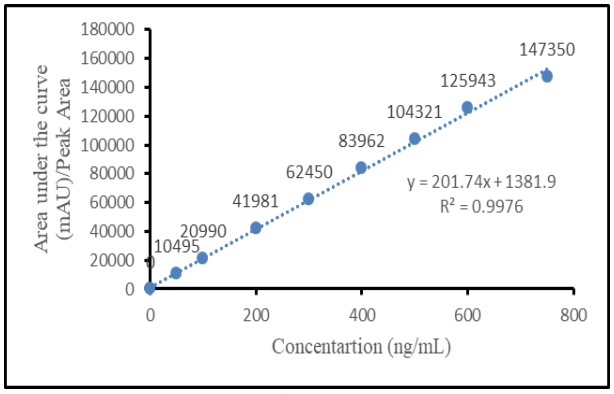

Figure. 2: Calibration curve of DDEA at 276nm wavelength

Preliminary Study:

Two days before the study a 2×2cm area on the abdominal of each rabbit was trimmed using a scissors one day before the study, a depilatory cream was applied for 15 min to the trimmed on the back and to both ears and then thoroughly washed off, to ensure complete removal of the hair in the respective areas[11].

Skin Irritation Test:

Skin irritation test was evaluated by the Draize method. Six healthy rabbits were used in this test. The rabbits were trimmed at abdominal skin both left and right side of the rabbit[12]. An aqueous solution of sodium lauryl sulfate (SLS) of 10% w/v was used as an irritant for the skin of the rabbit and its area was calculated according to the following law Area = πr^2. The desired amount of drug containing microsponges gel was applied on the right side of the rabbit, standard irritant (10% w/v SLS) was applied at the left side of the rabbit, then the microspongial gel was removed after a period of 24 hrs, 48hrs, 72hrs. Scores were graded after removal of microsponges gel from the skin and observed for the development of erythema or any sign of edema for each rabbit compared with standard irritant[13].

Sample Collection:

Albino male rabbits of bearing weight 1.75 to 1.85kg were used in the study. During *in vivo* pharmaceutical analysis rabbits were given for free access to food and normal water, until night, prior to dosing fasted for 10 hrs, before the experiment weight of each rabbit was measured[14]. An *in vivo* pharmacokinetic study was conducted in accordance with the ethical guidelines for investigations in laboratory animals and approved by the Institutional Animal Ethics Committee (IAEC), at Vaagdevi Institution of Pharmaceutical Sciences Reg No. 1663/PO/Re/S/2012/CPCSEA.

The experiments were designed as a crossover trial for this the same rabbit was used twice in the experiments including marketed gel (Reference) and finalized microsponge gel (Test). Initially, rabbits were divided

into the two groups, each group contains three rabbits. Group-1 (n=3) were subjected to the application of a Marketed gel of diclofenac diethylamine as a reference drug for both the groups at the abdominal side of the body. Group-2 (n=3) were subjected to the application of microsponge gel containing diclofenac diethylamine (optimized formulation) for both the groups, before the gel was applied, rabbits were trimmed and cleaned with normal saline. The skin of the rabbit was examined in case of any damage[15]. Each rabbit in the group was received a marketed gel and medicated microsponge gel containing DDEA 58 mg. The sample collected points were 1, 2, 3, 4, 6, 8, 10, 12, 24, 48 hrs. About 0.5mL of blood was collected from the marginal ear vein and placed in the K3EDTA tubes[16]. Plasma samples were separated by centrifugation at 3000rpm for 10 min at room temperature and stored at -20°C until analysis.

Preparation of Sample for Injection into HPLC:

Blank plasma sample (rabbit which is not treated) preparation method was validated from taking 10µL of internal standard (1000ng/mL of carbamazepine) and 100µL of acetonitrile was added into 10µL plasma sample in the centrifuge tube. the mixture was vortexed for 2 mins and extracted with 0.5mL of supernatant was taken after centrifugation of the Eppendorf tube at 10,000rpm for 10 min[17]. into another empty tube and 15 µL of the sample were injected into the HPLC for the analysis as a blank[18].

10µL of plasma was taken from the previously stored plasma sample (rabbit which is treated) which is subjected to drug analysis of that 10µL internal standard carbamazepine (1000ng/mL) was added followed by the addition of 100µL of ACN. The entire mixture was mixed with Cyclomixer (Remi, cm 101) and separation was done by centrifugation for 10 min at 1000rpm, about 15µL of supernatant was collected and injected into HPLC[19].

Table. 2: Comparative *in vivo* plasma drug concentration

	Marketed gel	Formulated gel
Time (hrs)	**Plasma Concentration (ng/mL)**	
0	0	0
1	174.4367	80.0513
2	235.5383	151.4824
3	299.2667	196.0589
4	291.61	262.1312
6	246.7033	229.4923
8	203.6017	185.7894
10	181.9767	144.9577
12	151.265	111.4987
24	6.635333	9.324939

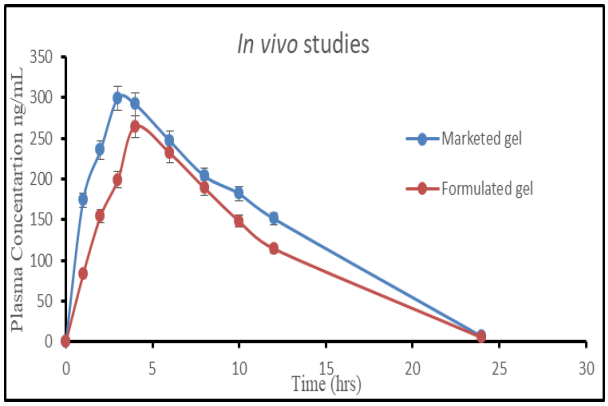

Figure. 3: Drug penetration profile of MG and FMG

Table. 3: Intra-day and Inter-day precision for quantification of DDEA

Concentration (ng/mL)	**Intraday**			**Mean**	**S.D.**	**% RSD**
250.00	52296.50	52450.30	+-56421.32	53722.70667	2338.332537	4.35
350.00	73212.00	74545.23	69587.33	72448.18667	2565.687278	3.54
550.00	115052.33	121458.30	100032.65	112181.0933	10997.61881	9.80
Concentration (ng/mL)	**Interday**			**Mean**	**S.D.**	**% RSD**
250.00	54569.66	56206.75	51097.05	53957.82	2609.218209	4.83
350.00	75487.99	77752.63	70684.21	74641.61	3609.419573	4.83
550.00	121548.32	125194.77	113813.43	120185.5067	5811.76997	4.83

Table. 4: LOD and LOQ data for detection of DDEA

Limit Of Detection (LOD)	**38.25 ng/mL**
Limit Of Quantification (LOQ)	**115.91 ng/mL**

Pharmacokinetic Analysis:

Phoenix WinNonlin Version 8.1 (Certara USA, Inc., Princeton, NJ) Pharmacokinetic parameters of Diclofenac diethylamine was performed with a comparison with Marketed gel to Microspongial gel, plasma samples were estimated in each rabbit using a computer program, WinNonlin software. Non-compartmental analysis (NCA) with mainly four terminal points was selected for calculation of pharmacokinetic parameters C_{max} (ng/mL), T_{max} (hrs), and Area Under curve AUC (ng-hr/mL), AUMC (hr.hr-ng/mL) etc[20].,

Table. 5: Pharmacokinetic Profile of Marketed gel (Reference)

Parameters	Units	R1	R2	R3	R4	R5	R6	Mean ±SD
C_{max}	ng/mL	252.46	320.08	357.99	388.27	296.98	292.63	318.06±48.83
T_{max}	hrs	4	3	3	3	4	3	3.33±0.51
$T_{1/2}$	hrs	2.82	2.59	2.67	3.64	2.53	2.25	2.75±0.47
AUC $_{(0-24)}$	ng-hr/mL	3101.38	3645.35	3417.07	4116.74	3329.56	3449.23	3509.88±345.92
AUC $_{(0-\infty)}$	ng-hr/mL	3126.25	3663.23	3435.35	4204.33	3346.34	3458.84	3539.05±369.52
Vd	mL	65180.95	51131.21	56222.71	62567.12	54579.24	47003.85	56114.18±6841.67
Clearance	mL/hr	15993.6	13649.15	14554.55	11892.5	14941.68	14455.69	14247.86±1383.64
AUMC	hr.hr ng/mL	25195.04	26518.27	25115.49	35556.38	26656.46	26825.52	27644.53±3947.37
MRT	hrs	8.05	7.23	7.31	8.45	7.96	7.75	7.79±0.464
K_E	hrs^{-1}	0.245	0.266	0.258	0.190	0.273	0.307	0.256±0.038
C_{last}	ng/mL	6.103	4.774	4.732	16.65	4.595	2.958	6.63±5.00

K_E = Elimination Rate Constant, C = Concentration,

Table. 6: Pharmacokinetic profile of Formulated Microspongial gel (Test)

Parameters	Units	R1	R2	R3	R4	R5	R6	Mean ±SD
C_{max}	ng/mL	253.99	257.50	282.18	248.14	255.61	275.33	262.13± 13.43
T_{max}	hrs	4	4	4	4	4	4	4± 0
$T_{1/2}$	hrs	3.463	2.721	2.213	4.102	3.898	4.169	3.42±0.8
AUC $_{(0-24)}$	ng-hr/mL	2719.66	2874.26	2816.71	2648.86	2825.73	2781.03	2777.70±81.40
AUC $_{(0-\infty)}$	ng-hr/mL	2764.66	2893.50	2823.42	2725.64	2898.80	2865.06	2828.51±7097
Vd	mL	90361.05	67857.33	56555.00	108570.03	97022.92	104972.9	83353.86±20381
Clearance	mL/hr	18085.36	17280.06	17708.98	18344.25	17248.47	17451.61	17686.46±448
AUMC	hr.hr ng/mL	22497.83	23060.42	21622.94	23265.19	25032.46	24801.17	23380±1321.45
MRT	hrs	8.13	7.96	7.65	8.53	8.63	8.65	8.258±0.410
K_E	hrs^{-1}	0.20	0.25	0.31	0.16	0.17	0.16	0.208±0.06
C_{last}	ng/mL	9.00	4.90	2.10	12.97	12.99	13.97	9.321±4.90

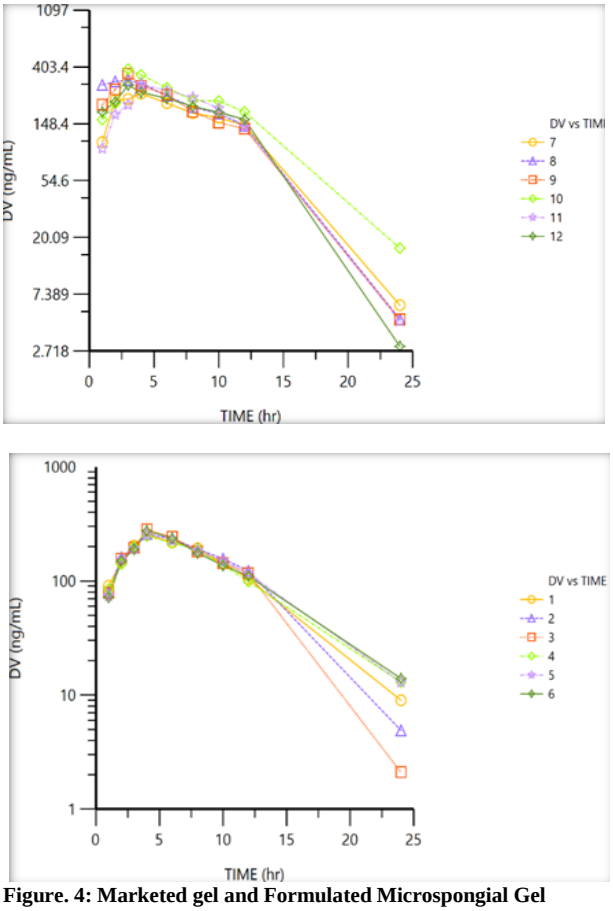

Figure. 4: Marketed gel and Formulated Microspongial Gel

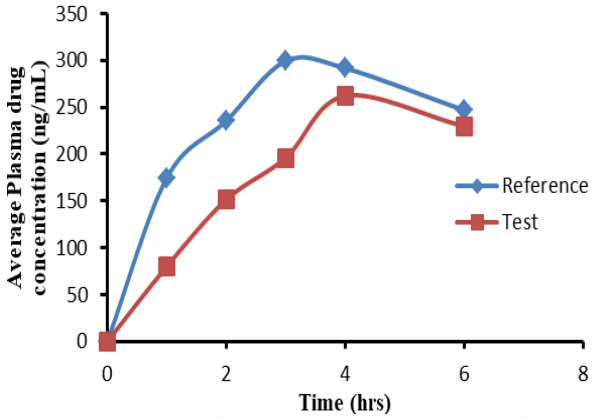

Figure. 5: Similarity factor between concentrations of MG and FMG

RESULTS AND DISCUSSION:

Since microsponges are prepared by quasi-emulsion solvent diffusion technique the drug has slightly soluble in the solvent and remaining drug is entrapped into the microsponges due to its porous nature of the carrier the drug is slowly released from the matrix to outer environment gel, from gel to a deeper layer of the skin through sweat pores which act as the main entrance of the drug into the skin, as a carrier called microsponges are larger in their structure which cannot penetrate into the pores and are trapped in the hooks of the skin thereby release the drug slowly into the pore, as the drug is absorbed into the pores, passes into the deeper layers of the skin through the pore column. At the end of the

column several bloods vessels are surrounded which helps in absorption of the drug into systemic circulation[21].

After 72 hrs of treated DDEA based gel, animals show no skin irritation such as redness of skin or inflammation at the site of application, both the areas, when applied with vehicle or selected formulation were found to be free of any sign of irritation thus it is conducted that is the following are safe for topical application of gel products, hence it is needed to confirm safety after repeated application also.

Pharmacokinetics parameters calculated by WinNonlin using a Non-compartmental model (NCM) from the pharmacokinetic studies appear that there is a change in C_{max} AUC $_{(0-24)}$, AUMC after microsponge gel treatment, there is a considerable decrease in C_{max} with prolonged release of DDEA after formulating into microsponges, this work was carried out in rabbit and that same results may also be excepted in humans.

Due to emulsion diffusion method of preparation of microsponges the excepted prolonged release was occurred compared to marketed gel which is required during the several joint pains and continuous release of inflammatory mediators, the absorption of the drug into systemic circulation is very close to marketed formulation due to the presence of diethyl ammonium salts of diclofenac act as a permeation enhancer, designed for topical application moreover the presence of triethanolamine help in faster penetration of the drug into the systemic circulation.

The area under the curve was measured by trapezoid rule in which the drug has an action for longer period of time. The prolonged-release of drug is due to entrapment of microsponges in nooks of the skin surface, the novel delivery system of microsponges act as reservoir system by maintaining sink condition, slowly drug is release from microsponges into the gel, from there to a deeper layer of skin, the study was conducted for 24 hrs. in this study we measure pharmacokinetic parameters of plasma concentration of diclofenac diethylamine.

In this study we measured DDEA concentration in plasma for both marketed and formulated microsponges gel at a maximum time points up to 24 hrs, the C_{max} after topical application of marketed gel and formulated microsponges were reported as 318.06±48.83 ng/mL and 261.13±13.43ng/mL, a decrease in C_{max} is due to slow release of medicament from the microsponges thereby preventing sudden increases in the concentration of drug in the plasma which leads to allergic reactions. Slower absorption may be the reason which is clear from the shift in T_{max} of the formulated gel was found to 4.0±0 hrs, has slight increase in T_{max} when compared with marketed gel 3.33±0.51 as given in table 5 and 6. The sensitivity of developed method for quality control analysis of DDEA proved by LOD and LOQ values were found to be 38.25ng/mL, 115.91ng/mL as given in table 4. Percentage RSD was used to determine precision for quantification of DDEA for intra-day and inter-day as shown in the table 3. the repeatability of newly method is proved by very less values are obtained as % RSD confirming the method is satisfactorily precise under the same conditions.

The $T_{1/2}$ value of the plasma drug concentration for marketed gel and formulated microsponges gel were 2.75±0.47 hrs and 3.42±0.8 hrs the increased in volume of distribution found to be 83353.86 ± 20381.68mL this is due to active absorption of the drug into deeper layers of the skin and into the systemic circulation. The mean residence time for marketed formulation and formulated was found to be 7.79±0.464 hrs and 8.258±0.410 hrs the long stay of the drug in plasma is due to its high protein binding nature of the drug, microsponges as a carrier system act as a depot for DDEA, the C_{last} the concentration found in the formulated microsponges gel was more when compared to marketed gel, still, there is a drug to delivery into the systemic circulation. So it must be important to design a study after repeated application of microsponge gel.

Since diclofenac diethylamine is having a high tendency to penetrate into deeper layers of the skin, the pharmacokinetics property has shown a difference in their parameters, due to a formulation difference since we required less amount of the drug that enter into systemic circulation for a prolonged period of time since there is the larger difference was observed with *in vitro* and *in vivo* studies in term of action of the drug for 24 hour's period of time. Finally, the similarity factor was applied between marketed formulation and formulated microsponge gel was found to be dissimilarity due to the values obtained as f2 = 12.6889 and f1 = 23.70427 respectively as shown in figure 5. A significant changed was observed between marketed gel and formulated microspongial gel, where drug absorption was rapid in plasma drug concentration of marked gel compared to formulated gel as shown in table 2 and figure 3 and 4. The basic points which shows prolonged action of the drug is due to its protein binding and found to be 99.7 % from the literature survey. the duration of action of the drug from marketed formulation and formulated microsponge gel is observed for 24 hrs in rabbit model that same can be estimated in the human model.

The purpose of this study is to identify the drug in blood for a prolonged period of time due to its high level of protein binding. The purpose of design of formulation

into novel microsponges are to prevent its sudden increase in plasma drug the concentration which leads to severe adverse effects likes gastrointestinal problems, allergic reactions including kidney damage, this can be prevented by a novel delivery system which releases the drug slowly into the systemic circulation for a prolonged time.

CONCLUSION:

On the present the study, with respect to the *in vivo* studies conducted. Gel administration of Diclofenac diethylamine produces better prolonged release when compared to topically applied marketed gel. This could be due to the slow and continuous supply of Diclofenac diethylamine at a desirable rate to the systemic circulation by microsponge gel without sudden increase in concentration.

Further the slow and sustained release of the drug from the transdermal system might reduce the release of inflammatory mediators for a prolonged time. The present study shows that topical application of DDEA gel exhibits better control of inflammations more effectively reversing the complication associated with inflammation release than normal gel application.

CONFLICT OF INTEREST:

There is no conflict of interest in publishing this article.

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RESEARCH ARTICLE

Comparing the Physico-Chemical Characteristics of Formulated and Marketed Yashada Bhasma

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ABSTRACT:
The present study is aimed to observe the difference in the Physico-Chemical characteristics of the marketed and formulated bhasma samples through X-Ray Diffraction analysis (XRD), Dynamic Light Scattering (DLS), Zeta potential, Thermo-Gravimetric analysis (TGA), Scanning Electron Microscopy (SEM) and Energy Dispersive X-Ray analysis (EDAX), apart from organoleptic methods. Inductively Coupled Plasma Mass Spectroscopy (ICPMS) analysis was also done to observe the presence of trace and heavy metals so that the safety of all these samples could be ensured. XRD shows variation in oxide nature of zinc as well crystallite size in all bhasma samples. DLS and SEM results show difference in particle size of marketed bhasma samples as compared to formulated Yashada bhasma. EDAX and ICPMS also confirm the alteration in elemental composition of all these bhasma samples. Thus, it can be concluded that these ayurvedic medicines should be prepared strictly using the formulation methods as mentioned in the Ayurvedic texts. This will help the prepared products to adopt the inherent quality of the ancient system of medicine, which shall be useful and devoid of any side effects for human consumption.

KEYWORDS: Ayurvedic formulation, Yashada bhasma, X-ray diffraction, Scanning Electron Microscopy.

INTRODUCTION:
According to WHO, about 70% of world population extensively use traditional and alternative medicines for the healthcare[1]. "Ayurveda" which exactly means "knowledge of life". This traditional form of medicine is thought to be thousands of years old, Ayurveda remained as applicable as ever then and now—and it is given great importance every day as individuals globally use its timeless knowledge in their everyday lives.

Bhasma which is an ancient medicinal trend is a beautiful gift of Ayurveda given to the humankind. The bhasma is the incinerated state of metals which is prepared taking a lot of care to remove the metallic qualities of the original metal and transform it into nano size particles, which makes it to adopt medicinal qualities and used for treatment of human beings[2,3,4,5,6].

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According to the experts of these shastras, the process of preparation should be strictly followed to get the exact component. It means all these metals with plant and herbal extracts added to it, should be prepared with the same lengthy process propounded for it and incinerated in the same manner as mentioned in the shastras. This result in the metals to lose its metallic qualities and toxicity and change into such compounds which have medicinal and healing qualities of very high grade which when taken as medicines by human beings is absolutely safe and works as a curative drug without any side effects.

In Ayurveda, Yashada bhasma has great medicinal value, and had been in use since hundreds of years for various medicinal purposes like eye disorder, diabetes, blood disorder etc. It is very useful in treatment of Leucorrhoea, eye disorder, Eczema, anemia, respiratory disorder, wound healing etc[7]. But, validation of this biological formula has not been introduced, due to which the quality control of this product has not been taken care of. It is believed that standardization is the

capacity for confirming the quality and is used to describe all methods, which are taken during the manufacturing process and quality control leading to a reproducible quality. Thus, standardization method should be introduced to ensure its qualities and also to describe all its measures[8]. The quality control and manufacturing process of this product needs a great attention to make it consumable for all humankind [9,10].

The present study has been carried out to analyze and compare the formulated and marketed samples of Yashada bhasma through some physico chemical and modern standardization techniques like XRD, DLS, Zeta Potential, TGA, SEM, EDAX and ICP MS. The opinion propounded was that either the bhasma preparation did not follow the proper process as mentioned in the text or else a shortcut method may have been followed due to certain reasons which raise a question on their safety and efficacy[11,12] and render it unacceptable as approved drugs on international level[13,14]. Thus, with an objective to find out more regarding the same and to look towards the standardization of the bhasma, the present study has been carried out.

MATERIAL AND METHODS:

Preparation of Yashada bhasma:

Yashada bhasma was prepared according to the methodology laid down in Ayurvedic texts [15-18] in the analytical lab of National Institute of Ayurveda, Jaipur, Rajasthan.

Yashada bhasma was prepared by three step process i.e. Shodhana (purification), Jarana (roasting) and Marana (calcination). Shodhana was carried out by heating and quenching of Yashada (Zinc) in five different liquid medias i.e. Tila Taila (sesame oil), Kanji (fermented ayurvedic preparation), Takra (buttermilk), Kulattha Kwatha (decoction of horse gram), and Gomutra (cow urine) for seven times. For Vishesha Shodhana same process was repeated in Nirgundi herbal extract (*Vitex negundo*) for three times[19]. Shodhita metal is converted into powdered form. For Jarana process, shuddha Yashada and apamarga churna (*Achyranthes aspera*) was taken in an iron pan and was allowed to melt at high temp, metal was converted in to powder form and thus, no part of the metal remained in metallic form. This powder is further processed for Marana, triturated with kumari swarasa (aloe vera juice) to form chakrikas and subjected for calcination process in muffle furnace at temperature 500^0.The same procedure was repeated using fresh aloe vera juice every time until the bhasma, which passed the classical test characterization was obtained. The other samples of marketed Yashada bhasma were procured from local market of Jaipur and their documentation regarding their batch no, date and manufacturing was done[20].

Characterization with Comparative Assessment of Formulated and Marketed Yashada Bhasma:

Details of formulated Yashada bhasma (prepared in National institute of Ayurveda, Jaipur) and marketed Yashada bhasma (procured from Jaipur market) samples were given specific codes of BYB1, BYB2 and CYB whereas the formulated bhasma was coded as OMM. These formulations were characterized through classical methods of analysis as mentioned in the text, at National Institute of Ayurveda, Jaipur. Physical characterization was carried out at Ayushraj Institute, Jaipur and Manipal University Jaipur. Modern characterization techniques like XRD, TGA, DLS, Zeta potential and ICPMS was done at Sastra University, Thanjavur and SEM and EDAX analysis at MNIT, Jaipur.

Classical characterization[21] and Physical characterization of Bhasma:

The qualitative evaluation of bhasma particles forms the basic analytical validation technique, where different bhasma samples are examined for physical properties like color, taste, lusterness, fineness and floating properties etc. The quantitative analysis of bhasma includes physical and chemical properties of bhasma samples[22, 23]. This is carried out by finding Total Ash value, Acid insoluble Ash, Water soluble Ash, Loss on drying and pH value.

Modern Characterization of Bhasma:

The modern techniques of characterization of bhasma give a clear picture on the safety and efficacy of the bhasma samples[24, 25].

XRD Analysis[26, 27]: XRD of formulated and purchased samples were carried out for detection of crystalline or amorphous nature of compounds and phases of Zinc metal. Crystallite size, structural identification, and purity of samples were also determined by X-ray diffractometer (Bruker D8 focus) using Cu K_α radiation of wavelength λ=1.5418 A^o. The 2Θ value ranged from 20^o to 80^o.

DLS and Zeta potential analysis: Particle size and Zeta potential analyzer determined Particle size, surface charge, polydisperse index (PDI) and stability of all Yashada bhasma samples. Nano-ZS series 633 nm laser (Malvern Instruments Limited, UK) was used for nano particle size distribution.

TGA Analysis: Thermo gravimetric is a technique that measures the variation of mass of a sample during thermal treatment. An SDT Q600 TGA/DSC of TA instrument is used to record the loss in weight of samples with temperature range 0^0- 800^0.

Table 1: Ancient and Physical characterization of BYB1, BYB2, CYB, OMM

Sample	Varna (Color)	Niswadu (Taste	Nishchandrata (Lusterless)	Varitara (Lightness)	Nirdhuma (Fumes)	Rekhapurnata (Fineness)
BYB1	Yellow	Tasteless	No luster	Positive	No fumes	Positive
BYB2	Yellow	Tasteless	No luster	Positive	No fumes	Positive
CYB	Yellow	Pungent	No luster	Positive	Mild fumes	Positive
OMM	Creamish	Tasteless	No luster	Positive	No fumes	Positive
Samples	**Total Ash Value**	**Acid Insoluble Ash**	**Water Soluble Ash**	**Loss on Drying**	**pH value**	
BYB1	95.20%	62.33%	9.55%	0.78%	9.23	
BYB2	96.13%	66.21%	11.78%	0.80%	10.00	
CYB	85%	67%	15%	2.5%	11.00	
OMM	99.76%	46.10%	1.66%	0.01%	8.58	

SEM Analysis[28]: SEM analysis is useful for determining the three-dimensional configuration and surface morphology of all formulations. Small quantity of samples was sprinkled on to a double-sided carbon tape and mounted on aluminum stubs, to get electron image for SEM and EDAX analysis. Morphology of particles, size of particles and elemental composition of Yashada bhasma samples were carried by using SEM-EDAX instrument, NOVA NANOSEM 450 (XT Microscope server, FEI Netherlands).

ICPMS Analysis: ICPMS is carried for determination of heavy metals present in bhasma samples. Thermo Series-II, NWR 213 (Thermo Fisher Scientific, Germany) was used for detection of heavy metals present in samples. The ICP source converts the atoms of the elements in the sample to ions. These ions are then separated and detected by the mass spectroscopy.

RESULTS:

Ancient and Physical characterization:

Ancient characterization like Varna, Taste, Nishchandrata, Varitara, Nirdhuma and Rekhapurnata of all samples and Physical characterization of bhasma samples like Total Ash value, Acid Insoluble ash, Water soluble ash, Loss on drying and pH value of all samples are shown in Table 1.

Modern characterization of Bhasma:

X-ray Diffraction:

XRD result of formulated Yashada bhasma and marketed Yashada bhasma samples are illustrated in Fig.1. BYB 1 sample shows the diffraction peaks at angle 2θ= 31.70°, 34.36°, 36.18°, 47.47°, 56.51°, 62.79°, 66.29°, 67.87°, 69.00°, 72.36° ,76.88° , and 81.27°, corresponding to the hkl value (1 0 0), (0 0 2),(1 0 1), (1 0 2),(1 1 0),(1 0 3), (2 0 0),(1 1 2),(2 0 1),(0 0 4),(2 0 2) and (1 0 4) with reference to the JCPDS File No (89-1397). It confirms the presence of ZnO (zinc oxide) in hexagonal $p6_3$ mc symmetry. The XRD pattern of BYB2 is similar to that of BYB1 and again this result confirms the presence of ZnO (zinc oxide) with JCPDS File No (89-1397). In CYB sample XRD peaks at angle 2θ= 31.72°, 34.39°, 36.25°, 47.49°, 56.53°, 62.82°, 66.31°, 67.89°,69.02°,76.90 corresponding to the hkl value (1 0 0), (0 0 2),(1 0 1), (1 0 2),(1 1 0),(1 0 3), (2 0 0),(1 1 2),(2 0 1),(2 0 2) matches with JCPDS File No (89-1397) confirming the presence of zinc oxide(ZnO). Some peaks of CYB sample are at angle 2θ= 20.98°, 25.41°, 31.32°, 38.60°, 40.79°, 43.23, 48.64°, 52.22°, 55.69°, 65.42°, corresponding to value (0 1 1), (2 0 0), (1 0 2), (0 2 2), (1 2 2), (3 1 1),(3 0 2),(0 4 0), (3 2 2),(0 2 4) confirms the presence of $CaSO_4$ as it matches with JCPDS File No (72-0916).

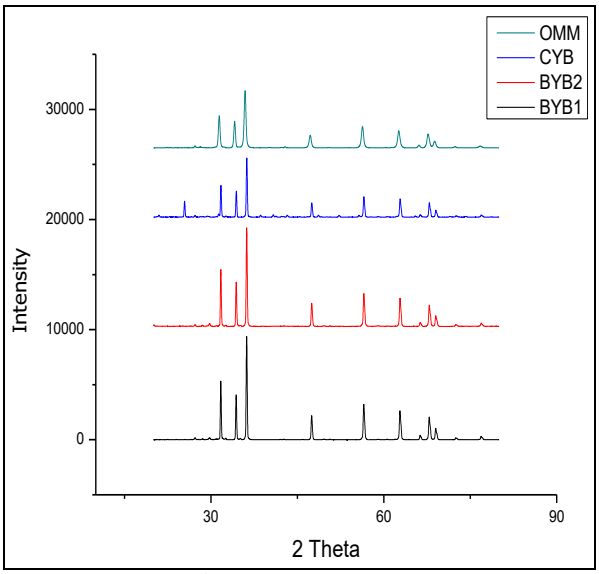


Fig. 1: XRD spectra of BYB1, BYB2, CYB and OMM

XRD analysis of OMM pattern shows hexagonal ZnO (zincite phase) crystalline phase, with JCPDS card 79-0208 and P63mc space group, located at 2θ= 31.42°, 34.09°, 35.91°, 47.22°, 56.28°, 62.58°, 66.09°, 67.67°, 68.80°, 72.36° and 76.71°. Crystallite size of all Yashada bhasma samples were calculated by the prominent peak of corresponding XRD pattern by using Debye Scherrer's formula (D=0.89λ/βcosθ), Where D is the crystallite diameter, λ is the x-ray wavelength (0.15418 nm), β is the full width at half maximum intensity (FWHM) of the diffraction peak, and θ is the diffraction angle of the peak pattern of the of bhasma sample. Crystallite sizes of all the samples are as shown in Table 2.

Table 2: Crystallite size of BYB1, BYB2, CYB, OMM

Sample	2θ value	FWHM	Crystallite size(nm)
BYB 1	36.18	0.185	44.65
BYB 2	36.18	0.157	53.40
CYB	36.19	0.174	47.48
OMM	35.91	0.252	32

DLS and ZETA Potential:

Particle size of all bhasma samples as calculated by DLS method are shown in Fig. 2. BYB1 and BYB2 show mean particle diameter of 1397 and 1046 nm, CYB shows 858 nm while OMM shows very less particle size of diameter 339 nm. Zeta potential values of BYB 2, CYB and OMM are approximately same i.e., -18.3, -16.5, -20 but for BYB 1, zeta potential value is slightly higher (-8.4) than other samples as shown in Fig. 3, (a-d).

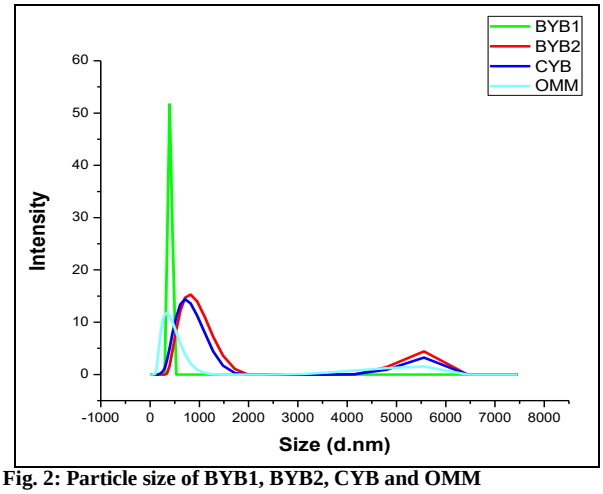


Fig. 2: Particle size of BYB1, BYB2, CYB and OMM

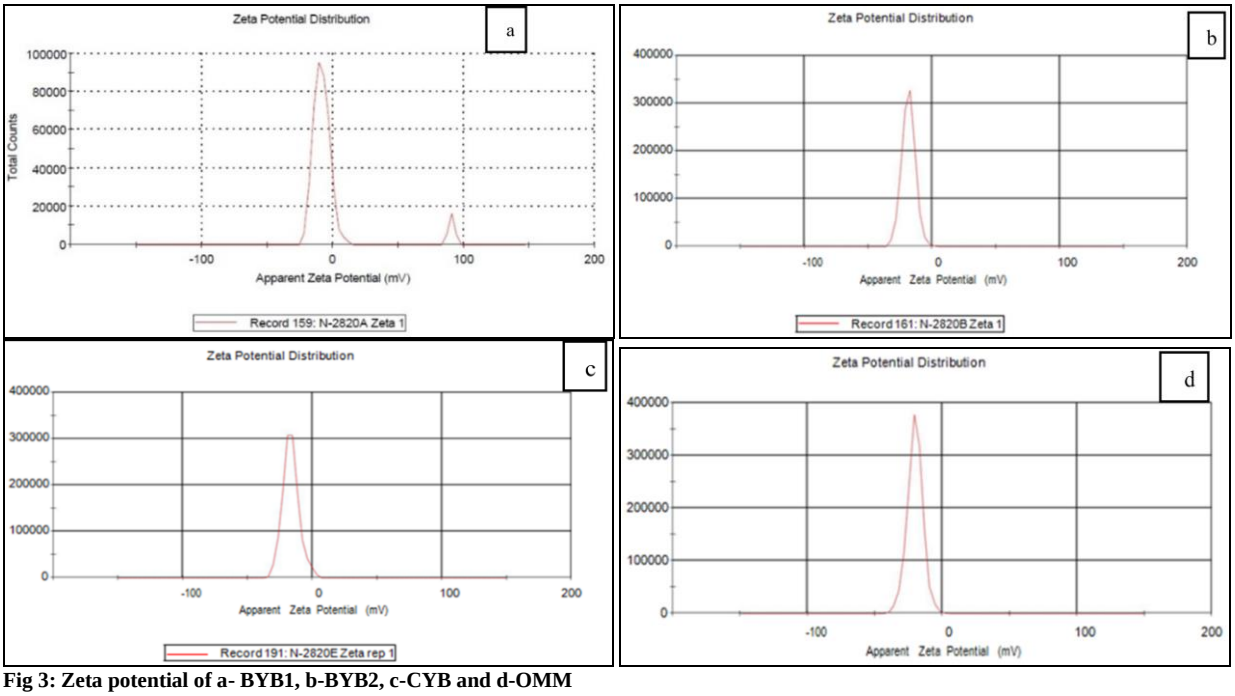


Fig 3: Zeta potential of a- BYB1, b-BYB2, c-CYB and d-OMM

TGA:

TGA analysis curves of all bhasma samples are presented in Fig. 4. Weight loss of sample BYB1, BYB2, and OMM is found to be 0%, while sample CYB shows 2.78% weight loss above 800^{0}C temperature.

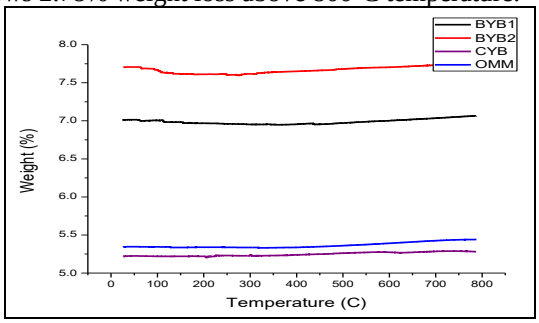
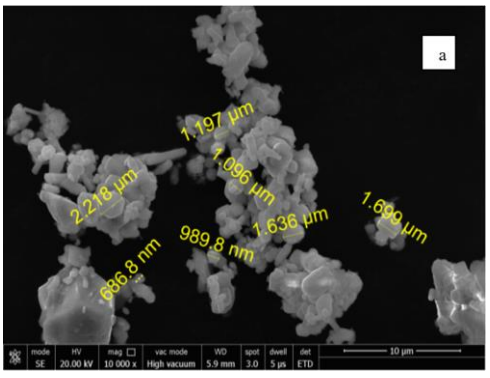


Fig. 4: TGA Spectra of BYB1, BYB2, CYB and OMM

SEM and EDAX analysis:

SEM micrographs and EDAX analysis of all bhasma samples are presented in Fig. 5 and Table 3 respectively.



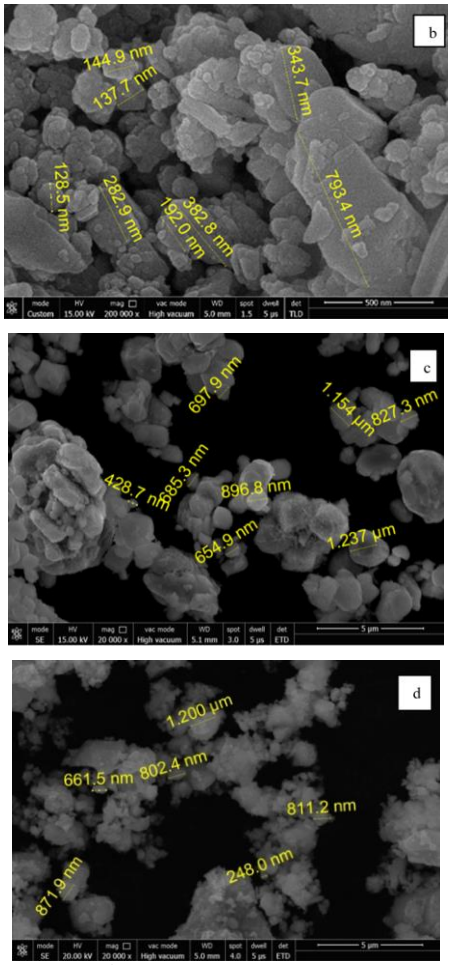


Fig. 5 SEM results of (a) BYB1, (b) BYB2, (c) CYB and (d) OMM

SEM image of BYB1, BYB2, CYB and OMM shows difference in particle size, agglomeration and morphology of particles as shown in Fig. 5. BYB1 shows spongy and globular particle with size ranges between 400nm-1.2µm, BYB2 shows spongy, non-symmetrical and non-specified particles with size ranging 250nm-1.2µm, in CYB particles are in rod shaped, non-porous and symmetrical structure with size ranging from 650nm-2.2 µm, while OMM shows globular, porous and symmetrical particles with size ranging from 130- 700nm.EDAX results shown in Table 3 represents different concentration of elements in each sample. OMM shows higher concentration of zinc and oxygen as compare to BYB1, BYB2, and CYB.

Table 3: Elemental analysis of BYB1, BYB2, CYB AND OMM

Elements	BYB1	BYB2	CYB	OMM
	Concentration (%w/w)			
Zn	49.28	38.58	35.50	65.18
O	20.92	20.72	18.50	30.45
C	28.97	39.49	40.30	-
Al	0.15	0.51	-	-
Ca	-	-	5.7	0.49
P	-	-		2.72
K	-	-		0.70
Fe	-	-		0.40

ICPMS Analysis:

ICPMS results show the presence of heavy metals in all bhasma samples, but their concentration in each sample is different. In BYB1, BYB2 and CYB the concentration of lead is 6442 ppm, 7738 ppm and 3226 ppm while in formulated sample OMM, the concentration of Pb is only 36 ppm.

DISCUSSION:

Zinc is a very important element for all physiological processes in human body, as it plays vital role in cell functioning, boosting immune system, promoting wound healing, control diabetes and regulates many more functions in human body[29]. Imbalance of zinc in human system causes appetite loss, eye and skin lesions, and diabetes and weight loss. Also, zinc deficiency causes anemia, low insulin level, as it is slowly absorbed by body. This deficiency of zinc cannot be fulfilled by taking zinc metal directly, as their direct consumption might create harmful side effects in human body. It may be noted that for its suitable consumption, they are formulated by proper incineration process as mentioned in classical texts. Different steps (Shodhana, Jarana, and Marana) of preparation of bhasma not only remove the impurities present in the metal but integrate much medicinal property which renders them suitable for treatment of many ailments.

Yashada bhasma may be prepared using various methods and raw materials as per texts laid down in many literatures. These may vary from one manufacturer to another as described in the Ayurvedic textbooks[30]. It is needed to deduce the chemical composition, check upon the physical properties and structure of these bhasmas which after its complete preparation eliminates the toxicity present in the metal resulting in giving excellent medicinal effect to the human body.

Firstly, all bhasma samples (formulated and marketed samples) were subjected to ancient tests. Color of OMM sample was found to be different from other marketed samples as color of bhasma sample depends upon the material used in Marana process. In OMM, Kumari swarasa was used as bhavana dravya, so that OMM has creamish color while BYB1, BYB2, CYB have yellow color. Varitara, Rekhapurnata are done to check the lightness and fineness of bhasma as fineness and lightness will enhance the absorption and curing property of bhasma. Similarly, Nishchandrata and Nirdhuma show the absence of metallic part in all bhasma samples as there was no luster and no fumes were found emerging out from bhasma samples except CYB as it shows mild fumes may have some metallic part remaining. Total ash content shows proper incineration of BYB1, BYB2 and OMM as they have

ash value >95%, while in CYB, ash content value is 85% shows that there may be some moisture and organic impurity present in bhasma sample. Greater acid insoluble value of all bhasma samples revealed that some inorganic matter is present in all bhasma samples which was insoluble in hydrochloric acid used as a solvent in this test. Water soluble ash value of OMM is very less (1.66%) as compared to BYB1, BYB2, CYB which shows that inorganic matter present in OMM is insoluble in water, comparatively high-water soluble ash value of BYB1, BYB2, and CYB confirms the presence of water-soluble matter in samples. Loss on drying measures the water content and volatile matter present in bhasma samples. Less value of LOD (<1%) in OMM, BYB1, BYB2 shows absence of water in bhasma samples. Increased value of LOD in CYB sample confirms the presence of water in it which may have been inserted by the use of bhavana dravya during Marana process. The pH value represents acid or basic nature of samples. All samples were found to be basic in nature.

Instrumental analysis using XRD confirms the presence of zinc oxide in all bhasma samples as their major portion. All bhasma samples have sharp peaks with different intensities reveals the crystallite nature of samples. OMM, BYB1, BYB2 have only ZnO crystallite peaks in it, and no peaks of zinc metal revealed that all zinc had been converted into its oxide form[31]. While XRD peaks of CYB shows some peaks of $CaSO_4$ matches with JCPDS File No (72-0916). Thus, Calcium may have been present in zinc metal already or may have come from some herbal juice and plant extract that get converted into $CaSO_4$ during preparation process. The average crystallite size of all bhasma samples is found to be less than 100 nm from Scherer's formula which confirms proper incineration of zinc metal and properly incinerated nano size bhasma particles can be easily absorbed by targeted site.

Particle size of samples BYB1, BYB2, and CYB is higher (1397, 1046, 858 d nm) as compared to size of OMM (339d nm) sample. The greater particle size of all samples obtained from DLS method as compared to XRD result because in DLS method, was attributed to the fact that when particles are dispersed in aqueous media, these particles colloid together and form suspension of negatively charged hydrophobic particles. This may be due to the repeated incineration method during Marana process[32], although OMM shows decreased particle size as compared to marketed samples. This may be due to proper incineration of bhasma sample. TGA result confirms the stability of all bhasma samples at high temperature.

Zeta potential value of all samples shows their stable condition. SEM result shows the average particle size of BYB1, BYB2, CYB and OMM bhasma samples are in micrometer range, whereas particle size of OMM bhasma sample is in nanometer range. ICPMS result shows the higher concentration of lead in all marketed samples as compared to formulated sample that may causes harmful effects in human body. SEM results are in good agreement with particle size obtained from DLS analysis. Less particle size of OMM may be attributed due to repetitive burning in Marana process. EDAX confirms the presence of zinc and oxygen as major components in all bhasma samples but the concentration is different in all samples. BYB1, BYB2, CYB had decreased amount of zinc and oxygen as compared to OMM which reveals that major part of zinc has converted into zinc oxide in OMM sample. Some trace elements like phosphorus, potassium and calcium present in OMM sample, may be attributed from some herbal juice or plant extracts (Kumari swarasa is rich with Ca, Na, Mg) during preparation method[33]. Fe might be introduced from iron pan used in Jarana process at high temperature.

CONCLUSION:

Variation in the results of the Marketed samples BYB1, BYB2, CYB clearly indicates that they have not been prepared strictly as per literature mentioned in Ayurvedic texts. Even there had been variation in the results of BYB1and BYB2 with the same brands but different batches. These raise a serious concern over not only safety of these formulations but their efficacy also. Thus, there is an urgent need of some standard operating protocol being made for the preparations of these bhasmas and strict regulations should be made that these ayurvedic medicines should be prepared strictly using the formulation methods indicated in the Ayurvedic texts only then it will adopt the quality which shall be useful and devoid of side effects for human consumption.

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CONFLICT OF INTEREST:

None.

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RESEARCH ARTICLE

Assessment of the convergence angle of teeth prepared for full crown by preclinical dental students

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ABSTRACT:

The objective of this study was to measure the buccolingual and mesiodistal convergence angles of six typodont teeth (# 26, 36, 45, 15, 21, and 13), prepared by preclinical dental students at Ajman University, for porcelain fused to a metal full crown and to compare them with the recommended convergence angle (6.5°). Additionally, we sought to compare the angles recorded for the six sets of teeth and relate the results according to the tooth position and surface and to know which one shows the greater tendency of straying from the normal convergence angle. **Materials and methods:** The angle of convergence of one hundred ninety-eight typodont teeth preparations was measured both buccolingually and mesiodistally by using a Dino-lite pro digital microscope (AM-413ZT Taiwan) with a Dinocapture (2.0 version 1.5.27.A, AnMo Electronics Corporation). All the results were recorded, and the data were analyzed by means of a one-sample t-test and one-way ANOVA. Results: The mean total convergence angle for this study was 11.29°± 6.66° from both surfaces, which is greater than the recommended value of 6.5° and statistically significant ($p<0.000$). Only 7.07% of teeth met the ideal convergence angle from both surfaces, and the one-sample test showed a statistically significant difference ($p<0.057$) from the recommended convergence angle, except for the mesiodistal convergence angle of the lower-right second premolar, which revealed no significant difference. The mean convergence angle for the buccolingual surface was 12.42°± 6.16°, which was higher than that of the mesiodistal surface (10.16°± 7°). One-way ANOVA showed a significant difference between all selected teeth ($p<0.000$), and a paired samples t-test showed a significant difference within two teeth only, the lower-right second premolar and upper-right canine ($p<0.000$), in which the mesiodistal measurement showed a lower convergence angle than the buccolingual angle. **Conclusions:** Preclinical students prepared teeth with a convergence angle higher than the recommended convergence angle. However, all the recorded angles were within the range of previous studies. It was concluded that the recommended convergence angle was difficult to achieve in preclinical practice.

KEYWORDS: Fixed prosthodontics, full crown preparation, angle of convergence, tooth preparation, preclinical students.

INTRODUCTION:

The elements of a successful full crown preparation are multiple and interacting, but most of them are related to correct tooth preparation. One of these elements that has to be taught to dental students in the preclinical fixed prosthodontics course is the minimum convergence angle [1, 2]. This angle is formed by two opposing axial walls of the prepared tooth for a full crown or fixed

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dental prosthesis[3]. This angle is essential in every external coronal indirect restoration to allow proper seating of the wax pattern and later the final restoration. The smaller the convergence angle is, the fewer the paths for removal and the better the retention and resistance of the restoration. Overtapered preparation may result in unnecessary weakening of the tooth structure, an increase in stresses in the cement interface between the tooth and restoration, retention and resistance reduction, and subsequent restoration failure [2, 4-5].

In textbooks, the recommended convergence angle for all types of indirect restorations ranges between 2.5 and 6.5 degrees [1,2]. Nevertheless, a large number of studies have revealed that this taper criterion is unrealistic. Some studies recommended a more achievable angle of 10° - 20° [6]; however, this angle may be difficult to achieve, especially for preclinical students in their first attempt at indirect restoration preparation[7-11]. Most studies showed that when a student, trained dentist, or even prosthodontist finishes his or her preparation of a full crown, the resulting high convergence angle is far from the ideal range. The recorded angles in former studies vary depending on the tooth anatomy, length, position, accessibility, experience, crown material, and type of indirect restoration [6,8,10-18]. Several studies interrelated the angle with the tooth type and location, which revealed dissimilar results. Some found the convergence angle for anterior teeth to be greater than that for posterior teeth[7,13-20]. Mandibular teeth were prepared with a larger convergence angle than that of maxillary teeth[19-21,23], mandibular molars, higher than any other teeth [20, 21, 24], and maxillary and mandibular molars, more than other teeth[12,16-25], whereas El-Mubarak et al recorded the highest convergence angle for the upper canine [26].

Other studies addressing tooth surfaces showed dissimilar results, and buccolingual surfaces had greater convergence than mesiodistal surfaces [15,16,26]. Virdee et al showed that the mesiodistal convergence angle was greater than the buccolingual one [21]. Abdulla et al found that distal and buccal tapers were larger on posterior teeth than on anterior teeth, whereas lingual tapers were higher on anterior teeth [27].

Recent studies showed an improvement in crown preparation for preclinical students through the development of dental materials [28] and the aid of digital technology by 3D scans together with assessment learning software, which assesses tooth preparation objectively and might reduce the bias of traditional laboratory evaluation methods. Many studies have ascertained many improvements in tooth preparation by which students can overcome their mistakes, including overtapering, before attending clinics [9, 29-31].

This study aimed to first assess and compare the buccolingual and mesiodistal convergence angles of six sets of typodont teeth prepared for porcelain fused to a metal full crown by preclinical dental students at Ajman University, concerning its recommended values (6.5°). Second, we compared the angle recorded for six sets of teeth (# 26, 36, 45, 15, 21, and 13) and related the results according to the tooth position and surface to determine which one shows the greater tendency of straying from the normal convergence angle.

MATERIALS AND METHODS:

After receiving approval from the ethical committee at Ajman University (RD-2017/2018-05-S), one hundred ninety-eight samples were collected from the work of preclinical students. Inclusive criteria were randomly selected typodont teeth of 33 sets of six teeth, # 26, 36, 45, 15, 21, and 13, prepared by 33 male and female students for porcelain fused to a metal full crown. The exclusion criteria were coarse irregularities that may disturb the angle measurement, an unclear finish line and preparation with an undercut at any measured surface.

At the College of Dentistry, Ajman University, preclinical dental students are taught full crown preparation in the fixed prosthodontic lab in the second semester of their third year. Senior prosthodontists conduct live demonstrations under a fixed mounted video camera that transfers an enlarged image to a television monitor before each student, after which they are given a minimum of 90 minutes to finish a plastic typodont (Frasaco GmbH, Postfach 1244 Germany) tooth preparation that is fixed onto an educational dental model Frasaco AG-3, mounted on a mannequin phantom head (Frasaco 7001>ABS <). No special instructions were given to either supervising staff or the students before the initiation of this study that the teeth would be tested. Students used the same type of bur preparation set (11312 DIATECH Inlay and Crown Preparation Kit, Coltène/Whaledent AG), Switzerland). They were trained to prepare all teeth according to the textbook of [1], with a convergence angle maximum of 6.5°.

To measure the convergence angle, a Dino-lite pro digital microscope (AM-413ZT Taiwan) and the Dinocapture (2.0 version 1.5.27.A, AnMo Electronics Corporation) software were used. Each prepared tooth was mounted on a custom jig made from silicon putty (to ensure a reproducible position for each tooth group), and the putty ended away from the cementoenamel junction (to expose any accidentally placed subgingival finish line). The tooth was placed against a black background, with 100× Dino-lite magnification (figure 1).

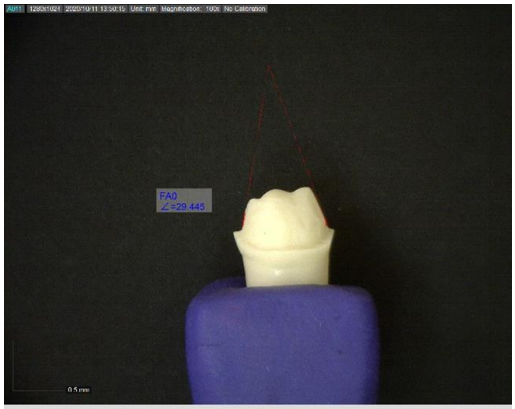

Figure 1: Measuring the angle of convergence by dinolite microscope

The buccolingual convergence angle was measured from the mesial surface, and the mesiodistal convergence angle was measured from the buccal surface by joining the taper of two opposing lines starting from the finish line internal angle extended coronally in a straight line tangentially with the axial wall. The convergence angle was recorded by one operator (prosthodontist), who measured the angles five times at different times, with the average of five readings being considered. One-sample t-test was used to compare the difference in mean convergence angle value for every surface with its recommended value (6.5°), and one-way ANOVA was used to compare the difference in angles for 12 surfaces for the six teeth. The statistical analysis was performed using SPSS-14.0.

RESULTS:

The total sample was composed of 40 sets, six teeth each, with seven sets excluded because of the presence of undercuts in one or more surfaces or teeth in the set, leaving 33 sets and 198 teeth. The average values of the recorded convergence angles are presented in Table 1.

The mean of the total convergence angle for this study was 11.29°± 6.66° from both surfaces, which is greater than the recommended value of 6.5° and statistically significant (p<0.000). The recoded convergence angle showed high diversity, with a minimum of 0.60° and a maximum of 33.96°. The overall mean convergence angle for the mesiodistal surface was 10.16°± 7°, with the highest being that for the upper-left first molar 13.49°± 6.64° and the lowest average convergence angle being that for the lower-right second premolar (8.30°± 5.22°). The mean convergence angle for the buccolingual surface was 12.42°± 6.16°, where the highest reported average was on the upper-right canine (17.12°± 7.83°) and the lowest was exhibited by the lower- left first molar (8.88°± 4.82°). The buccolingual convergence angles in the upper-right canine, upper-left central incisor, upper-left first molar and lower-right second premolar were greater than the mesiodistal convergence angle, while the lower-left first molar and upper-right second premolars showed the opposite trend, with higher mesiodistal convergence angles than the buccolingual ones. Comparing the individual average convergence angle for 12 teeth surfaces with the ideal, a one-sample t-test showed a statistically significant difference (p<0.057) from the recommended convergence angle, except for the mesiodistal convergence angle of the lower-right second premolar, which revealed no significant difference.

One-way ANOVA was used to compare the buccolingual and mesiodistal convergence angles recorded for six sets of teeth (# 26, 36, 45, 15, 21, and 13) and relate the results according to the tooth position and surface, in which there was a significant difference between all selected teeth (p<0.000). Out of the 396 surfaces inspected, only 115 surfaces (29.04%) met the ideal convergence angle, 62 teeth (31.32%) from the buccolingual aspect, 53 (26.77%) teeth from the mesiodistal aspect, and 14 teeth from both surfaces (7.07%). Table 2 shows the number and percentage of specimens falling within the ideal convergence angle.

Table 1 Mean convergence angles

Surface	No.	Mean	Std. Deviation	Std. Error	min	max
Buccolingual #36	33	8.88°	4.82°	0.839	1.33	22.15
Mesiodistal #36	33	9.90°	5.31°	0.92	1.07	20.89
Buccolingual #26	33	14.13°	5.91°	1.03	2.42	25.12
Mesiodistal #26	33	13.49°	6.64°	1.16	3.35	31.53
Buccolingual #21	33	9.58°	6.88°	1.20	0.82	28.36
Mesiodistal #21	33	8.42°	5.03°	0.88	1.04	21.81
Buccolingual #45	33	14.16°	6.60°	1.15	3.43	26.55
Mesiodistal #45	33	8.30°	5.22°	0.91	1.09	22.72
Buccolingual #13	33	17.12°	7.83°	1.36	0.60	33.96
Mesiodistal #13	33	8.61°	5.42°	0.94	0.85	17.42
Buccolingual #15	33	10.64°	5.98°	1.04	2.38	22.24
Mesiodistal #15	33	12.23°	7.23°	1.26	0.72	25.45
Total	396	11.29°	6.66°	0.33	0.60	33.96

Table 2 Number and percentage of convergence angles that met the recommended angle

Tooth number	Buccolingual		Mesiodistal		Both surfaces	
	No	%	No	%	No	%
# 36	13	6.57%	10	5.05%	3	1.52%
# 26	6	3.03%	6	3.03%	1	0.51%
# 21	16	8.08%	11	5.56%	2	1.01%
# 45	6	3.03%	12	6.06%	3	1.52%
# 13	2	1.01%	14	7.41%	2	1.01%
# 15	10	5.05%	9	4.55%	3	1.52%
Total	53	26.77%	62	31.31%	14	7.07%

An evaluation of different convergence angles within the same tooth mesiodistally versus buccolingually analyzed by a paired samples t-test showed a significant difference between two teeth only: the lower-right second premolar ($p<0.000$), in which the mesiodistal measurement (8.30°±5.22°) showed a lower convergence angle than the buccolingual angle (14.16°±6.60°), and the upper-right canine ($p<0.000$), in which the mesiodistal measurement (8.61°±5.42°) showed a lower convergence angle than the buccolingual angle (17.12°±7.83°)

DISCUSSION:

The recommended small convergence angle in textbooks is based mainly on a conservative approach with minimal tooth structure removal together with maximization of the retention and resistance for future restoration, rather than operator capability.

One of the aims of the present study is to evaluate the ability of preclinical students, provided with an ideal environment, to finish crown preparation with an advisable convergence angle. The results showed that the total average convergence angle for 396 surfaces was 11.29°± 6.66°, which is higher and significantly different from the target value of 6.5°. The average convergence angles ranged from 17.12° to 8.30°, which are both greater than the ideal angle; nevertheless, these results are within the clinical range suggested by Goodacre et al between 10° to 20° [6] and close to most previous studies that evaluated students' laboratory preparation. Marghalani reported a mean convergence angle of 11.11° [9], which is slightly less than the one obtained in this study; this difference may exist because he measured the angle for only maxillary first premolars prepared by fifth-year students with better experience than the third-year students involved in this study. Another in vitro study conducted by Rafeek et al achieved a mean convergence angle of 16.8° [7], which is higher than the mean of this study, but they did not report any undercuts, unlike our study, in which we excluded seven sets out of 40 because of the presence of undercutting. Ayad et al compared the convergence angle achieved by preclinical students at three universities [8]; they found a range of 19.8°-14.1°, which is higher than the average of this study, while Yoon et al found results for a set of three typodont teeth, the range of which was 6.1°-21.2° [11].

Other clinical studies conducted by Abdulla et al [27], Virdee et al [22], Annerstedt et al [14] and Aleisa et al [15] obtained total occlusal convergences of 28.6°, 24.2°, 19.4°, and 18.56°, respectively, which are higher than the results of this study. This is mainly because they collected their data from clinical cases for dental students and practitioners, who usually stress reducing undercuts that will interfere with restoration insertion and may lead to walls over tapering.

For this study, only 14 teeth (7.07%) met the recommended convergence angle from both surfaces, 26.77% from the buccolingual surface only and 31.31% from the mesiodistal surface only. This result may be considered a low percentage, though still comparable to those of other studies; the present result is higher than the percentage (0.4%) achieved by students in the Noonan et al study with a similar ideal convergence angle of 6° [17]. Four other previous studies recorded a higher percentage of teeth that met the recommended value, mainly because all these studies considered a higher convergence angle limit. Al-Omari and Al-Wahadni, and Annerstedt et al achieved 9% [7] and 28% [14] per surface, respectively, because they targeted a high ideal convergence angle limit of 14°. Aleisa et al reported that 32.7% of their recorded convergence angles were within the recommended convergence angle, which was set as 12°. However, 26.4% of undergraduate students involved in Virdee'set al study achieved the recommended value of 15° [22].

Other results of this study revealed that the recorded angles demonstrated significant differences between all twelve surfaces of the selected teeth, with large variability among the different tooth types or even two surfaces of the same tooth. The highest average convergence angle measured was for the upper-right canine buccolingually (17.12°), which may be because of its anatomy, especially the palatal surface, together with indirect vision. This result is close to the finding from other studies that documented the highest buccolingual converges for anterior teeth [7, 26]. The lowest average convergence angle for this study was for the lower-right second premolar (8.30°), the only surface that had an insignificant difference with respect to the ideal value; this may be due to its good accessibility, direct vision, and relatively simple tooth anatomy. In a recent systematic review of 12 studies of preparations performed by dental students, the convergence angle for the mesiodistal surface of the lower premolar range was between 13.35° and 38.96° [13], which is higher than the average recorded in the current study.

Otherwise, the wide range of recorded convergence angles for all 12 surfaces included in this study is steady, in agreement with most similar studies that concluded that the tooth type, surface and position influenced the convergence angle [9-12, 14-16, 25, 26]. Analysis of the tooth-by-plane interaction for each tooth showed no significant difference except for two teeth only, i.e., the upper-right canine and lower-right second premolar, with that mesiodistally less than that buccolingually. This may be due to a lack of operator experience with tooth anatomy and instrument accessibility, and the control of the taper for the mesial and distal surfaces may be easier than for buccal and lingual surfaces, by which the operator can place the mirror lingually and view the proximal surface inclination, which may not be applicable for buccal and lingual surfaces.

Although the introduction of resin cement minimized the effect of overtapering on the retention and resistance of indirect restoration [32], the high convergence angle is still against the main principle of tooth preparation for tooth structure preservation. This emphasizes the need for digital self-assessment software, which has been shown to improve students' performances with crown preparation technology by providing immediate feedback and a reliable and precise evaluation of students' work, thus showing much promise for improving crown preparation [27, 29,30] .

CONCLUSIONS:

Within the limitations of this study, the preclinical students at Ajman University were unable to prepare teeth with the ideal convergence angle; however, the results were comparable to those of previous studies and within the acceptable range suggested by Goodacre and comparable or even less than those of most other previous studies. The mean total convergence angle for this study was 11.29°± 6.66°, and the recorded angles ranged between 17.12° and 8.30°; only the mesiodistal surface of the lower-right second premolar showed an insignificant difference. Moreover, 7.07% of all examined teeth met the ideal 6.5° convergence angle.

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DECLARATION OF COMPETING INTEREST:

The authors declared that there is no conflict of interest.

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RESEARCH ARTICLE

Antitubercular study on stem bark of *Albizia procera* (ROXB.) BENTH using Microplate Alamar Blue assay (MABA)

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ABSTRACT:
Albizia procera commonly known as white siris is a large deciduous tree of the family Mimosideae, widely distributed throughout India. Traditionally the plant is used in convulsions, pain, delirium, cancer and septicemia. The decoction of bark is given for rheumatism, hemorrhage and is considered useful in treating pregnancy problems, for stomach ache and sinus.The preliminary phytochemical screening of ethanol extract of *Albizia procera* shows the presence of active constituents like flavonoids, phenolic compounds and tannins. Earlier studies show the flavonoids and phenolic compounds play major role in antitubercular activity. Based on this, we made an attempt to evaluate the antitubercular potential of this plant. The ethanolic extract and its fractions (Hexane and Ethyl acetate) were investigated for antitubercular activity using Alamar Blue dye method (MABA), in which *Mycobacterium tuberculosis* was sensitive upto 3.12µg/ml.The extract and fractions show better anti tubercular activity than streptomycin and potentially equal to pyrazinamide and ciprofloxacin. The observed activity may be due to the presence of flavonoids and phenolic compound which is confirmed by HPTLC. This investigation on stem bark of *Albizia procera* has the potential to be developed further into a natural Anti-TB drug.

KEYWORDS: Flavonoids, antitubercular, *Albizia procera*, pyrazinamide, ethyl acetate.

INTRODUCTION:

Tuberculosis (TB) is a severe infectious socially significant disease that often attacks lungs caused by *Tubercle bacillus* bacteria or *Mycobacterium tuberculosis*[1,2.] However, TB can occur in any parts of the body including lymphatics, brain, spine and kidneys[3]. The dangerous phenomenon associated with TB is the spread of infection through air from person to person[4]. There are actually two types of Tuberculosis: Latent TB disease and Active TB disease. Conventional treatment of TB includes antibiotics, which should be taken for a much longer time than typically required for other bacterial infections. Drug resistant forms of TB have created additional and unacceptable dangers that include global security risks. Unfortunately, over the past few years little progress only has been done in the investigation of new natural products against mycobacterial targets[5,6,7,8].

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Albizia procera is a large deciduous tree with tall cylindrical boles and rather small, elongated crown. Flowers sessile, in numerous small heads. Leaves abruptly 2-pinnate, pinnae 4-12, leaflets 12-24. Pods thin flexible, 10-20 cm long, bright orange-brown. Chemical constituents alpha spina sterol, hentriacontane and hexacosanol have been reported from leaves. Isoflavones biochanin A and formononetin, genistein and daidzein have been isolated from Heartwood and bark. A new pterocarpan – demethylmedicarpin, biochanin A, formononetin, genistein, daidzein and ß-sitosterol have been isolated from bark. Seeds contain procerogenin A, mechaerinic acid, proceric acids, proceranin A, (hypotensive in animals), oleanolic acid and saponin. Root contains a-spinasterol and oleanolic acid[9].

Traditionally the plant is used in convulsions, pain, delirium, cancer and septicemia. The decoction of bark is given for rheumatism, hemorrhage and is considered useful in treating pregnancy problems, for stomach ache and sinus[10]. They were reported to exhibit various pharmacological activities such as CNS activity,

Cardiotonic activity, Lipid lowering activity, Antioxidant activity and Hepatoprotective activity[11,12]. Seeds are powdered and used in amoebiasis. It cures urinary tract infections including glycosuria, hemorrhoids, fistula and worm infestation. It also suppresses skin diseases. Fruits of *Albizia procera* acts as astringent and diminishes Kapha and Sukra[13]. The antitubercular potential of this plant has not been studied and also the preliminary phytochemical screening reveals the presence of flavonoids and phenolic compounds which play a major role in the antitubercular potential reported by earlier studies[14].

Flavonoids harm to the bacteria cells in different ways[15]. Their effects on bacteria might be related to ability against microbial adhesins, cell-wall or transport proteins[16,17]. It is reported that the flavonoids have regulatory effect on drug metabolizing enzymes[18,19,20]. Some flavonoids show antagonistic effect by activating aryl hydrocarbon receptor by increasing CYP1A1 transcription such as diosmetin, quercetin, chrysin and genistein[21,22]. Hence, in the present study, an attempt has been made to evaluate the antitubercular potential of *Albizia procera*.

MATERIALS AND METHODS:

Plant material:

The Bark of *Albizia procera* was procured from Uthukottai, Tiruvallur (Tamil Nadu) in April 2018.The plant material was identified and authenticated by Dr. K.N. Sunil kumar, Research officer and HOD of Pharmacognosy, Department of Siddha Central Research Institute, Arumbakkam, Chennai -600. The Bark was dried under shade, segregated, pulverized by a mechanical grinder and passed through a 40- mesh sieve and stored in airtight container and used for extraction.

Preparation of Extract and Fractions:

Dried powder of Bark was extracted by Soxhlet apparatus, using ethanol as solvent. The extract was concentrated by using rotary evaporator. The extract was further dried under room temperature and kept in desiccator for further drying. The ethanol extract was fractionated by liquid- liquid partition using solvents hexane and ethyl acetate. Both the fraction was evaporated and dried to get the residue.

Antitubercular activity:

The antitubercular activity of compounds were assessed against *M. tuberculosis* using microplate Alamar Blue assay (MABA). This methodology is non-toxic, uses a thermally stable reagent and shows good correlation with proportional and BACTEC radiometric method.Briefly, 200µl of sterile deionized water was added to all outer perimeter wells of sterile96 wells plate to minimize evaporation of medium in the test wells during incubation.The 96 wells plate received 100µl of the Middlebrook 7H9 broth and serial dilution of compounds were made directly onplate. The final drug concentrations tested were 100 to 0.2µg/ml.Plates were covered and sealed with parafilm and incubated at 37ºC for fivedays.After this time, 25µl of freshly prepared 1:1 mixture of Alamar Blue reagent and 10% tween 80 was added to the plate and incubated for 24hrs.A blue color in the well was interpreted as no bacterial growth, and pink color was scored as growth.The MIC was defined as lowest drug concentration which prevented the color change from blue to pink[23,24,25].

Standard Strain used:
Mycobacteria tuberculosis (Vaccine strain, H37 RV strain):
ATCC No - 27294.

Standard values for the Anti-Tb test which was performed.
*Pyrazinamide - 3.125µg/ml
*Ciprofloxacin -3.125µg/ml
*Streptomycin - 6.25µg/ml

High Performance Thin Layer Chromatography (On fractions):
HPTLC studies were carried out by following the method of Harborne and Kpoviessia. Chromatographic Conditions Chromatogram was developed on 5 x 10cm aluminum TLC plate precoated with a 0.2mm layer of silica gel 60F254 (E. Merck Ltd, Darmstadt, Germany) stored in a desiccator. The application was done by Hamilton micro syringe (Switzerland), mounted on a Linomat V applicator. Application of bands of each extract was carried out using spray technique. The sample was applied in duplicate on precoated silica gel 60F254 aluminum sheets (5 x 10cm) with the help of Linomat 5 applicators attached to CAMAG HPTLC system, which was programmed through WIN CATS software (Version 1.3.0) at λmax 254 and 366nm is using Deuterium light source, the slit dimensions were 6.00 X 0.45mm and at λmax 620nm using Tungsten light source.

Developing Solvent System:

The spotting was done on the TLC plate, ascending development of the plate, migration distance80mm (distance to the lower edge was 10mm) was performed at 20°C with Chloroform: Methanol: Glacial acetic acid: water (3:7:0.1:0.1) as a mobile phase in a camag chamber previously saturated with solvent vapour for 30 mins. The concentration of the sample (2.5µL) was applied in the track as 8 mm bands at a spraying rate of 15s/L. After development, the plate was dried at 60°C in an oven for 5 mins. Densitometric scanning was then

performed with a Camag TLC Scanner 3 equipped with the win CATS Software.2.3.3.

Development of Chromatogram:

After the application of the sample, the chromatogram was developed in Twin trough glass chamber 10 x 10 cm saturated with solvent Chloroform: Methanol: Glacial acetic acid: water (3:7:0.1:0.1)

Detection of Spots

The air-dried plates were viewed under ultraviolet radiation in midday light. The chromatograms were scanned by the densitometer at 254 and 366 nm with or without staining with Permanganate, Potassium dichromate, Phosphomolybdic acid, Anisaldehyde-sulfuric acid stains, and Iodine vapor. The Rf values and finger print data were recorded by WIN CATS software. Documentation of chromatograms was carried out with digital camera SNRand Lens. DXA252: 223971607. Computer,12 mm, 14.0[26].

RESULTS AND DISCUSSION:

Table no.1 Results of Antitubercular activity

Sl. No.	Sample	100µg/ml	50µg/ml	25µg/ml	12.5µg/ml	6.25µg/ml	3.12µg/ml	1.6µg/ml	0.8µg/ml
01	Ethanolic	S	S	S	S	S	S	R	R
02	Hexane	S	S	S	S	S	S	R	R
03	Ethyl acetate	S	S	S	S	S	S	R	R

Note: S – Sensitive; R – Resistant

As it was observed from the results, the extract and fractions (hexane and ethyl acetate) show good antitubercular activity, in which *Mycobacterium tuberculosis* was sensitive upto 3.12µg/ml. The extract and fractions show better anti tubercular activity than streptomycin. The activity was equal to pyrazinamide and ciprofloxacin.

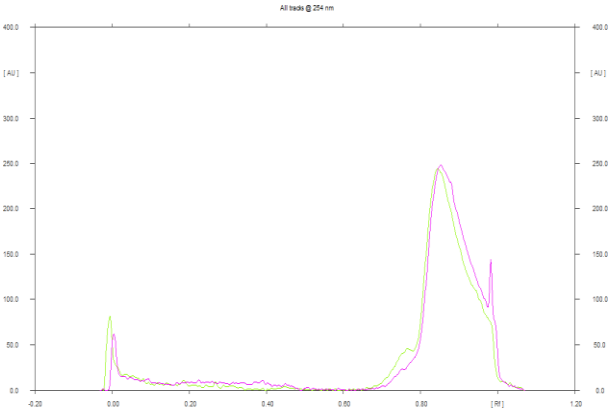

Figure 1: Ethyl acetate fraction of bark scanning at 254 nm

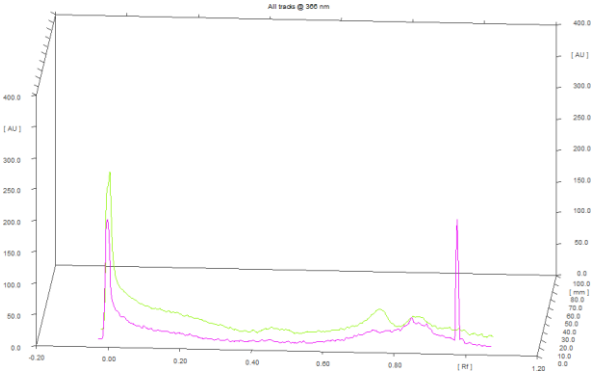

Figure 2: Ethyl acetate fraction of bark scanning at 366 nm

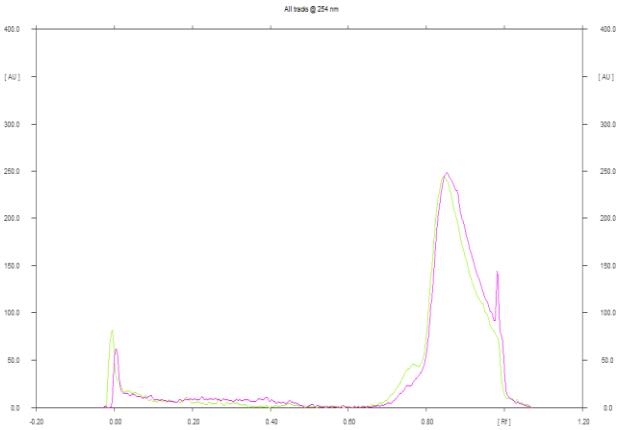

Figure3: Hexane fraction of bark scanning at 254 nm

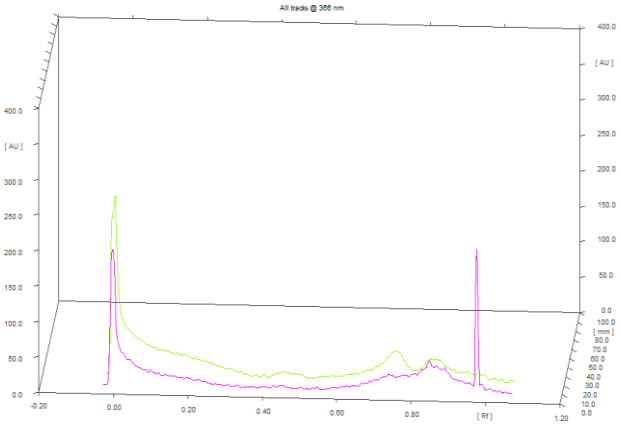

Figure 4: Hexane fraction of bark scanning at 366 nm

From the results obtained in the preliminary phytochemical screening and the antitubercular activity, the hexane and ethyl acetate fractions were carried out for HPTLC finger print analysis (Fig 1-4). It was observed that, both fractions at the solvent system

Chloroform: Methanol: Glacial acetic acid: water (3:7:0.1:0.1) shows the dark color spot at Rf value 0.78 at shorter wavelength 254nm and at longer wavelength at 366nm the blue florescent spot appears at Rf value 0.87. The dark color spot in 254 nm shows the presence of violet color when the plate is dipped in anisaldehyde in sulfuric acid, which indicates the presence of flavonoids. Hence the observed antitubercular activity may be due to the presence of the flavonoids.

CONCLUSION:

The observed antitubercular activity of the extract and fractions of bark of *Albizia procera* may be due to flavonoids which is confirmed by HPTLC using detecting agents. For a better understanding of synergistic behavior and the mechanisms of action of flavonoids drug combinations against TB, there is need to isolate and characterize new flavonoids from plants and to investigate their mode of action against microorganisms.

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RESEARCH ARTICLE

Evaluation of the Vasoprotective Effects of Metformin versus Glibenclamide in Type 2 Diabetic Patients

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ABSTRACT:
Adiponectin (APN) is an adipokine with anti-inflammatory and anti-atherogenic properties decreased in type 2 diabetes mellitus (T2DM) that may influence endothelial function by regulating serum nitric oxide (NO) levels. The current study aimed to investigate the effect of two oral hypoglycemic drugs, Metformin and Glibenclamide (GLC), on circulating APN and NO levels and to find a correlation between APN and NO levels in type 2 diabetic patients. Fifty males and females previously diagnosed with T2DM were conducted in this trial and classified into groups: Group A involved 18 untreated patients with T2DM, group B involved 16 patients receiving Metformin monotherapy (1000 mg/day) for up to 1 year and group C involved 16 patients receiving GLC (5 mg/day) for up to 1 year. Circulating APN and NO were measured. Compared to GLC, Metformin therapy showed a significant increase in APN and NO levels in type 2 diabetic patients. Our findings established that Metformin has a protective effect on endothelial function, including increased APN and NO bioavailability, beyond its glucose-lowering effect.

KEYWORDS: Adiponectin, Diabetes, Glibenclamide, Metformin, Nitric oxide.

INTRODUCTION:
Approximately one in 11 adults worldwide are living with diabetes, 90%-95% of whom have type 2 diabetes mellitus[1]. Asia is a main region of the global epidemic of diabetes[2]. Type 2 diabetic patients are at high risk for cardiovascular events[3]. Metformin is still the best recommended oral hypoglycemic agent in treating type 2 diabetes[4]. The European Association for the Study of Diabetes (EASD) and American Diabetes Association (ADA) recommended Metformin use regardless of patient's body mass index, age and blood glucose level, because of its desirable control of lipids, blood glucose and weight[5]. On the other hand, GLC is an effective oral hypoglycemic agent that is commonly used in type 2 diabetes for years[6].

According to the United Kingdom Prospective Diabetes Study , treatment with Metformin showed a reduction in myocardial infarction in obese diabetic patients[7]. A persisted benefit with Metformin use was observed after a follow-up study of 10 years[8]. In addition, Metformin use revealed a reduction in mortality in diabetic patients with atherosclerosis in an observational study[9]. Endothelial dysfunction is a main feature of diabetes which is associated with insulin resistance and increased blood glucose [10]. A critical mediator to sustain proper endothelium function is NO and its bioavailability [11]. NO, well-known potent vasodilator, is generated by endothelial nitric oxide synthase (eNOS) in the vascular endothelium via oxidation of substrate L-arginine to L-citrulline[12]. NO plays a critical role in maintaining vascular function and blood pressure regulation in healthy persons, whereas a reduction in NO bioavailability previously reported in patients with type 2 diabetes[13].

In addition to its direct reduction in glucose availability in diabetic patients, Metformin can improve endothelial dysfunction by glucose-independent mechanism in prediabetic patients. Although the underlying

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mechanism behind the beneficial effect of Metformin on endothelial function remains undefined, few data exist. Several studies have suggested the potential role for Metformin in cardiovascular protection by increasing AMP-activated protein kinase (AMPK), resulting in activation of eNOS[16-18].

Even though the promising results by UKPDS[19]. There was a theoretical adverse cardiovascular effect of GLC[20]. A prospective study for diabetic patients with atherosclerotic heart disease, impairment of left ventricular function was higher in patients received GLC than in those received insulin only[21]. Two studies revealed higher mortality rate in diabetic patients undergoing angioplasty for myocardial infarction on GLC[22,23]. However, theoretically GLC has lower cardiovascular risk than the old-generation sulphonylurea[24]. These variations suggest the requirement for additional demonstration of the cardiovascular safety of hypoglycemic agents.

Among the adipokines, APN hormone showing reduced circulating levels in obesity, type 2 diabetes and myocardial infarction[25]. Moreover, APN demonstrated as a direct endogenous inhibitor for vascular inflammatory response and tumor angiogenesis[26]. In addition, the correlation between APN and vascular function is complex, and studies have showed discrepant results with respect to the effect of Metformin and GLC on APN levels.
In this study, we compared between Metformin versus GLC effect on APN and NO in patients with T2DM.

MATERIALS AND METHODS:

This comparative case-control study comprised of 50 patients with T2DM aged between 32 and 58 years (both sexes), were classified into three groups; Group A: 17 type 2 patients (newly diagnosed as controls), Group B: 15 Metformin-treated patients (1000 mg/day) and Group C: 14 GLC-treated patients (5 mg/day) for a period of less than one year, achieved in Al Waffaa Centre for Diabetes/Mosul from May to November 2019. It was approved by the Ethical Committee of Nineveh health institution. Patients were diagnosed as type 2 diabetes based on ADA and WHO criteria. This present study exclude pregnant and breastfeeding women, patients taking other medications, dietary supplements-taking patients and patients have acute or chronic health conditions other than T2DM, patients undergoing medication changes over the study year, smokers and alcoholic patients. In all patients, body mass index (BMI) was calculated based on anthropometric data (height and weight).

After at least 10 hrs overnight fasting, blood samples were taken from patients with T2DM and incubated in water bath for 10 minutes at 37°C, then centrifuged for 10 mins at 4,000x g. Except for fasting serum glucose (FSG) which was measured instantly, samples were stored at -20°C for later use.

FSG was determined via enzymatic colorimetric method, using a kit supplied from BIOLABO kit (France). Enzyme linked immunosorbent assay machine was used to determine serum insulin, using a kit supplied from Monobind kit (USA). Fasting serum insulin and glucose were used to calculate insulin resistance through the equation:
HOMA-IR = Serum Insulin × Serum Glucose / 22.5.

APN hormone was determined by ELISA technique, using a kit supplied by USBIOLOGICAL (USA) . Serum NO was assessed by Greiss reagent . Briefly, 200 µl of supernatant and Griess reagent were added, then absorbance was measured at 540 nm by ELISA. NO concentration was estimated according to the standard curve of sodium nitrite.

Statistical analysis:
Statistical analyses were performed using GraphPad Prism. Two or multiple comparisons were used by performing Mann Whitney test and Kruskal-Wallis test, then by a Dunn's multiple comparisons test. All quantitative *results* were expressed as the mean value ± SD and statistical significance was set at $p < 0.05$.

RESULTS:

Demographic profile of untreated and treated patients with T2DM:
The current study included 50 patients with T2DM, with percentage of male and female (46% and 54%) in the ages between 35 and 54 years. Table 1 shows the demographic profile of patients with T2DM in our study. No significant differences have been found among three groups.

Table 1: The demographic profile of patients with T2DM

Parameter (unit)	Untreated	Metformin	GLC
Age (years)	45.51±5.723	47.11±6.134	42.68±6.019
BMI (kg/m^2)	25.11±0.976	24.44±1.078	24.43±0.199
Duration of treatment (month)	-	8.09±2.901	8.111±3.132

Effect on glucose, serum insulin and HOMA-IR:
Compared to untreated and GLC-treated diabetic patients, Metformin-receiving patients revealed a significantly reduced HOMA-IR index and FSG level. Nevertheless, insulin was significantly higher in GLC-receiving group in comparison group A and B as shown in Table 2

Table 2: Diabetic profile of the study groups of patients with T2DM

Parameter (unit)	Group A	Group B	Group C
FSG (mmol/l)	11.99± 0.971	9.079± 0.5171 a****b**	11.01± 0.8123
Insulin (µu/L)	9.039±0.7301	8.971±0.5029	8.989± 0.4939 a*
HOMA-IR	5.041±0.5051	3.581± 0.1923 a****b****	4.429± 0.3161

Results are set as mean value ± SD. [a] represents a differences between group B and C in contrast to group A; [b] represents a differences between group B and C.

Effect on plasma adiponectin and nitric oxide levels:

Untreated patients had a mean APN level of 5.976 µg/mL and mean NO of 8.506 µmol/L. The plasma APN levels were significantly higher in Metformin-treated patients (7.953 µg/mL) compared to newly diagnosed diabetic patients (5.976 µg/mL) and GLC-treated patients (5.564 µg/mL) (Figure 1A). Moreover, Metformin-receiving patients showed significantly higher concentration of plasma NO levels (10.44 µmol/L) compared to newly diagnosed (8.506 µmol/L) and GLC-treated patients (9.393 µmol/L) (Figure 1B). No significant correlations were noted between ADP and NO in all groups.

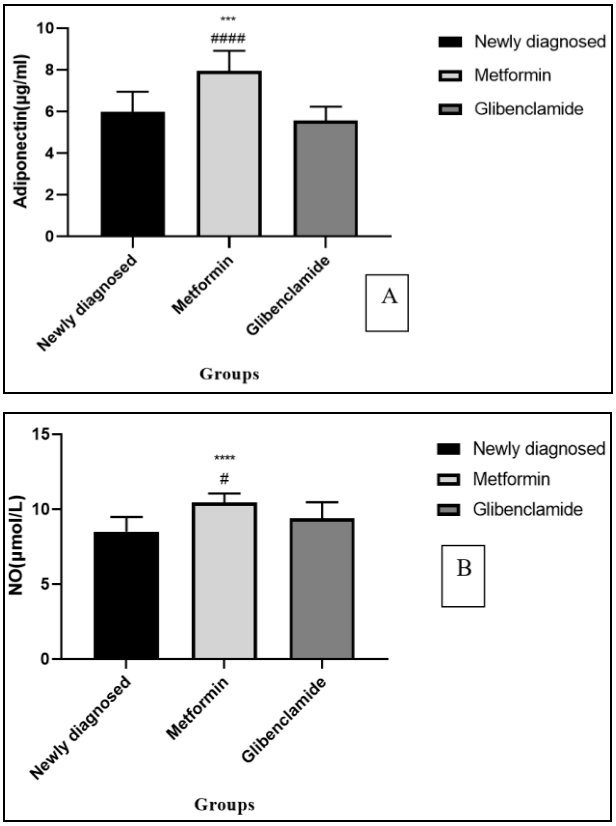

Figure 1: Effects of Metformin and GLC on serum (A) APN levels (B) NO levels. Values are represent as mean ± SD. Variations are statistically significant with (*p < 0.001; ****p < 0.0001) in comparison of group B and C in contrast to group A; whereas, # sets statistically significant differences in comparison between group B and C (#p < 0.05; ####p < 0.0001).**

DISCUSSION:

The comparative effect of Metformin versus GLC on plasma APN and NO levels has not yet been evaluated in type 2 diabetic patients. In the current study, we demonstrated that Metformin improved the cardiovascular biomarkers more significantly than GLC and this effect was independent of changes in insulin level. Although GLC has been significantly increased insulin production, APN and NO levels has been increased with Metformin-treated patients.

In recent years, APN has been the focus of intense research. Increased circulating levels of APN have been previously established in Metformin-treated patients . The main findings of our study confirmed that APN was significantly higher in Metformin-receiving patients relative to that of GLC group is in line with results of Zulian et al. and Emini-Sadiku et al. . However, findings of other studies in type 2 diabetic patients treated with Metformin revealed no effect on serum APN levels [32,33].

APN protein has been shown to possess anti-diabetic, anti-inflammatory and anti-atherogenic properties . However, it is still uncertain whether APN has a vasoprotective effect under pro-inflammatory conditions like untreated diabetes. *In vivo* and *in vitro* studies have supported this idea, which reported an inverse association between APN and insulin resistance[35,36]. In our study, Metformin-treated patients had this negative correlation which suggested APN as a possible protective protein [37].

Moreover, the beneficial effect of Metformin on circulating levels of NO have been demonstrated in Metformin-treated patients . In the current study, patients receiving Metformin showed significantly improved NO levels compared with those receiving GLC is supported with the findings of Liu et al. and Jojima et al.[38,40]. In another study conducted by Kato et al, the in vitro inhibitory action of Metformin on lipopolysaccharide-dependent NO production has been established in macrophage cell line . Nevertheless, a recent study suggested that Metformin has a beneficial direct effect on endothelial function by increasing NO production and decreasing nitroxidative stress in rats, independent on its hypoglycemic effect . These variations indicating the diversity of NO pathways that should be taken into account when linking with endothelial function.

CONCLUSIONS:

In addition to its well demonstrated glucose lowering properties, Metformin treatment provides new insight for treatment of underlying vascular defect associated with diabetes mellitus. In future studies, it will be important to achieve a direct measurement for in vivo

endothelial function and to determine whether and how agents targeting overall parameters to preserve endothelium.

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CONFLICT OF INTEREST:

The authors declare no conflict of interest.

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RESEARCH ARTICLE

Evaluation of Sertraline as an antioxidant, anti-inflammatory and anticataract

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ABSTRACT:

Sertraline can also protect against environmental causes of free radicals such as smoking. Cigarette tar is a source of free radicals which has been found to damage erythrocyte membranes. It was also found that Sertraline and its conjugate metabolites could protect erythrocytes from the membranous damage that is caused by smoking. The ability of Sertraline is claimed to exert many beneficial effects on health, including protection against various diseases such as osteoporosis, lung cancer, and cardiovascular disease. The studies showed that there has been a reduction in the risk of cardiovascular disease in subjects, who had a high intake of flavonoids. Progressive disorder of the lung parenchyma and airways or also known as chronic obstructive pulmonary disease (COPD) which happens to be the third-leading cause of death in the USA. Therapies thus far for COPD, unfortunately, is said to be partially effective with possibilities of side effects.

KEYWORDS: Anti-cataract, Anti-oxidant, Anti-inflammatory and sertraline.

INTRODUCTION:

Cataract is a clouding of the lens in the eye which leads to decrease in vision. Cataracts often develop slowly; symptoms may include blurry vision and trouble seeing at night[1,2,3]. Cataracts are most commonly due to aging but May also occur due to trauma or radiation exposure, be present from birth, or occur following eye surgery for other problems. Prevention includes wearing sunglasses and not smoking. Early on the symptoms may be improved with glasses. If this does not help, surgery to remove the cloudy lens and replace it with an artificial lens is the only effective treatment. Surgery is needed only if the cataracts are causing problems and generally results in an improved quality of life. Cataract surgery is not readily available in many countries, which is especially true for women, those living in rural areas, and those who do not know how to read[4,5].

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About 20 million people are blind due to cataracts. It is the cause of approximately 5% of blindness in the United States and nearly 60% of blindness in parts of Africa and South America. Blindness from cataracts occurs in about 10 to 40 per 100,000 children in the developing world, and 1 to 4 per 100,000 children in the developed world. Cataracts become more common with age. More than half the people in the United States had cataracts by the age of 80. Risk factors include diabetes, smoking tobacco, prolonged exposure to sunlight, and alcohol[6,7]. The underlying mechanism involves accumulation of clumps of protein or yellow-brown pigment in the lens that reduces transmission of light to the retina at the back of the eye. Diagnosis is by an eye examination [8].

MATERIALS AND METHODS:

***in-vitro* Antioxidant activity:**

DPPH radical scavenging activity

Procedure:

The stable 1, 1-diphenyl-2-picrylhydrazyl radical (DPPH) was used for determination of free radical

scavenging activity of the extract. The reaction mixture contained ml of different concentration of ascorbic acid and 5 ml of 0.04%(w/v) solution of DPPH I n 80% methanol. After 30 min at room temperature, the absorbance was recorded at 517 nm using spectrophotometer (HITACHI U-1900 spectrophotometer 200V). The commercial known antioxidant, ascorbic acid was used as a positive control. The experiment was performed in triplicate[9]. The percentage of the DPPH free radical was calculated using the following equation:

DPPH scavenging effect (%) = [(A0-A1)]×100

Scavenging of Hydrogen Peroxide:
Procedure:
The scavenging of hydrogen peroxide. A solution of hydrogen peroxide (20 mM) was prepared in phosphate buffer saline (pH 7.4), different concentration of plant extract and standard ascorbic acid solution viz. 10,20,40.60,80 and 100 mg/ml in methanol (1ml) were added to hydrogen peroxide solution (2ml). Absorbance of hydrogen peroxide at 230 nm was determined after 10 min against a blank solution containing phosphate buffer without hydrogen peroxide[10]. The percentage inhibition activity was calculated using the following equation:

Scavenging activity (%) =[(A0-A1)/A0]×100
Where A0 is the absorbance of the control and A1 is the absorbance of extract/standard.

***In-vitro* Anti Cataract Activity:**
Procedure:
Lens culture:
A fresh goat lens were obtained from the slaughter house and immediately transported to the laboratory at 0-4˚C. The lens were removed by extra capsular extraction and incubated in artificial aqueous humor (NaCl 140mM, KCl mM, $MgCl_2$ 2mM, $NaHCO_3$ 0.5mM, $NaHPO_4$ 0.5mM , $CaCl_2$ 0.4mM and glucose 5.5mM) at room temperature and maintain pH 7.8 by addition of $NaHCO_3$). Penicillin G 32% and streptomycin 250 mg% added to the culture media to prevent bacterial contamination. At high concentration glucose in the lens was metabolized through sorbitol pathway and accumulation of polyols causing over hydration and oxidative stress. This leads to carctogenesis[11].

Induction of in vitro cataract:
Glucose at a concentration of 55mM was used to induce cataracts. At high concentrations, glucose in the lens metabolizes through the sorbitol pathway. Accumulation of polyols (sugar alcohols) causes over hydration and oxidative stress. This generates cataractogenesis. These lens were incubated in artificial aqueous humor with different concentration of glucose (5.5 mM) served as normal control and 55mM served as toxic control) for 72 hours[12].

Photographic Evaluation:
Lenses are placed on a wire mesh with the posterior surface touching the mesh, the pattern of mesh number of squares clearly visible through the lens was observed to measure lens opacity[13].

Anti-inflammatory models used:
Rate paw edema Model:
Procedure:
1. Standard drugs and test compounds were dissolved in minimum amount of dimethyl sulfoxide (DMSO) and diluted with phosphate buffer (0.2M, pH 7.4).
2. Final Concentration of DMSO in all solution was less than 2.5%.
3. Test solution (1ml) containing different concentrations of drug was mixed with 1ml of 1%mM egg albumin solution in phosphate buffer and incubated at 27º C± 1ºC in BOD incubator for 15min.
4. Denaturation was induced by keeping the reaction mixture at 60º C± 10 ºC in water bath for 10min.
5. After cooling the absorbance of turbidity was measure at 660nm on UV-visible spectrophotometer.
6. Percentage of inhibition of denaturation was calculated from control where no drug was added. Each experiment was done in triplicate and average was taken. The Sertraline was used as standard drug[14].

% Inhibition of denaturation = 100 *(1- A2/A1)
Where,
A1= Absorption of control sample
A2 = Absorption of test sample

RESULT AND DISCUSSION:

Antioxidant activity:
In vitro antioxidant activity by DPPH radical scavenging activity and scavenging of hydrogen peroxide and reducing power assay, Sertraline as lasted at various concentration and the IC_{50} values had been determined for each compound and compared with standard antioxidant. Ascorbic acid (AA) was used as the standard antioxidant[15].

DPPH (2, 2-diphenyl -1- picrylhydrazyl) radical scavenging activity:

Table 1: Antioxidant activity by DPPH radical scavenging activity. Values in parenthesis are expressed as mean +- S.D (n=3).

	% Free radical scavenging effect	
Conc.(mg/ml)	STD (AA)	L-Carnitine
10	27.13+ 1.46	49.69+ 1.54
20	29.15+ 1.67	52.6+1.27
30	34.1+1.72	54.25+0.64
40	39.89+2.58	58.66+2.41
50	45.8+0.43	73.88+0.93
IC50	62.5	59.5

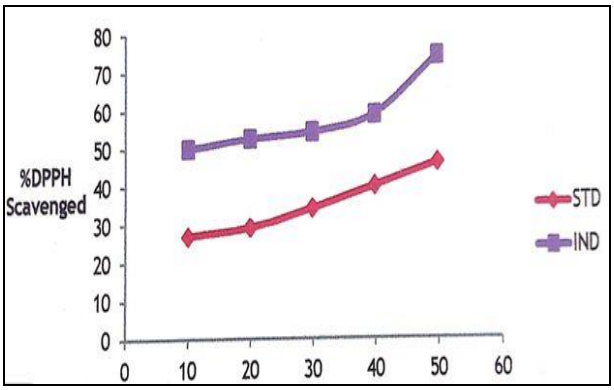


Figure 1: Scavenging effect of test compound on DPPH radicals compared with Ascorbic acid.

Scavenging of hydrogen peroxide[16]

Table 2: Anti-oxidant activity by Hydrogen peroxide radical scavenging Activity. Values in parenthesis are expressed as mean +S.D (n=3).

Conc.(g/ml)	% Free radical scavenging effect	
	Standard (Ascorbic acid)	Sertraline
10	3.36+1.61	9.84+1.12
20	3.77+1.27	7.98+1.26
40	20.6+1.17	27.21+1.28
60	31.97+1.23	32.51+1.46
80	34.57+1.92	37.51+1.67
100	38.44+0.70	46.58+1.41
IC_{50}	110	103

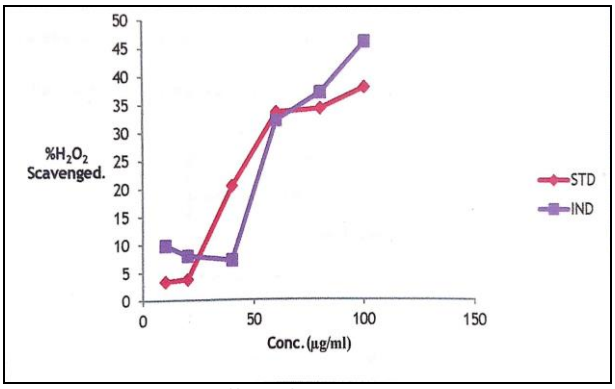
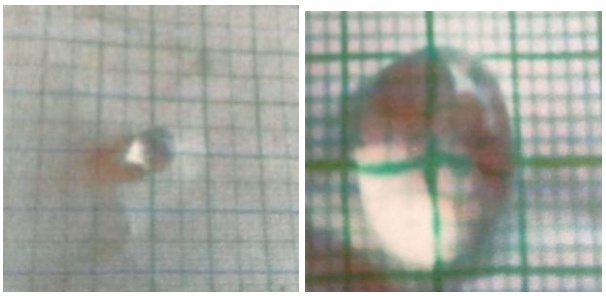
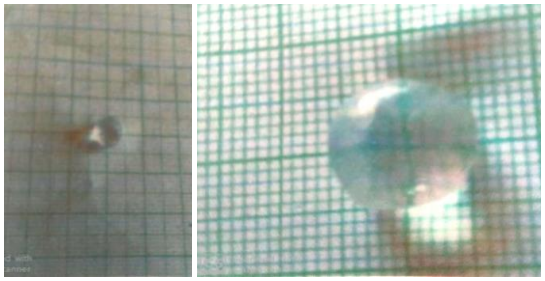


Figure 2: Scavenging effect of the test compound on hydrogen peroxide radical compared with Ascorbic acid.

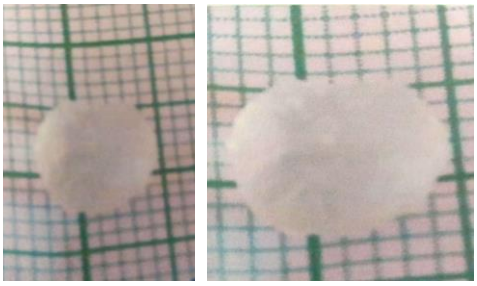
Photographic evaluation [17]



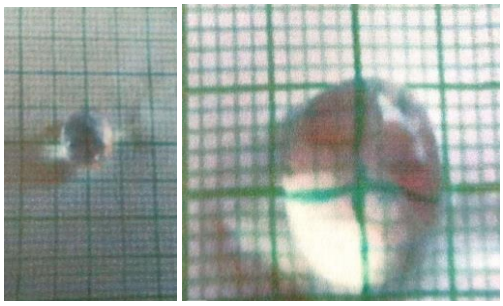
Normal view **Zoom view**
Aqueous humor only (Normal control)



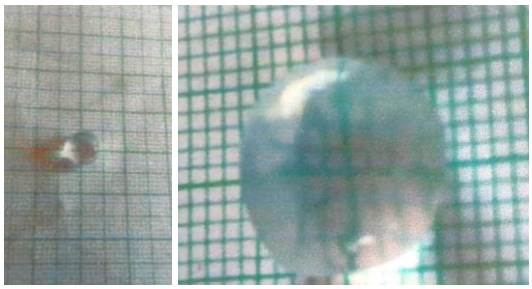
Normal view **Zoom view**
Aqueous humor + 5.5 mm glucose (Negative control)



Normal view **Zoom view**
Aqueous humor + 55mm glucose (Negative control)



Normal view **Zoom view**
Aqueous humor + 55mm glucose + 20 µg/ml Test compound



Normal view **Zoom view**
Aqueous humor + 55mM glucose + 20 µg /ml standard compounds (Positive control).

Figure 3: Photographic evaluation of anticataract activity

Estimation of total protein (TP)

Table 3: Total Protein Content

Group	Dose	Total Protein Content
Normal control	------	103.6±8.89
Glucose control	55 mM	36.64±1.26
Std	20 µg/ml	86.22±3.12
Test 1	10 µg/ml	64.24±3.62
Test 2	20 µg/ml	82.22±3.72
Test 3	30 µg/ml	83.23±3.10

Anti-inflammatory activity[18]

Table 4: Percentage Inhibition of Protein (%), IC50 ± SEM

Conc. of Drug[ppm]	5	10	15	20	25
Standard Drug (Baicalein)	129 ± 0.24	140 ± 0.16	157 ± 0.45	194 ± 0.26	198 ± 0.14
Test Drug (Sertraline)	110 ± 0.45	136 ± 0.63	151 ± 0.34	190 ± 0.18	191 0.16

CONCLUSION:

Sertraline is a flavonoid with antioxidant properties. The ability of Sertraline is claimed to exert many beneficial effects on health, including protection against various diseases such as osteoporosis, lung cancer, and cardiovascular disease. The studies showed that there has been a reduction in the risk of cardiovascular disease in subjects, who had a high intake of flavonoids. Flavonols is the most prominent flavonoids in fruits and vegetables and of these, Sertraline is the most commonly consumed in the human diet. Although a wide number of drugs are available today for effective treatment of diabetes associated dyslipidemia, statins are gold standard drugs and the growing evidences of their pleiotropic effects establish their supremacy over other available lipid lowering agents, as they are most effective, best tolerated and can provide additional benefits like the antioxidant effect in diabetic cataract as evidenced in the present in vitro study. Even though, in contrast to other workers' results, we did not find any activity of HMG-CoA-reductase in our experiments with goat lenses and also their incubation in a normal lens does not seem to be associated with an increased risk of cataract, the preventive role of statins in cataract was proved. With the increasing trend for initiating statin therapy among diabetics with or without hyperlipidemia, the need to assess the effect of long-term and high dose exposure on eye in various other animal models as well as clinical trials including post marketing surveillances remains.

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CONFLICT OF INTEREST:

The authors declare no conflict of interest.

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RESEARCH ARTICLE

Synthesis and pesticidal activity of some 4-Azolylmethyl-1,3-dioxolanes, based on cyclic ketones

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ABSTRACT:
In vitro tests of substituted 1,3-dioxolanes, based on cyclic ketones and substituted with 1H-1,2,4-triazole and 1*H*-imidazole showed a fungicidal activity lower than the activity of the triadimefon, and spiroxamine. Studied compounds in the entire concentration range: from 0.001 to 10 mg/L showed noticeable retardant properties. The target compounds were derived by cyclization of substituted cyclohexanones with epichlorohydrin followed by alkylation of the derived 2-chloromethyl-1,3-dioxolanes of sodium salts of imidazole or 1,2,4-triazole.

KEYWORDS: Alkylation, 1,3-dioxolane, growth-regulating activity, imidazole, fungicidal activity, ketalization, ketals, 1,2,4-triazole.

INTRODUCTION:
Among numerous azole fungicides, substituted 2-azolylmethyl-1,3-dioxolanes: propiconazole and diphenoconazole occupy the most important place on the market.

In the mid-1990s, the full range of plant protection chemicals was supplemented by alkylaminomethyl-substituted 1,4-dioxaspiro[4.5]decane — *spiroxamine*, which, in terms of the mechanism of action, is also an inhibitor of steroid biosynthesis.

The combination of two fragments in a single molecule: toxophore azole, as well as relatively sensitive to hydrolysis 1,3-dioxolane, while preserving the fungicidal properties, reduces the hydrolytic stability and, therefore, accelerates the degradation of the drug in the environment. The use of the analog method allows to develop and synthesize 4-azolylmethyl-1,3-dioxolanes with high fungicidal activity and reduced persistence.

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It should be noted the earlier synthesized similar analogs substituted 4-azolylmethyl-1,3-dioxolanes, based on cyclic ketones: 1-[(1,3-dioxolan-4-yl)methyl]-1*H*-1,2,4-triazoles and 1-[(1,3-dioxolan-4-yl)methyl]-1*H*-imidazoles demonstrated a wide range of biological activities: mainly fungicidal[1-6] and antimycotic[7,8], as well as antimycobacterial[9], antioxidant, antiradical[10-15], antibacterial[16], cytotoxic[17], antimicrobial[18-26], antidiabetic[27] and other wide range of biological activity[28-33].

A search for new biologically active compounds was carried out among analogues of spiroxamine – 1,3-dioxolanes, based on cyclic ketones and substituted with 1H-1,2,4-triazole and 1*H*-imidazole, in which the ethylpropylamine group was replaced with 1,2,4-triazole or imidazole fragments, and alkyl substituents in cyclohexane ring were subjected to variation.

MATERIALS AND METHODS:
^{1}H NMR spectra were recorded on Bruker AM-300 instrument (300.13 MHz). IR spectra were recorded on a Specord M-80 instrument (Nujol). The course of reaction was monitored and the purity of the compounds was checked by TLC (Silufol UV-254).

Substituted 2-chloromethyl-1,4-dioxaspiro[4.5] decanes. (General procedure). Boron trifluoride etherate (0,002 mol) was added to a stirred solution of substituted cyclohexanone **1-4** (0,1 mol), in CCl_4 (250 ml). The mixture was stirred at room temperature for 20 min. Then epichlorohydrin (0,08 mol) was added at 25-30 ºC and stirring was continued at room temperature for 2 h. The reaction mixture was washed with 3% NaOH (100 ml) and water (100 ml) and dried over anhydrous $MgSO_4$. The solvent was removed and the residue was fractioned *in vacuo*.

2-Chloromethyl-1,4-dioxaspiro[4.5]decane (1a). Yield 67%, n_D^{20} 1.4755. NMR^{1}H ($CDCl_3$, δ, ppm, *J*/Hz): 1.41-1.59 (m, 10H, cycl.); 3.48 (d.d, 1H, CH_2Cl, ^3J = 8.0, ^2J = 8.4); 3.60 (d.d, 1H, CH_2Cl, ^3J = 6.6, ^2J = 8.4); 3.87 (d.d, 1H, CH_2O, ^3J = 5.0, ^2J = 8.0); 4.15 (d.d, 1H, CH_2O, ^3J = 6.4, ^2J = 8.0); 4.34 (q, 1H, CHO, ^3J = 5.9). IR (Nujol, ν/sm^{-1}): 1245, 1225, 1170, 1115, 1085 (COCOC); 768 (CCl).

2-Chloromethyl-8-methyl-1,4-dioxaspiro[4.5]decane (2a). Yield 45%, n_D^{20} 1.4685. NMR^{1}H ($CDCl_3$, δ, ppm, *J*/Hz): 0.92 (d, 3H, CH_3, ^3J = 6.2); 1.09-1.32 (m, 2H, cycl.); 1.32-1.51 (m, 2H, cycl.); 1.55-1.78 (m, 5H, cycl.); 3.46 (d.d, 1H, CH_2Cl, ^3J = 7.7, ^2J = 8.4); 3.59 (d.d, 1H, CH_2Cl, ^3J = 5.2, ^2J = 8.4); 3.89 (d.d, 1H, CH_2O, ^3J = 4.8, ^2J = 8.2); 4.11 (d.d, 1H, CH_2O, ^3J = 6.2, ^2J = 8.2); 4.31 (q, 1H, CHO, ^3J = 5.8).. IR (Nujol, ν/sm^{-1}): 1242, 1220, 1172, 1115, 1085 (COCOC): 772 (CCl).

8-*Tert*-butyl-2-chloromethyl-1,4-dioxaspiro[4.5]decane (3a). Yield 88%, n_D^{20} 1.4763. NMR^{1}H ($CDCl_3$, δ, ppm, *J*/Hz): 0.85 (s, 9H, $C(CH_3)_3$), 1.02 (t, 1H, CH, ^3J = 11.9); 1.38-1.89 (m, 8H, cycl.); 3.47 (d.d, 1H, CH_2Cl, ^3J = 7.4, ^2J = 8.1); 3.58 (d.d, 1H, CH_2Cl, ^3J = 6.5, ^2J = 8.1); 3.80 (d.d, 1H, CH_2O, ^3J = 6.9, ^2J = 8.8); 4.10 (d.d, 2H, CH_2O, ^3J = 7.9, ^2J = 8.8), 4.45 (q, 1H, CHO, ^3J = 5.9). IR (Nujol, ν/sm^{-1}): 1245, 1225, 1170, 1118, 1088 (COCOC); 778 (CCl).

2-Chloromethyl-7,7,9-trimethyl-1,4-dioxaspiro[4.5]decane (4a). Yield 85%, n_D^{20} 1.4671. NMR^{1}H ($CDCl_3$, δ, ppm, *J*/Hz): 0.90 (s, 6H, $(CH_3)_2$); 1.01 (s, 3H, CH_3); 1.06-1.14 (m, 1H, cycl.); 1.15-1.30 (m, 2H, cycl.); 1.32-1.50 (m, 2H, cycl.); 1.32-1.50 (m, 2H, cycl.); 3.46 (d.d, 1H, CH_2Cl, ^3J = 7.3, ^2J = 8.2); 3.57 (d.d, 1H, CH_2Cl, ^3J = 5.1, ^2J = 8.0); 3.89 (d.d, 1H, CH_2O, ^3J = 4.6, ^2J = 8.3); 4.08 (d.d, 1H, CH_2O, ^3J = 6.4, ^2J = 8.3); 4.26 (q, 1H, CHO, ^3J =5.9). IR (Nujol, ν/sm^{-1}): 1245, 1220, 1175, 1115, 1085 (COCOC); 774 (CCl)

Substituted 1-(1,4-Dioxaspiro[4.5]dec-2-ylmethyl)-1*H*-1,2,4-triazoles and 1-(1,4-Dioxaspiro[4.5]dec-2-ylmethyl)-1*H*-imidazoles (general procedure).
A mixture of 0,03 mol a substituted 2-chloromethyl-1,4-dioxaspiro[4.5]decane (1a-4a) and 0,03 mol a sodium salt of 1,2,4-triazole or imidazole [34] in was refluxed in 50 ml DMF for 16 h, filtered and evaporated. The residue was chromatographed on silica gel by gradient eluation in acetone-hexane with a concentration gradient of acetone from 10% to 40%.

1-(1,4-Dioxaspiro[4.5]dec-2-ylmethyl)-1*H*-1,2,4-triazole (1b). Yield 12%, n_D^{20} 1.4874. NMR^{1}H ($CDCl_3$, δ, ppm, *J*/Hz): 1.32-1.44 (m, 2H, CH_2 cycl.); 3.77 (d.d, 1H, CH_2O, ^3J = 8.0, ^2J = 8.9); 4.11 (d.d, 1H, CH_2O, ^3J = 8.0, ^2J = 8.9); 4.32 (d, 2H, CH_2N, ^3J = 6.2); 4.46 (q, 1H, CHO, ^3J = 5.8); 7.94 (c, 1 H, C^3H triaz.); 8.19 (s, 1H C^5H triaz.). IR (Nujol, ν/sm^{-1}): 1270 (β CH triaz.); 1248, 1228, 1172, 1120, 1072 (COCOC).

1-[(8-Methyl-1,4-dioxaspiro[4.5]dec-2-yl)methyl]-1*H*-1,2,4-triazole (2b). Yield 44%, n_D^{20} 1.4930. NMR^{1}H ($CDCl_3$, δ, ppm, *J*/Hz): 0.90 (d, 3H, CH_3, ^3J = 6.2); 1.04-1.32 (m, 3H, CH, CH_2 cycl.); 1.33-1.82 (m, 6H, CH_2 cycl.); 3.77 (d.d, 1H, CH_2O, ^3J = 6.0, ^2J = 8.8); 4.09 (d.d, 1H, CH_2O, ^3J = 7.1, ^2J = 8.8); 4.31 (d, 2H, CH_2N, ^3J = 6.0); 4.42 (q, 1H, CHO, ^3J = 5.8); 7.94 (s, 1 H, C^3H triaz.); 8.16 (s, 0.6H C^5H triaz.); 8.18 (s, 0.4H C^5H triaz.). IR (Nujol, ν/sm^{-1}): 1270 (β CH triaz.); 1243, 1223, 1171, 1128, 1078 (COCOC).

1-[(8-Methyl-1,4-dioxaspiro[4.5]dec-2-yl)methyl]-1*H*-imidazole (2c). Yield 39%, n_D^{20} 1.5035. NMR^{1}H ($CDCl_3$, δ, ppm, *J*/Hz): 0.88 (d, 3H, CH_3, ^3J = 6.2); 1.02-1.31 (m, 3H, CH, CH_2 cycl.); 1.31-1.80 (m, 6H, CH_2 cycl.); 3.70 (d.d, 1H, CH_2O, ^3J = 6.2, ^2J = 8.8); 4.05 (d.d, 1H, CH_2O, ^3J = 7.2, ^2J = 8.8); 4.28 (d.d, 2H, CH_2N, ^3J = 6.0, ^2J = 8.8); 4.38 (q, 1H, CHO, ^3J = 5.8); 6.97 (s, 1 H, C^4H imidaz.); 7.18 (s, 1H C^5H imidaz.); 7.68 (s, 1H C^2H imidaz.). IR (Nujol, ν/sm^{-1}): 1281 (β CH imidaz.); 1240, 1220, 1170, 1130, 1080 (COCOC).

1-[(8-*Tert*-butyl-1,4-dioxaspiro[4.5]dec-2-yl)methyl]-1*H*-1,2,4-triazole (3b). Yield 44%, m.p. 57-59ºC. NMR^{1}H ($CDCl_3$, δ, ppm, *J*/Hz): 0.84 (s, 9H, $C(CH_3)_3$); 0.99 (t, 1H, CH cycl., ^3J= 6.1); 1.09-1.22 (m, 2H, CH_2cycl.); 1.25-1.38 (m, 1H, CH_2cycl.); 1.39-1.54 (m, 2H, CH_2cycl.); 1.56-1.69 (m, 2H, CH_2cycl.); 1.71-1.80 (m, 1H, CH_2cycl.); 3.76 (d.d, 2H, CH_2O,^3J= 5.8, ^2J= 8.0); 4.02 (d.d, 2H, CH_2O,^3J= 5.6, ^2J= 8.0); 4.25-4.43 (m, 4H, CH_2N, CHO); 7.94 (s, 1 H, C^3H triaz.); 8.17 (s, 1HC^5H triaz.). IR (Nujol, ν/sm^{-1}): 1272 (βCH triaz.); 1245, 1225, 1170, 1125, 1075 (COCOC).

1-[(8-*Tert*-butyl-1,4-dioxaspiro[4.5]dec-2-yl)methyl]-1*H*-imidazole (3c). Yield 41%, n_D^{20} 1.4960. NMR^{1}H ($CDCl_3$, δ, ppm, *J*/Hz): 0.82 (s, 9H, $C(CH_3)_3$); 0.95 (t, 1H, CH cycl., ^3J= 6.1); 1.07-1.20 (m, 2H, CH_2 cycl.); 1.22-1.34 (m, 1H, CH_2 cycl.); 1.35-1.53 (m, 2H, CH_2 cycl.); 1.53-1.65 (m,2H, CH_2 cycl.); 1.69-1.78 (m, 1H,

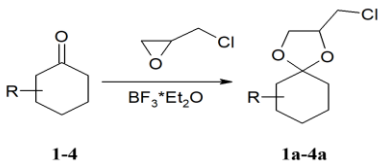
CH_2 cycl.); 3.72 (d.d, 2H, CH_2O, ^3J = 5.6, ^2J= 8.2); 4.00 (d.d, 2H, CH_2O, ^3J = 5.6, ^2J = 8.2); 4.22-4.40 (m, 4H, CH_2N, CHO); 6.92 (s, 1 H, C^4H imidaz.); 7.28 (s, 1H C^5H imidaz.); 7.78 (s, 1H C^2H imidaz.). IR (Nujol, ν/sm^{-1}): 1280 (β CH imidaz.); 1245, 1225, 1172, 1127, 1080 (COCOC).

1-[(7,7,9-Trimethyl-1,4-dioxaspiro[4.5]dec-2-yl)methyl]-1*H*-1,2,4-triazole (4b). Yield 27%, n_D^{20} 1.4885. NMR^{1}H ($CDCl_3$, δ, ppm, *J*/Hz): 0.82-1.06 (m, 9H, $(CH_3)_3$); 1.07-1.27 (m, 3H, CH, CH_2 cycl.); 1.29-1.61 (m, 2H, CH_2 cycl.); 1.66-1.93 (m, 2H, CH_2 cycl.); 3.69 (d.d, 0.44H, CH_2O, ^3J = 6.4, ^2J = 8.4); 3.79 (d.d, 0.56H, CH_2O, ^3J = 5.8, ^2J = 8.4); 4.0-4.15 (m, 1H, CH_2O); 4.22-4.49 (m, 2H, CHO, CH_2N); 7.94 (s, 1 H, C^3H triaz.); 8.16 (s, 0.56H C^5H triaz.); 8.18 (s, 0.44H C^5H triaz.).

RESULTS AND DISCUSSION:

For the synthesis of the target compounds, a two-stage scheme was selected, by which ketalization of the source cyclohexanones with epichlorohydrin during catalysis by trifluoride boron etherate synthesized intermediate 2-chloromethyl-1,4-dioxaspiro[4.5]decanes **1a-4a**, which then alkylated 1,2,4-triazole or imidazole sodium salts.

For the synthesis of 2-chloromethyl-1,4-dioxaspiro[4.5]decanes, the Petrov method served as the prototype. We tested this method on 4-methylcyclohexanone **2**, however, having reduced the excess of 4-methylcyclohexanone to twofold. The yield of the target 8-methyl-2-chloromethyl-1,4-dioxaspiro [4.5]decane **2a** was only 16%, and the main share among the reaction products was a resin of unidentified structure, apparently formed during the polymerization of epichlorohydrin, since the conversion of 4-methylcyclohexanone **2** almost coincided with the yield of **2a** (fig. 1). When epichlorohydrin was added to the mixture of ketone and catalyst, the reaction temperature increased sharply, possibly due to the side reaction of polymerization of epichlorohydrin. In this regard, the methodology has been modified: 4 mol% of boron trifluoride etherate were added to a twofold excess of ketone with stirring, cooled to 10–15ºC, after which epichlorohydrin dropwise at 30-40ºC. With this method, the yield increased to 34% and the proportion of by-products of polymerization of epichlorohydrin decreased.



1-4 1a-4a

Fig. 1

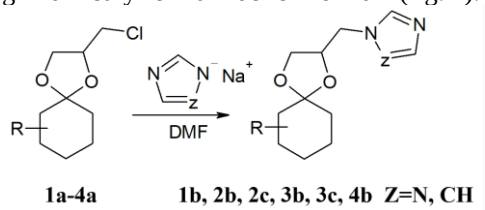
Due to the fact that various authors describing the use of boron trifluoride etherate as a catalyst in the synthesis of 1,3-dioxolanes, varied its amount from 1 to 10 mol%, we conducted an additional study to identify the effect of the amount of catalyst on the yield of chloromethyldioxolane **2a**. It was found that the highest yield of **2**a – 45% was achieved in the case of reducing the amount of catalyst from 4% to 2%. Further reducing the amount of catalyst to 1 mol% led to a decrease in the yield to 38%:

According to the method modified for the product **2a**, 2-chloromethyl-1,4-dioxaspiro[4.5]decanes **1a**, **3a, 4a** were synthesized with average yields of 67%, 88% and 85% respectively. In the case of the synthesis of 8-*tert*butyl-2-chloromethyl-1,4-dioxaspiro[4.5]decane **3a** from crystalline 4-*tert*butylcyclohexanone, the reaction was carried out in a solvent, carbon tetrachloride.

In the ^{1}H NMR spectra of 2-chloromethyl-1,4-dioxaspiro[4.5]decanes **1a-4a**, protons of the cyclohexane fragment were present in the form of several multiplets at 1.01–1.89 ppm, and characteristic signals of 2,2,4-tri-substituted dioxolane were observed: two doublets of doublets of protons of the chloromethyl group at 3.47–3.48 and 3.57–3.60 ppm, as a rule, two doublets of doublets of the dioxolane ring methylene protons at 3.80-3.89 and 4.08–4.15 ppm, and dioxolane methine proton quintets at 4.26–4.45 ppm.

In the IR spectra of chloromethyldioxolanes **1a-4a**, there was no signal from the carbonyl group of the source ketone, and five characteristic bands of the dioxolane ring were observed in the range of 1085–1245 cm^{-1}.

Target substituted 1,3-dioxolanes, based on cyclic ketones and substituted with 1H-1,2,4-triazole and 1*H*-imidazole: 1-[(1,4-dioxaspiro[4.5]dec-2-yl)methyl]-1*H*-1,2,4-triazoles and 1-[(1,4-dioxaspiro[4.5]dec-2-yl)methyl]-1*H*-imidazoles **1b; 2b,c; 3b,c, 4b** were synthesized with a yield of from 12 to 44% by alkylation of the sodium salt of 1,2,4-triazole or imidazole with 2-chloromethyl-1,4-dioxaspiro[4.5]decanes **1a-4a** with boiling in dimethylformamide for 16–20 h (fig. 2).



1a-4a 1b, 2b, 2c, 3b, 3c, 4b Z=N, CH

Fig. 2.

The alkylation reaction of sodium azolates with 2-chloromethyl-1,4-dioxaspiro[4.5]decanes in lower boiling solvents: acetonitrile or tetrahydrofuran, did not

lead to the formation of the target azole derivatives. No positive results have been achieved when using imidazole as a base and imidazole melt solvent, or in the case of using potassium carbonate as a base.

According to ^{1}H NMR spectroscopy and chromatography-mass spectrometry, the reaction masses contained in addition to target substituted 1-[(1,4-dioxaspiro[4.5]dec-2-yl)methyl]-1*H*-1,2,4-triazoles up to 10% of substituted 4-[(1,4-dioxaspiro[4.5]dec-2-yl)methyl]-1*H*-1,2,4-triazoles, 15% of the mixture of hydrolyzates of the target derivatives 3-(1*H*-azol-1-yl)propan-1,2-diols and source cyclohexanones. Since the derivatives **1b; 2b,c; 3b,c, 4b** in the technical condition were viscous oily liquids with high boiling points; it was not possible to purify them by recrystallization or vacuum distillation. Therefore, we used column chromatography to isolate individual 1-[(1,4-dioxaspiro[4.5]dec-2-yl)methyl]-1*H*-1,2,4-triazoles. For this purpose, a column with a diameter of 30 mm was used; Acros silica gel (particle size 35–70 μm) was eluted with chloroform: methanol (10:1) system. With this method, the target derivatives were successfully separated from the impurities of the source cyclohexanones, by-products of 4-substitution, bis-alkylation, as well as 3-(azol-1-yl)propane-1,2-diols.

In ^{1}H NMR spectra of substituted 1-[(1,4-dioxaspiro[4.5]dec-2-yl)methyl]-1*H*-1,2,4-triazoles and 1-[(1,4-dioxaspiro[4.5]dec-2-yl)methyl]-1*H*-imidazoles **1b; 2b,c; 3b,c, 4b**, characteristic signals of the protones of 2,2,4-trisubstituted dioxolane are observed: at 3.69-3.77 and 4.02–4.11 ppm, there are two doublets of doublets of methylene protons of the dioxolane cycle, at 4.22–4.49 ppm, there are two doublets of doublets of the azolylmethyl group and the quintet of the methine proton of dioxolane, both in the form of separate signals and in the form of multiplets [4]. In comparison with 2-chloromethyl-1,4-dioxaspiro[4.5]decanes, the signals of the protons of the exocyclic methylene group of substituted 1-[(1,4-dioxaspiro[4.5]dec-2-yl)methyl]-1*H*-1,2,4-triazoles and 1-[(1,4-dioxaspiro[4.5]dec-2-yl)methyl]-1*H*-imidazoles are shifted to a weaker field, and their chemical shift values are greater than those of protons.

The target compounds were tested for fungicidal activity *in vitro* on six common fungal phytopathogens: *Sclerotinia sclerotiorum (S.s.)* – causative agent of white rot, *Fusarium oxysporum (F.o.)*, *Fusarium moniliforme (F.m.)* – causative agent of Fusarium wilt, *Bipolaris sorokiniana (B.s.)* – causative agent of helminthosporium root rot, *Rhizoctonia solani (R.s.)* – causative agent of brown rot – Rhizoctonia rot and *Venturia inaequalis (V.i.)* – causative agent of apple scab. Effect of compounds on the radial growth of mycelium was studied at a concentration of 30 mg/L. Solutions of the test substances were prepared in acetone, their aliquots were added to the molten sterile potato-sucrose agar, and the resulting media were poured into aseptic conditions in Petri dishes, in which case a final concentration of acetone in all media, including test medium, did not exceed 1%. Pieces of fungus mycelium were placed on the consolidated nutrient medium, thermostated in the dark at 25±0.5ºC, and the radial growth was measured after 72 h. The experiment was repeated three times. The percent of mycelial growth inhibition (I) was calculated by Abbott:

$$I = \frac{D_c - D_t}{D_c} \cdot 100\%$$

D_c – is the diameter of the fungus colonies in the test medium (control), D_t – is the diameter of the fungal colonies in the medium with the test substance.

The known 1,2,4-triazole fungicide – *triadimefon* and the prototype – dioxaspirodecane – *spiroxamine* were used as standards. The research results are presented in table 1.

Investigated 1-[(1,4-dioxaspiro[4.5]dec-2-yl)methyl]-1*H*-1,2,4-triazoles and 1-[(1,4-dioxaspiro[4.5]dec-2-yl)methyl]-1*H*-imidazoles showed a fungicidal activity, significantly lower than the activity of the widely triazole fungicide triadimefon, and the closest to them in the structure of dioxaspirodecane – spiroxamine. Compounds **3b** and **3c**, differing from spiroxamine only by the fact that the propylethylamine group is replaced with a 1,2,4-triazole or imidazole group, also showed activity significantly lower than the standards.

Table 1. Inhibition (I) of mycelial growth of phytopathogenic fungi under the action of test compounds.

Compound	I, %						logP*
	V.i.	*R.s.*	*F.o.*	*F.m.*	*B.s.*	*S.s.*	
1b	7	1	5	16	7	10	0.64±0,60
2b	0	14	8	21	2	9	1.13±0,60
2c	0	7	8	16	11	8	1.78±0,46
3b	26	0	9	0	13	8	2.36±0,61
3c	21	0	14	26	16	17	3.01±0,47
4b	26	0	15	12	1	12	2.16±0,62
triadimefon	58	40	82	89	54	57	-
spiroxamine	81	70	16	59	70	59	

* Calculated values of logP

A series 1-[(1,4-dioxaspiro[4.5]dec-2-yl)methyl]-1*H*-1,2,4-triazoles was tested for growth-regulating activity on germs of cucumber of Zozulya variety (hybrid F_1).

In the work, an express method was used, which consisted in growing cucumber seeds *in vitro* for 8 days. The seeds were cultivated on Murashige and Skoog (MS) agar medium free of substances belonging to the auxin and cytokinin classes. The drugs were subjected to cold sterilization (solutions were passed through bacterial filters) and then they were added to the pre-autoclaved nutrient medium MS, and poured into sterile culture bottles. Cucumber seeds were superficially sterilized with a 0.1% solution of mercuric chloride for 10 minutes, then washed three times with sterile distilled water and placed on the nutrient medium MS. The bottles with plant material were covered with foil and transferred to the light room, where the temperature of 22°C, constant illumination with white fluorescent lamps, with an intensity of 3 thousand lux, at a humidity of 70% were maintained. In the experiment, each drug was studied in 5 concentrations: 0.001; 0.01; 0.1; 1 and 10 mg/l. Control was a variant that did not contain the drug. For each variant, 50 seeds were sown.

At the end of the 8th day, the linear and weight indicators of cucumber germ plants were studied: the length of the root system (cm), the length of the aboveground part of the germ plant (cm), dry and wet weight of the germ plant (g). The experimental results were statistically processed using Straz and Excel software packages. The analysis of variance was carried out and the standard error or the error of the sample mean was found.

The growth-regulating activity of compounds was judged by the following criteria: the length of the aboveground part of the germ plant and root, the dry mass of the aboveground part of the germ plant and root. The research results of the growth-regulating activity are presented in table 2.

Table 2. Growth-regulating activity on germs of cucumber under the action of test compounds.

No.	Conc., mg/l	length of the root system		length of the aboveground part		wet weight		dry weight	
		ℓ, cm	Δℓ,%	ℓ, cm	Δℓ,%	m, g	Δm,%	m, g	Δm,%
3b	0,001	2,1±0,4	**-40,0**	7,5±1,2	-16,7	3,99	-0,5	0,21	-47,5
	0,01	0,7±0,3	**-80,0**	5,2±1,0	-42,2	2,89	-27,9	0,47	17,5
	0,1	2,1±0,5	**-40,0**	7,3±1,2	-18,9	3,52	-12,2	0,34	-15,0
	1,0	1,6±0,6	**-48,5**	8,1±1,1	-10,0	3,99	-0,5	0,41	2,5
	10,0	1,5±0,4	**-57,1**	7,0±1,0	-22,2	3,16	-21,2	0,39	-2,5
4b	0,001	1,6±0,3	**-48,5**	10,3±2,0	14,4	2,63	-34.4	0,31	-22,5
	0,01	0,6±0,2	**-82,8**	3,3±0,6	-63,3	2,21	-44.9	0,27	-32,5
	0,1	1,9±0,6	**-45,7**	4,5±1,3	-50,0	3,54	-11.7	0,47	17,5
	1,0	1,3±0,5	**-62,8**	5,6±0,9	-37,7	2,61	-34.9	0,28	-30,0
	10,0	1,0±0,2	**-71,4**	5,5±0,6	-38,9	2,71	-32.4	0,32	-20,0
Control (water)		3,5±0,4	0	9,0±1,1	0	4,01	0	0,40	0

Most of the studied compounds in the entire concentration range: from 0.001 to 10 mg/l showed noticeable retardant properties, reducing the length of the aboveground part with significantly less inhibitory effect on root growth.

CONCLUSION:

The substituted 4-azolylmethyl-1,3-dioxolanes, based on cyclic ketones: 1-[(1,4-dioxaspiro[4.5]dec-2-yl)methyl]-1*H*-1,2,4-triazoles and 1-[(1,4-dioxaspiro[4.5]dec-2-yl)methyl]-1*H*-imidazoles showed no fungicide activity, but proved good retardant properties. That is why new 4-azolylmethyl-1,3-dioxolanes, based on cyclic ketones synthesis and their retardant activity evaluation is to be done further.

CONFLICT OF INTEREST:

The authors declare no conflict of interest.

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RESEARCH ARTICLE

Effect of beetroot supplementation on 10 km time trial performance of distance runners

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ABSTRACT:

Objective: To find out the effect of 15 days of beetroot juice (BRJ) supplementation on 10 km time trial performance in trained distance runners of University level.**Methods**: Thirty trained athletes,15 males age = 26.3 y ± 1.52, height 170.5 ± 0.2 cm, and 15 females, age = 25.2 y ± 1.30, height 157.8 ± 0.3 cm were selected for the present study. Two experimental and two control groups were made consisting of males and females separately. The first group of male and female (Experimental Group) consumed the BRJdaily 250 ml/dayand the second group (Control Group) did not consume beetroot juice. Both groups underwent a regular athletics training programme. All the subjects were tested on Ten Km Time Trial (TT)performance before supplementation of BRJ and after 15 days of supplementation of BRJ. **Results**: The significant effect of BRJ supplementationwas observed ($p < 0.05$) between pre and post measures of 10 km TT in experimental group. BRJ supplementation significantly improved performance in 10 km TT in both groups (respectively male; P< 0.006; F=11.09, ES = .480, female; P < 0.000, F=40.45, ES = .771.**Conclusion***:* Consumption of BRJ250 ml/day in improved 10 km time trial performance in traineddistance runners.

KEYWORDS: Nitrate supplementation, Nitric oxide, Running exercise, nutritional science, Endurance.

INTRODUCTION:

Beetroot is a cultivated form of Beta vulgaris,as a naturally occurring root vegetable and a rich source of phytochemicals and bioactive compounds[1] (Wruss 2015). As a rich and nutritious source, it is believed to hold health-promotional characteristics, anti-oxidant and anti-inflammatory effects[2] (Georgive 2010) BRJ is an excellent source of iron and vitamin C, which is considered good for anemia. Whereas, leaves of beetroot are useful for liver and spleen diseases." It also helps in repairing and reactivating the red blood cells (RBC) in the human body, which increases the oxygen supply to all parts of the body[3] (Kavitha,2020).

Beetroot juice (BRJ) supplementation has been extensively studied and proofed that inorganic nitrate consumption increased plasma concentrations of NO_3 and nitrite (NO_2) in a dose-dependent manner[4,5] (Nyakayiru et al.2017; Wylie et al. 2013). NO_3 also help to decrease blood pressure (BP) to the same level, affirm a positive linkage among NO_3 content and the observed bp-lowering effect of beetroot[6] [Velmurgan 2015). BRJ consumption along with decreasein BP, causes increasein the plasma NO_2 level, improve endothelial function and systemic production of NO[7,8] (Kapil 2010,Ormesher 2018).

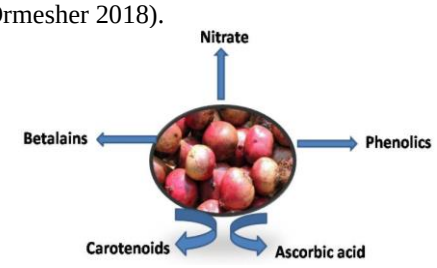

Figure 1: Properties of fresh Beetroot

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NO plays an important role in many physiological processes that may impact sports performance, including the regulation of blood flow, mitochondrial respiration, muscle contractility, and biogenesis, favoring oxidative metabolism[9,10] (Jones, 2014; Dejam et al., 2004). Many authors reported that NO_3- supplementation reduces the O_2 cost of exercise and increases the efficiency of exercise, [11-15] (Whitfield et al. 2016; Lansley et al. 2011; Larsen et al. 2007, 2011; Vanhatalo et al. 2010), It has been demonstrated that BRJ can modify physiological variables which are associated with endurance performance[16] (Jones, 2014) such as cardiorespiratory endurance, maximal oxygen uptake (VO_{2max}), the kinetics of pulmonary oxygen uptake (VO_2) and exercise efficiency or economy[17] (Dominguez et al., 2017). However, the direct effects of BRJ supplementation on time trial performance tests that are closer to a competitive reality, especially in running, need to be further investigated.

Most of the studies reporting that the ingestion of acute or chronic NO_3 gives positive effects on TT performances included tests with a duration of less than minutes of moderate or high intensity [18,19] (Peeling et al., 2014; Cermak et al., 2012;)However, recent evidence indicates that the ingestion of NO_3 can improve running endurance performance in 5-km and 10-km TT[20,21] (Shannon et al. 2017; de Castro et al. 2018).Nitrate rich BRJ supplementation improved one km time trial performance of University Runners[22] (Tirkey et al. 2016). Shannon et al. (2017) and Tirkey and Venugopal (2014) [20,23] conducted a study and reported a significant improvement in 5-km TT (performance improvement: 1.9%) after acute BRJ supplementation compared to PLA condition. Similarly, de Castro et al. (2018)[21] showed significant improvement in 10-km TT after BRJ supplementation in recreational endurance runners compared to PLA condition (performance improvement: 1.9%). These improvements in TT performance after acute or chronic BRJ supplementation were comparable and greater than those reported in studies with endurance running training protocols prescribed with VO_{2max} and Vpeak (improvement of performance in 3-km and 10-km TT: 1% and 1.4%, respectively) [24,25] (Smith et al. 2003; Manoel et al. 2017). "Besides, most studies that have examined these effects have used the time to- exhaustion protocols with exercise being performed at a constant work rate[12] (Lansley et al. 2011), which do not turn to the normal athletic competition.

It is believed that the supplementation of BRJ is less expressive in trained athletes when compared to moderately or active trained individuals[26,27,28] (Boorsma et al., 2014; Wilkerson et al.,2012; Cermak et al., 2012), even though the athletes classified as "responders" [29] (Jonvik et al., 2015). Thus, taking into consideration the different responses of BRJ supplementation in performances with different intensities and durations as well as in the direct impact of BRJ supplementation on performance in individuals with varying training levels, the purpose of this study were to investigate the effects of BRJ supplementation on 10 km time trial performance in trained distance runners of Universitylevel.

METHODOLOGY:

Participants and design:

Thirty trained athletes,15 males age = 26.3 y ± 1.52, height 170.5 ± 0.2 cm, and 15 females, age = 25.2 y ± 1.30, height 157.8 ± 0.3 cm who represented the University in National/Zonal competition, were selected for the present study. Two experimental and two control groups were made consisting of males and females separately. The first group of male and female (Experimental Group) consumed the beetroot juice daily 250 ml/day in *natura* (5.00 mmol NO_3- day− 1) and the second group (Control Group) did not consume beetroot juice. The subjects had at least 2 year of experience in running;all are physically fit and reported no use of medication or nutritional supplements during the study. Prior to testing, written informed consent was taken from all participants to participate in the study. The experimental protocol was approved by the Institutional Ethics Committee for Human Research (071/IEC/PRSU/2014).

Table 1. Experimental and control group of trained athletes selected for present study

Gender	Group	No. of Subjects
Males	Experimental Group	08
	Control Group	07
Females	Experimental Group	08
	Control Group	07

After familiarization with the protocol, nutritional orientation (i.e., dietary procedures to be adopted throughout the testing period) and anthropometrical assessment (i.e., height and body weight), Performance of Ten km run were measured of subjects of all the groups before the start of the experiment. The experimental group was administered 250 ml beetroot juice/day for 15 days. All the subjects were involved in a regular athletics training program. After 15 days all the subjects were tested for performance of Ten km run. The experimental protocol is illustrated in Table2.

Table 2. Experimental protocol of 10- km Running Performance.

Group	Pre –Test	15 Days Training Programme	Post -Test
Experimental Group (16 Subjects) Male=8 Female= 8	Ten Km TT performance	Beetroot Supplementation (250 ml/day at 2 pm) and regular training programme (Morning and evening session)	Ten Km TT performance
Control Group (14 Subjects) Male=7 Female= 7	Ten Km TT performance	Regular training programme (Morning and evening session) without any Supplementation.	Ten Km TT performance

Dietary standardization and Training program:

The dietary assessment was carried out by a Nutritional Professional for estimating the foods consumption in NO_3-, iron, vitamin C, betaine and other compounds present in BRJ that could interfere the results of the tests. Throughout the testing period participants received written guidelines regarding dietary procedures, which involved no consumption of foods rich in NO_3- hours before each test procedure, the subjects were instructed to consume a standardized meal with the minimum amount of protein and carbohydrate calculated individually, respecting the eating habits of subjects. It was advised to replicate the meals to avoid interference of any food factorbefore the tests. Before the s

The subjects performed of 10-km running performance on a 400 mt standard athletics track of Pt.Ravi Shankar Shukla University, Raipur, India without the presence of opponents in the track preceded by a self-determined warm-up of 10 min. All participants were motivated to give their best performance. Participants freely choose their pacing strategy during the performance and the time was recorded every from starting to the end of 10 km. Mineral water was provided in bottles throughout trials, so that runners could hydrate themselves as they were used to do in long-distance races.Participants were advised to attend the well-hydrated test local, performed a stanardized meal three hours before the start of testing and instructed not to use any type of mouthwash during the testing period because of their potential inhibitory effect on the conversion of NO_3- NO_2[33] (Govoni et al., 2008). They were also instructed to abstain from caffeinated or alcoholic beverages and strenuous physical exercise within 24 hours prior to testing and to consume the same diet and to maintain the same physical exercise regimen 48 hours prior to testing.

Table 3. 15 Days Training Programmefor trained athletes

Days	Morning Session	Evening Session
1	Warm Up and exercise – 30 min Slow Continuous Running for 60 min with a pace of 4 min/km for men and 4.30 min/km for women	Warm Up and exercise – 30 min Stride of 80 mts. – 10 repetitions 1 Km of 5-8 repetitions Intensity – 80%
2	Warm Up and exercise – 30 min Fast continuous running - 8 km (Men) and 6 km (women) with a pace of 3.30 min/km. for men and 3.45 min/km for women	Warm Up and exercise – 30 min Slow Continuous Running for 60 min with a pace of 4 min/km. for men and 4.30 min/km for women
3	Warm Up and exercise – 30 min Fartlek running for 30 minutes Intensity – 80%	Warm Up and exercise – 30 min Recreational game
4	Warm Up and exercise – 30 min Slow Continuous Running for 45 min with a pace of 4 min/km for men and 4.30 min/km for women	Warm Up and exercise – 30 min Stride of 80 mts. – 10 repetitions 2 Km of 3-5 repetitions Intensity – 80%
5	Warm Up and exercise – 30 min Sand running for 30 minute Intensity – 80%	Warm Up and exercise – 30 min 400 mts – 8-10 repetitions Intensity – 80%
6	Warm Up and exercise – 30 min Fast continuous running - 8 km (Men) and 6 km (women) with a pace of 3.30 min/km. for men and 3.45 min/km for women	Warm Up and exercise – 30 min Complete stretching exercise
7	Warm Up and exercise – 30 min 5 km time trial	Warm Up and exercise – 30 min Lead up activity
8	Warm Up and exercise – 30 min Slow Continuous Running for 45 min with a pace of 4 min/km for men and 4.30 min/km for women	Warm Up and exercise – 30 min Stride of 80 mts. – 10 repetitions 1 Km of 5-8 repetitions Intensity – 90%
9	Warm Up and exercise – 30 min Stride of 80 mts. – 10 repetitions 2 Km of 3-5 repetitions Intensity – 90%	Warm Up and exercise – 30 min Slow Continuous Running for 30 min with a pace of 4 min/km for men and 4.30 min/km for women
10	Warm Up and exercise – 30 min Fast continuous running – 10 km (Men) and 10 km (women) with a pace of 3.15 -3.30 min/km. for men and 3.45 - 4.00 min/km for women	Warm Up and exercise – 30 min Complete stretching exercise
11	Warm Up and exercise – 30 min Sand running for 30 minuteIntensity – 80%	Warm Up and exercise – 30 min Lead up activity

12	Warm Up and exercise – 30 min Slow Continuous Running for 45 min with a pace of 4 min/km for men and 4.30 min/km for women	Warm Up and exercise – 30 min 10 km time trial
13	Warm Up and exercise – 30 min Fartlek running for 30 minutes	Warm Up and exercise – 30 min 400 mts – 8-10 repetitions
14	Warm Up and exercise – 30 min Fast continuous running – 10 km (Men) and 10 km (women) with a pace of 3.15 -3.30 min/km. for men and 3.45 - 4.00 min/km for women	Warm Up and exercise – 30 min Recreational game
15	Warm Up and exercise – 30 min Slow Continuous Running for 45 min with a pace of 4 min/km for men and 4.30 min/km for women	Warm Up and exercise – 30 min Complete stretching exercise

Statistical analyses:

All statistical analyses were performed using the Statistical Package for the Social Sciences software (v.20.0,SPSS Inc., Chicago, IL, USA). Descriptive statistics (Mean ± SD) were calculated for all the variables. Data normality was verified by Shapiro–Wilk test and the variables are presented as mean ± standard deviation (SD). The results of the 10-km TT performance determine of the Experimental and control group were compared by ANCOVA. To calculate the effect size (ES) and percentage difference (Dif.%), a comparison was made between the means. The ES was used to estimate the (standardized) magnitude of the difference, and the values were classified according to Cohen (1988) [34] in: ≤ 0.20 (trivial), 0.21–0.50 (small), 0.51–0.80 (moderate) and > 0.80 (large). Each time, $P < 0.05$ was considered a significant difference.

RESULTS:

Table 3 shows the comparisons between the MV for 10-km running performance for the EG and CG of male trained athletes. Statistically significant differences was found in 10-km ($P < 0.05$), running performance respectively (P < 0.007; F= 10.79, ES = .474; P < 0.006; F=11.09, ES = .480. Analysis based on the magnitude of effect indicated a "small" effect 480 sizes in favor of supplementation with EG male trained athletes in the total time of the 10-km running TT.Table 4 shows the comparisons between the MV for the 10-km running performance for the EG and CG of female trained athletes. Statistically significant differences wasfound in 10-km ($P < 0.05$), running performance P < 0.006, F=11.26, ES = .484; P < 0.000, F=40.45, ES = .771. Analysis based on the magnitude of effect indicated a "moderate" effect 771 sizes in 10 km in favor of supplementation with EG female trained.

Table 4 - Mean values ± standard deviation (SD) of the variables obtained during10-km TT running performance in male trained athletes (n = 16)

Variables	EG (M ± SD)		CG (M ± SD)		F value	P value	Effect size (f2)
	Pre Test	**Post Test**	**Pre Test**	**Post Test**			
10 km performance (min)	35.92 ± 1.35	34.41.22 ± 1.34	37.14 ± 2.51	36.92 ± 2.20	11.09*	.006	0.48 (Small)

SD= Standard Deviation, [a] significance differences from pre test (p< 0.05), Δ (%)=positive effect on experimental group when compared to control group, p value = Statistically significant values are in bold characters.

Table 5 - Mean values ± standard deviation (SD) of the variables obtained during10-km TT running performance in femaletrained athletes (n = 14).

Variables	EG (M ± SD)		CG (M ± SD)		F value	P value	Effect size (f2)
	Pre Test	**Post Test**	**Pre Test**	**Post Test**			
10 km performance (min)	47.18 ± 5.34	43.61.22 ± 3.22	54.24 ± 5.07	53.81 ± 4.93	40.45*	.000	0.77 (Moderate)

SD = Standard Deviation, [a] significance differences from pre test (p< 0.05), Δ (%)=positive effect on experimental group when compared to control group,p value = Statistically significant values are in bold characters.

DISCUSSION:

The aim of the present study was to investigate the effect of 15 days beetroot juice (BRJ) supplementation on 10 km time trial performance in trained distance runners of University level. The result of the present study showed increased performance in 10 km time trial, hence increase in the performance of endurance may not be directly related to increase in various parameters of blood. The increase in performance after beetroot juice supplementation can be attributed to the high nitrate (NO_3^-) concentration, which can be converted to bioactive NO_2^- and nitric oxide. However, there are multiple bioactive components in beetroot juice that are potential sources of these beneficial effects. The main finding was that the 15-day supplementation of BRJ increased 10 km time trial performance. To our knowledge, this was the first study to evaluate the effects of BRJ supplementation on 10 km time trial in both male and female in trained distance runners

It has already been shown that supplementation of NO_3- can improve cardio respiratory endurance in athletes by increasing efficiency and by improving performance in time to exhaustion tests at submaximal intensities and graded exercise tests [35,36] (Dominguéz et al. 2017; McMahon et al. 2016). These results are significantly notable in active and trained individuals with a VO_{2max} < 60 ml kg− 1 min− 1[37,38] (Carriker et al. 2016; Hoon et al. 2014). However, some studies shows controversial results, have investigated the effects of both $NaNO_3$- [39,40] (Bescós et al. 2011; Larsen et al. 2010) and BRJ supplementation[41,42,43] (Lansley et al. 2011a, b; Vanhatalo et al. 2010; Bailey et al. 2009) on the VO_2max response during exercise. Differing to our study, both Bescós et al. (2011) and Larsen et al. (2010)[39,40] find a reduction in VO_{2max} by maintenance or a small increase in time to exhaustion, after a single dose of (10 mg) 3 hour before exercise and chronic dose (0.1 mg) for 2 days before the test. In these both studies, authors recommend that there may be 2 distinct mechanisms involved: one can improve muscles efficiency and energetic function in the muscles involved and the other supplement can reduce VO_{2max} (Bescós et al. 2011; Larsen et al. 2010) [39,40]. Similar to our study, Waldron et al. (2018)[44] observed supplementation of 350 mL of NO_3- rich (≅ 20.5 mmol) improvements in the energy cost of exercise, resting mean arterial pressure, recovery of VO_2, and blood markers.Vanhatalo et al. (2010) [42] found a statistically significant increase in VO_{2max} (≅ 140 mL min− 1)after 15 days of chronic supplementation of BRJ (5.2 mmol NO_3- day− 1) in physically active men.

The result of the present study shows an increase in final time of the 10-km running performance test between the EG and CG in male and female groups was 4.2% and 7.5% indicating an improvement with a “small” and “moderate”(female) effect size. Our findings are in concord with previous studies that investigate the effects of BRJ on long duration endurance performance[20,27] (Shannon et al., 2017; Wilkerson et al., 2012) and with a literature review and meta-analysis that found a "trivial" but not significant effect in favor of dietary supplementation of NO_3- , which can be translated into improvement of 0.8% in the final performance[45] (McMahon et al., 2017). Although the result of the present study may seem small in the competitive environment in which athletes have very similar levels of performances, the improvement of 0.5 to 1.5% is considered sufficient to make a difference between the competitors [46,47] (Dominguez et al., 2018; Paton and Hopkins, 2006) as well as the final result of the performance. The improvement in the exercise economy will allow an athlete to exercise at a higher power output or running speed for the same VO_2 and reduced time required to complete the10-km performance[48] . (Jones, 2014b).

It has already been shown that BRJ can increase VO_{2max} in physically active men and it is believed that both its vasodilatory properties, as well as its additional action on improved muscle contraction efficiency and mitochondrial respiration, may be involved in this process [46,49,50] (Dominguez et al., 2018; Fergunson et al., 2015; Vanhatalo et al., 2010).

Although in the present study EG changed the MV in the final phase of the performance. Our results are in agreement with the data reported in the literature, which show that supplementation of NO_3- gives a positive effect on TT performances lasting less than 30 minutes [51,52,53] (Porcelli et al., 2015; Peeling et al., 2014; Cermak et al., 2012b) when compared to longer tests of lower intensities [54] (Glaister et al., 2015). The exact reasons for this difference between the results in performances with different durations are still unclear; however, one of the hypotheses supported is the difference in the predominance of recruitment of muscle fibers in these two types of activity[46,20] (Dominguez et al., 2018; Shannon et al., 2017). It is known that the effects of NO_3- can enhance type II muscle fibre contractility and blood flow[54,55] (Ferguson et al. 2013a, 2013b) suggesting the possibility that BRJ supplementation.

Although the mechanistic bases for the reduction in the O_2 cost of sub-maximal exercise and enhancements in exercise tolerance following acute dietary BRJ remain unclear, these results provide important practical information which may underpin the potential use of BRJ/NO_3 supplementation for improving cardiovascular health in the general population, and for enhancing exercise performance in athletes.

CONCLUSION:

In conclusion, the once daily consumption of BRJ 250 ml/day improved in 10 km time trial in trained athletesof University level.Since these areimportant variables for the prediction, monitoring, and prescriptionof endurance training, trained athletes maybenefit from the effects of BRJ if they add this supplementto their dietary routine.

CONFLICT:

The authors have no conflicts of interest to report.

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RESEARCH ARTICLE

Stability of Curcumin Improved in Hydrophobic Based Deep Eutectic Solvents

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ABSTRACT:

Turmeric is a commonly known natural spice that contains many phytoconstituents. Among which Curcumin is a polyphenol present in turmeric responsible for many pharmacological actions. Curcumin is still used as a traditional medicine in fields such as Ayurvedic, Siddha, and Unani. Though Curcumin has a large number of activities, it has disadvantages, such as small shelf life due to poor chemical stability, poor absorption results in less bioavailability, less water solubility, rapid metabolism results in quick elimination from the systemic circulation. A Deep eutectic solvent (DES) is a new class of solvents. Hydrophobic DES can be used for dissolving water-insoluble compounds. DES can be prepared when two solid components mixed in a particular proportion are converted into liquid. DES can be used as a solvent for dissolving water-insoluble compounds and to increase the stability. In this work initially, curcumin linearity studies were conducted in different buffers. A buffer showing maximum absorbance was selected from the linearity studies. Then, DES was prepared by combining Camphor:Menthol (1:1) (CM-DES), Camphor:Thymol (1:1) (CT-DES) and, Menthol:Thymol (1:1) (MT-DES). The stability of curcumin in different DES was determined from the stock and working solutions in benchtop condition (room temperature) and, refrigerator condition (5±3°C). Only working solution stability was determined in the in vitro media temperature (37±2°C). From this study, it was concluded that 50 mM Sodium dihydrogen orthophosphate with 0.5% SLS at pH 5.5 showed maximum absorbance value compared with other buffers, so it was selected for further studies. From stability studies, it was found that curcumin in CM-DES was found to be stable in both stock and working solutions compared to the other two CT-DES and MT-DES.

KEYWORDS: Curcumin, Deep eutectic solvent, Camphor, Menthol, Thymol, Stability studies.

INTRODUCTION:

Toxic effects of synthetic medications have drawn awareness to turn toward natural medicines[1]. The demand for herbal medicines is increasing rapidly due to their lack of side effects. Further as healthcare costs continue to escalate, the attraction for low-cost remedies has stimulated consumers to reevaluate the potential of alternatives[2]. Turmeric (*Curcuma longa*)

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belongs to the Zingiberaceae family, is broadly utilized as a zest, food, additive and traditional medication and as a family unit solution for different illnesses in Asian nations, including China and southeast Asia[3-5]. The name turmeric is derived from the Latin word terra merita or turmeryte[6]. Curcumin, the main constituent present in turmeric[7] along with other chemical constituents known as curcuminoids. The major curcuminoids present in turmeric are 77% Curcumin, 17% demethoxycurcumin, and 3% bisdemethoxycurcumin (curcumin III), and the recently identified cyclocurcumin[8]. Curcumin (1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione), also called diferuloylmethane, is the main natural polyphenol present in turmeric[9].

Many of the research work recommended curcumin has been used in the avoidance and curing of various many illnesses[10-12]. Curcumin can be used to the following illness like anti-proliferative, anti-invasive, and anti-angiogenic agent, chemopreventive agent, as a mediator of chemoresistance and radioresistance. As a curative effect in wound healing, cardiovascular diseases, pulmonary diseases, parkinson's disease, alzheimer's disease, and in various types of arthritis, atherosclerosis, chronic anterior uveitis, colon cancer, familial adenomatous polyposis, hypercholesteremia, inflammatory bowel disease, pancreatic cancer, pancreatitis, psoriasis, ulcerative colitis[12], potent anti-oxidant, anti-inflammatory, anti-cancer, antidiabetic, hepatoprotective, anti-spasmodic[13], cholesterol lowering[14], anti-coagulant[15] etc.

Even though Curcumin has a large number of action, its usage is limited in the foodstuff industry and pharma companies because of its poor solubility and low stability in different environment[16]. Its bioavailability is also poor and it remains a major challenge due to the presence of olefinic groups present in its structure this β-diketone of poor aqueous solubility rendering it of relatively low bioavailability[17]. Curcumin is sensitive to light and temperature, which limits its applications[16]. The degradation of curcumin is pH-dependent, at neutral and under alkaline conditions it degrades more speedily, at acidic conditions, it degrades slowly, with less than 20% the following 30 min to one hr[18-21]. Protecting the stability of curcumin is important for effective pharmacological action[22].

Eutectic solvents are useful in numerous areas in the pharmaceutical field like to increase the drug solubility, permeation, and absorption[23]. DES is a new class of neoteric solvents that are said to be "green solvent" made from low-cost ingredients, which entered the year 2003[24,25]. DES is defined as a combination of two or more ingredients normally solid at room temperature, but when combined at a particular molar ratio, converted into liquid at room temperature[26]. Among the two ingredients one is hydrogen bond donor (HBD) and another one hydrogen bond acceptor (HBA)[27]. Tomasz Jeliński et al[22], the previously improved curcumin stability with the help of hydrophilic based eight natural DES. In this study, an attempt was made to determine the stability of curcumin in three hydrophobic-based DES prepared with Camphor:Menthol, Camphor:Thymol and Menthol:Thymol in equal proportion (1:1) with the help of suitable buffer.

MATERIALS AND METHODS:

Curcumin was obtained from Sami laboratories limited. Camphor and Thymol were purchased from Sisco Research Laboratories Pvt. Ltd, Maharastra, Menthol was procured from Reachem Laboratory chemicals private limited, Chennai. Methanol was purchased from Himedia Laboratory Pvt. Ltd. Mumbai. Distilled water was purchased from Rajco Pvt. Ltd., Sivakasi. All other chemicals used in this study for preparing different buffers were of analytical grade.

Selection of buffer:

Curcumin has poor solubility and stability in aqueous media. So initially, the suitable buffer on the basis of good solubility and stability was determined. Different trials have been made with different buffers pH according to different works of literature. The standard curve for Curcumin was performed in methanol, acetate phosphate buffer pH 4.5, phosphate buffer pH 7.4, phosphate buffer pH 6.8, phosphate buffer pH 5.5, phosphate buffer pH 5.5 with 0.5% SLS, 50 mM Sodium dihydrogen orthophosphate with 0.5% SLS with a pH of 5.5. The linearity of concentrations ranges from 1–10 µg/mL was made with different above said solutions. The solution with maximum absorbance was taken for further studies as they have the maximum solubility of Curcumin. For analyzing the drug content, various analytical methods like UV spectroscopy, HPLC method, HPTLC method, etc., are in use. According to ICH guidelines, drug analysis can be determined by simple, sensitive, quick, and accurate. UV spectroscopy fulfills the above condition and it is acceptable for regular testing of drugs[28,29,30]. Therefore, in this study curcumin solubility studies were determined with the help of double beam UV-Visible spectrophotometer (Shimadzu- 1800, Japan).

Curcumin in methanol and in different pH:

Curcumin is freely soluble in methanol. According to different works of literature, methanol satisfied the conditions like relative to peak quality and noninterference at the specified wavelength[28,31]. 10-mg curcumin was initially dissolved in 10-mL methanol

(Curcumin stock solution of 1 mg/mL). From the curcumin stock solution 10 mL was pipetted out and make up to 100 mL with the same methanol (Curcumin intermediate solution of 100 µg/mL). From the curcumin intermediate solution, 1 to 10 mL was pipetted out in a separate 100 mL volumetric flask and make up the volume with respective buffer solutions to give a concentration of 1 to 10 µg/mL. The buffers used for this study are phosphate buffer pH 5.5[32a], phosphate buffer pH 6.8[32b], phosphate buffer pH 7.4, in acetate buffer pH 4.5, phosphate buffer pH 5.5 with 0.5% SLS and 50 mM sodium dihydrogen orthophosphate with 0.5% SLS.

Preparation of DES:

Three types of DES were prepared by combining Camphor:Menthol (1:1) (CM-DES); Camphor:Thymol (1:1) (CT-DES) and Menthol:Thymol (1:1) (MT-DES). About equal quantities of the eutectic forming ingredients are accurately weighed and transferred to a small beaker, and it was mixed well with the help of magnetic stirrer at 500 rpm until a clear liquid was formed.

Curcumin in DES:

10-mg Curcumin was initially dissolved in 10 mL of CM-DES (Curcumin stock solution of 1 mg/mL) and dissolved with the help of magnetic stirrer with slight heat. Applying slight heat increases the solubility of curcumin rapidly when compared to without heat. Similarly, a curcumin stock solution in other types of DES (CT-DES and MT-DES) was prepared. 10 mL of curcumin stock solution (prepared with CM-DES, CT-DES, and MT-DES) was further made up to 100-mL methanol (Curcumin intermediate solution of 100 µg/mL). From the curcumin intermediate solution, 1 to 10 mL was pipetted out in a separate 100 mL volumetric flask and make up the volume with the best buffer to give a concentration of 1 to 10 µg/mL. The stability of curcumin in best buffer was performed with the help of bioanalytical method templates and validation[34,35].

Benchtop stability:

Bench top stability for Curcumin in three types of DESs (CM-DES, CT-DES, and MT-DES) in stock and working solution was performed by keeping the solution at the bench top at room temperature for about 25±3°C at a time interval of 1, 2, 3, 4, 5, 6, 12, and 24 h in triplicate. The stock solution stability was determined by making a dilution according to preparation of Curcumin intermediate (50 µg/mL) solution in methanol and preparation of Curcumin working solution (5 µg/mL) in 50 mM sodium dihydrogen ortho-phosphate with 0.5%w/v SLS solution. 50 mM sodium dihydrogen ortho-phosphate with 0.5%w/v SLS solution was selected for the last dilution as this solution has better absorbance than other buffers solution at 430 nm. The sample readings were compared with freshly prepared stock solution and analyzed with the help of UV double beam spectrophotometer.

Refrigerator stability (5±3°C):

Curcumin (in three different DES) in stock and working solution was prepared according to the above said procedure and kept in refrigerator maintaining a temperature of 5±3°C for about 48 hrs. The reading was taken at 2, 4, 6 and 8 days in triplicates. The samples readings were compared with freshly prepared stock solution and analyzed with the help of UV double beam spectrophotometer.

Stability in *in-vitro* media temperature (37°C±2°C):

Curcumin (in three DES) in stock and working solutions was prepared according to the above said procedure and kept at 37±2°C (dissolution temperature) for about 14 h. The reading was taken at 1, 2, 4, 6, 8, 10, 12, and 14 h to check the stability of Curcumin.

RESULTS AND DISCUSSION:

Curcumin in Methanol:

Curcumin is freely soluble in methanol. Curcumin showed more amount of absorbance at a concentration of 1–10 µg/mL at 425 and 430 nm, respectively. The linearity for curcumin concentrations ranges from 1–10 µg/mL was made with methanol and different buffers. The absorbance of another buffer pH was compared with methanol absorbance. Acetate buffer pH 4.5, phosphate buffer pH 5.5 (with and without SLS), 6.8, 7.4, and 50 mM Sodium dihydrogen orthophosphate with 0.5 % SLS (pH 5.5) was performed and the reading was shown as in Fig. 1. The comparative readings of Curcumin in different pH are given in the table 1.

Phosphate buffer pH 5.5 with 0.5% SLS was initially prepared with potassium dihydrogen phosphate, disodium hydrogen phosphate, and SLS in sufficient distilled water. However, we are unable to get a clear solution, a precipitate formed this is due to the interaction between sodium present in SLS and potassium present in potassium dihydrogen phosphate. So we replaced potassium dihydrogen phosphate with sodium dihydrogen phosphate. When potassium dihydrogen phosphate was replaced with sodium dihydrogen phosphate, *we* got a clear liquid. There was no change in the pH. Buffer having maximum absorbance was selected for further stability studies. Among the different buffer pH, 50 mM Sodium dihydrogen orthophosphate with 0.5% SLS was selected for further studies due to maximum absorbance value compared to another pH.

Table 1: Comparative readings of Curcumin in different buffer pH solutions

Buffer	Absorbance (nm)	Concentration		R^2 value
		Initial (1 µg/mL)	Final (10 µg/mL)	
Methanol	425	0.619±0.022	2.973±0.018	0.9988
	430	0.629±0.010	2.980±0.017	0.9987
Acetate buffer pH 4.5	425	0.084±0.003	0.773±0.002	0.9989
Phosphate buffer pH 5.5	425	0.084±0.003	0.774±0.006	0.9996
Phosphate buffer pH 5.5 with 0.5 % SLS	425	0.197±0.017	1.501±0.052	0.9986
Phosphate buffer pH 6.8	425	0.071±0.004	0.745±0.008	0.9986
Phosphate buffer pH 7.4	425	0.050±0.002	0.348±0.003	0.9974
50 mM Sodium dihydrogen orthophosphate with 0.5 % SLS (pH 5.5)	430	0.304±0.010	1.935±0.066	0.9987

*Linearity was determined for three times

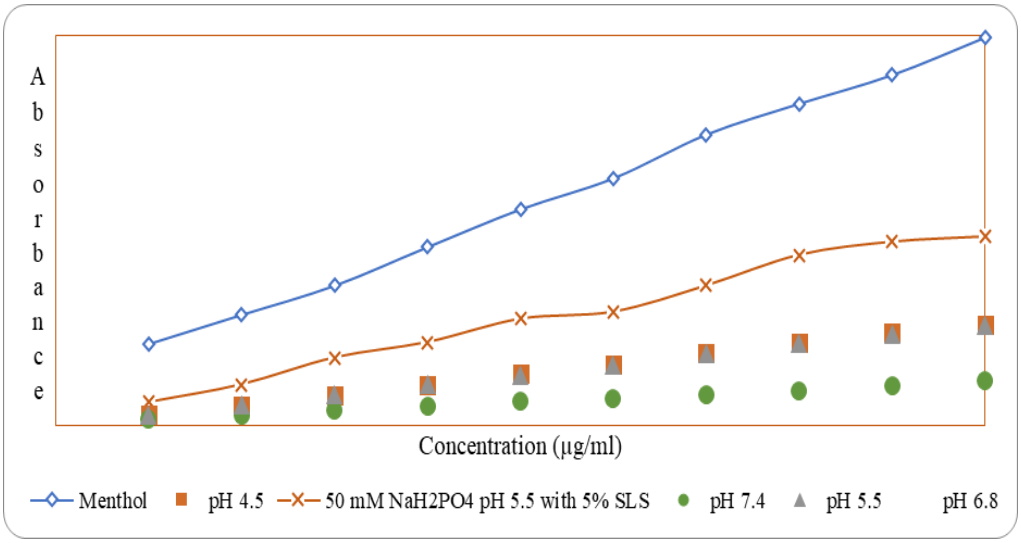

Fig. 1: Linearity of Curcumin in methanol, phosphate buffer pH 6.8, in phosphate buffer pH 7.4, in acetate buffer pH 4.5, in phosphate buffer pH 5.5, in phosphate buffer pH 5.5 with 0.5 % SLS and in 50 mM Sodium dihydrogen orthophosphate with 0.5 % SLS

Benchtop stability studies:

Benchtop stability for curcumin in-stock solution prepared from different DES (CM-DES, CT-DES, and MT-DES) and working solution in 50 mM Sodium dihydrogen orthophosphate with 0.5% SLS buffer was performed to determine curcumin stability while working in the laboratory temperature and compared with a freshly prepared stock solution. It helps in determining the curcumin stability in stock, the working solution while working at least for a while of 24 h at a period of 1, 2, 3, 4, 5, 6, 12, and 24 h.

Curcumin benchtop stability studies in CM-DES in the stock solution were found to be between 100.824±0.799 and 98.220±0.262% and for working solution it was found to be between 100.824±0.799 and 98.220±0.262. These values are within the acceptance criteria of 98%-102%. The percentage relative standard deviation (% RSD) was found to be 0.28 for a stock solution and 0.27 for the working solution. Based on the acceptance criteria results Curcumin in CM-DES was found to be stable for 24 h in both stock and working solutions.

Curcumin benchtop stability studies in CT-DES in the stock solution were found to be between 101.192±0.455 and 95.664±0.207% and for working solution it was found to be between 101.092±0.958 and 94.969±0.500%. These values are within the acceptance criteria of 98%-102%. The percentage relative standard deviation (% RSD) was found to be 0.47 for a stock solution and 0.92 for the working solution. Based on the acceptance criteria results Curcumin in CT-DES was found to be stable for 12 h in both stock and working solutions.

Curcumin benchtop stability studies in MT-DES in the stock solution were found to be between 101.035±1.028 and 83.367±1.056% and for working solution it was found to be between 100.401±0.601 and 75.351±1.102%. Based on the acceptance criteria results, curcumin in MT-DES was stable for 4 h in a stock solution and only 2 h in working solution.

The results of the benchtop stability of curcumin in different DES from stock and the working solution are shown in table 2.

Table 2: Benchtop stability of Curcumin in different DES from stock and working solution

Time (hrs)	Bench top (% stability)*					
	Stock solution % stability			Working solution % stability		
	CM-DES	CT-DES	MT-DES	CM-DES	CT-DES	MT-DES
Initial	100.989±0.198	101.192±0.455	100.902±1.028	100.824±0.799	101.092±0.958	100.401±0.601
1	100.890±0.171	100.828±0.448	100.050±1.231	100.692±0.713	100.960±1.160	99.165±1.053
2	100.725±0.206	100.497±0.553	99.349±1.099	100.396±0.785	100.861±1.261	98.263±1.108
3	100.429±0.659	100.132±0.607	98.798±1.071	100.363±0.644	100.430±1.319	97.128±1.360
4	100.396±0.198	99.834±0.510	98.096±0.612	100.264±0.853	100.099±1.119	95.157±0.711
5	100.363±0.151	99.636±0.676	95.190±0.931	99.901±0.785	99.735±0.980	92.184±0.626
6	100.330±0.374	99.305±0.525	92.735±0.351	99.439±0.919	99.073±0.752	88.811±0.587
12	100.165±0.318	98.246±0.250	87.926±0.514	99.077±0.887	98.080±0.207	83.434±0.352
24	99.769±0.249	95.664±0.207	82.866±1.056	98.220±0.262	94.969±0.500	75.351±1.102
Acceptance criteria	98-102%					

*Performed three times

Refrigerator temperature stability studies:

Curcumin stability in different DESs at 5±3°C was determined by placing the stock and working solution in a refrigerator for 8 days and samples were analyzed for every 2 days interval with a freshly prepared stock solution. Curcumin in CM-DES in the stock solution was found to be between 100.561±0.374% and 98.384±0.318% and for working solution, it was found to be between 100.495±0.799% and 98.154±0.228%. Based on the acceptance criteria range Curcumin in CM-DES was found to be stable for 8 days in both stock and working solutions.

Curcumin in CT-DES in the stock solution was found to be between 100.563±0.599% and 96.690±0.901% and for working solution it was found to be between 100.132±0.303% and 95.829±0.358%. Based on the acceptance criteria range Curcumin in CT-DES was found to be stable for 6 days in a stock solution and 4 days in the working solution.

Curcumin in MT-DES in the stock solution was found to be between 100.802±0.810% and 92.084±0.417% and for working solution, it was found to be between 100.301±0.459% and 91.850±0.810%. Based on the acceptance criteria range Curcumin in MT-DES was found to be stable for 4 days in a stock solution and 2 days in the working solution. The stability data of curcumin in different DES from stock and the working solution placed in a refrigerator at 5±3°C are shown in table 3.

Table 3: Stability data of Curcumin in different DES from stock and the working solution placed in refrigerator at 5±3°C

Time (hrs)	Bench top (% stability)*					
	Stock solution % stability			Working solution % stability		
	CM-DES	CT-DES	MT-DES	CM-DES	CT-DES	MT-DES
0	100.561±0.374	100.563±0.599	100.802±0.810	100.495±0.453	100.132±0.303	100.301±0.459
2	100.033±0.400	100.099±0.716	99.950±0.820	99.967±0.562	99.636±0.207	98.764±0.405
4	99.472±0.545	99.437±0.705	98.096±0.759	99.077±0.636	98.875±0.349	96.226±0.504
6	98.912±0.453	98.080±0.803	94.138±0.909	98.582±0.716	97.617±0.199	93.821±0.612
8	98.384±0.318	96.690±0.901	92.084±0.417	98.154±0.228	95.829±0.358	91.850±0.810
Acceptance criteria	98-102%					

*Performed three times

Stability studies in *in-vitro* media temperature:

Stability for curcumin in working solution in *in vitro* media temperature was performed to check the curcumin stability in the media at the time of *in vitro* release studies for a period of 24 hrs. It was checked in a different time interval of 1, 2, 4, 8, 12, 16, 20, and 24 h by comparing it with a freshly prepared working solution. The working solution prepared from CM-DES in 50 mM Sodium dihydrogen orthophosphate containing 0.5% SLS was found to be between 100.462±0.318% and 98.121±0.099%. Based on the acceptance criteria data the results clarify that Curcumin was stable for a period of 24 hrs at 37±2°C. The working solution was prepared from CT-DES and the solution was further diluted with 50 mM Sodium dihydrogen orthophosphate containing 0.5% SLS was found to be 100.397±0.455% and 86.759±1.098%. Based on the acceptance criteria data the results clarify that curcumin in CT-DES was stable for a period of 8 h at 37±2°C. The working solution was prepared from MT-DES and the solution was further diluted with 50 mM Sodium dihydrogen orthophosphate containing 0.5% SLS was found to be between 100.334±0.252% and 77.288±1.004%. Based on the acceptance criteria data the results clarify that curcumin in MT-DES was stable for a period of 2 h only at 37±2°C. The results of the stability of curcumin in the working solution in *in vitro* media temperature are shown in table 4.

Table 4: *In vitro* media temperature stability of curcumin in different DES from working solution

Time (hrs)	*In-vitro* media temperature (37°C±2°C)*		
	Working solution % stability		
	CM-DES	CT-DES	MT-DES
Initial	100.462±0.318	100.397±0.455	100.334±0.252
1	100.429±0.302	100.0990.358	99.499±0.265
2	100.198±0.262	99.636±0.401	98.564±0.417
4	99.835±0.206	99.139±0.303	96.460±0.904
8	99.472±0.151	98.213±0.199	93.654±1.009
12	99.110±0.099	95.631±0.813	91.617±0.963
16	98.714±0.171	92.916±0.845	87.375±1.017
20	98.384±0.151	90.798±0.745	82.398±0.852
24	98.121±0.099	86.759±1.098	77.288±1.004
Acceptance criteria	98-102%		

*Performed three times

CONCLUSION:

In this work, Curcumin standard curve was performed at different pH, based on the results it was confirmed that 50mM sodium dihydrogen ortho-phosphate with 0.5% SLS showed maximum absorbance compared to other pH. An attempt was made to determine the stability of curcumin in three hydrophobic based DES (CM-DES, CT-DES, and MT-DES) in a stock solution and in working solution prepared with 50mM sodium dihydrogen ortho-phosphate with 0.5% SLS. Curcumin in CM-DES was found to be stable in all stability parameters [benchtop (25±3°C), refrigerator (5±3°C), and *in vitro* media (37±2°C) temperature] both in-stock and working solution. Curcumin in CT-DES at benchtop condition was stable in stock solution and in working solution it was stable for 12 hrs. In refrigerator condition, curcumin in CT-DES was found to be stable for 6 days in a stock solution and 4 days in the working solution. In the *in vitro* media condition, it was stable for eight hrs only. Curcumin in MT-DES at benchtop condition was found to be stable for 4 h in a stock solution and only 2 h in the working solution. In refrigerator condition, curcumin was found to be stable for 4 days in a stock solution and 2 days in the working solution. In *in vitro* conditions, it was found to be stable for 2 h only. From this study, it was concluded that 50 mM sodium dihydrogen ortho-phosphate with 0.5% SLS is a best solution for making standard curve. In addition, also from the stability studies Curcumin was found to be stable in CM-DES in both stock and working solutions compared to the other two CT-DES and MT-DES.

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CONFLICT OF INTEREST:

The authors declare no conflict of interest.

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RESEARCH ARTICLE

Evaluation of Antidepressant-like Effect of *Clitoria ternatea* Linn.

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ABSTRACT:
Depressive disorder is a prevalent psychiatric disorder, which affects 21% of the world population. The presently using drugs can impose a variety of side-effects including cardiac toxicity, hypopiesia, sexual dysfunction, body weight gain, and sleep disorder. Ayurvedic medicine may be a powerful weapon given by our nature to cure disease. Considering the importance of plants as sources of drugs even today people are adopting different herbal drugs for the treatment of assorted diseases. During the last decade, there is a growing interest in the therapeutic effects of natural products on mental disorders. This study planned to assess antidepressant like activity of methanolic extract of *Clitoria ternatea* Linn. (fabaceae). Soxhlet extraction method was used for methanolic extraction. Antidepressant activity was studied using forced swimming test (FST) and tail suspension test (TST). Two doses 200 and 400 mg/kg of methanolic extract of flower were selected for testing. Imipramine (10 mg/kg, i.p.) were used as the reference standard drugs. Methanolic extract of *Clitoria ternatea* flower significantly reduced immobility time in both TST and FST. Extract increased the climbing behavior in FST, which is similar to effect observed with imipramine. The results of this study suggest that antidepressant like effect of *Clitoria ternatea* seems to be mediated by an increase in norepinephrine level in synapses. However further study is needed to understand mechanism of action and to isolate the active component responsible for antidepressant like activity.

KEYWORDS: Antidepressant-like effect, *Clitoria ternatea*, Forced swimming test, Tail suspension test, Depression.

INTRODUCTION:
Mood disorders are common in psychiatry and their characteristic is disturbance in regulating emotions. People affected with mood disorders, experience different emotions ranging from irritability to chronic depression. [1] Depression in late life is associated with significant morbidity, including deficits in a range of cognitive functions and considerable influence on functional impairment, disability, decreased quality of life and has a negative effect on the body's recovery from illness, increases the rate of suicide, increases use of health care services and expenses and can result in early death and disturbance in the general state of wellness. [2]

The exact cause of depression is not known. Many researchers believe it is caused by chemical changes in the brain. This may be due to a problem with your genes, or triggered by certain stressful events. More likely, it's a combination of both. [3] There are several types of depression. A person with major depression experiences symptoms of depression that last for more than two weeks. A person with dysthymia experiences episodes of depression that alternate with periods of feeling normal. A person with bipolar disorder, manic-depressive illness, experiences recurrent episodes of depression and extreme elation. A person with Seasonal Affective Disorder (SAD) experiences depression during the winter months, when day length is short. Although the exact cause of depression is unknown, research suggests that depression is linked to an imbalance of the neurotransmitters serotonin, norepinephrine, and dopamine in the brain. Factors that may contribute to depression include heredity, stress, chronic illnesses, certain personality traits (such as low self-esteem), and

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hormonal changes. [4] Depression is a significant contributor to the global burden of disease and affects people in all communities across the world. Today, depression is estimated to affect 350 million people. The World Mental Health Survey conducted in 17 countries found that on average about 1 in 20 people reported having an episode of depression in the previous year. [5] By 2020 it is expected to be the second-leading cause of disability globally. [6] At its worst, depression can lead to suicide, a tragic fatality associated with the loss of about 850 000 lives every year. [7] Animal model (Tail suspension test and Despair swim test) were used for evaluating antidepressant activity. There have many Ayurvedic plants/medicine which are effectively used for the psychiatric disorders.

Ayurveda, being a stream of treatment, can no way ignore the importance of psychic condition of a patient to effectively diagnosis and treat disorders. Medical science admits that the root cause of multiple disorders lies in psychic tensions. Ayurveda has a well-developed branch of psychiatry, which has clinically proven practices of treating mental ailments and also physical problems associated with psychological imbalances. [8]

Ayurveda the traditional system of Indian medicine mentions a number of plant products which can be used in the treatment of psychiatric disorders. The Ayurvedic concept of Rasayana consists of specialized class of drugs which prevent ageing, increase longevity, impact immunity, improve mental functions and vitality to body a number of synthetic drugs are being used as standard treatment for clinically depressed patient, they have adverse effects that can compromise the therapeutic treatment. Thus, it is worthwhile to look for antidepressant from plants with proven advantage and favorable benefit to risk ratio. A number of medicinal plants and medicine derived from these plants have shown antidepressant properties by virtue of combined effect of their medicinal constituents. [9] Synthetic antidepressants are often associated with their anticipated side effects like dry mouth, inability in driving skills, constipation and sexual dysfunction and majority of patients are reluctant to take this treatment. [10]

Clitoria ternatea L. belongs to family Fabaceae and it is commonly known as 'Butterfly pea'. It is a perennial twining herb, found throughout India in tropical areas. [11]Clitoral ternatea comprise 60 species distributed mostly within the tropical belt.[12]Flower is an important part of plant which contains a great variety of natural antioxidants, such as phenolic acids, flavonoids, anthocyanin and many other phenolic compounds. Flower part of plant contain excessive amount of Tannin. [13] The present study was aimed to perform a systematic study and to investigate the possible antidepressant activities of methanolic extracts of *clitoria ternatea* in normal and in experimentally induced depressant animal. Plant show a number of pharmacological effect that include- Antimicrobial effect, Anti-inflammatory antipyretic and analgesic effects, Antidiabetic Effect Wound healing effect, Hepatoprotective effect, Antidiarrheal activity, Immuno-modulator activity, Effects in CNS, Local anesthetic activity. Anticancer activity, Diuretic activity, Gastrointestinal effect, Anti Asthmatic activity, Hypolipidemic effect.[14]

MATERIAL AND METHOD:

Drugs:

Drug (Imipramine), chemicals, instruments, equipment's used in this study were procured from labs of Daksh Institute of Pharmaceutical Science, Chhatarpur (M.P.). All the other solvents and chemicals used are of analytical grade.

Plant material:

Flowers of *Clitoria ternatea* plant were collected from forest area of Bundelkhand region, distt- Chhatarpur (M.P.). They were authenticated by Dr. Pushpendra Kumar Khare, Assistant professor of Botany, Govt. Maharaja PG College, Chhatarpur (M.P.) and were given a specimen no. (041/Bot/2019).

Preparation of Extract:

The flowers of plant were shade dried and then powdered in a mechanical grinder for preparation of extract. The powdered materials of flowers were Soxhlet extracted with 90% methanol for about 8 hr. The extract was concentrated by distilling the solvent to obtain the crude extract. Finally, the extract was evaporated to dryness. The extract, on removal of solvent in vacuum, gave a dark greenish brown semisolid residue. The powdered material or the extracts of the flowers are used for the experimental procedure.

Experimental Animals:

Albino mice (weight 20–25 g) and Albino rats (160-200 g) of either sex are used. Animals were housed under standard conditions of temperature (24 ± 2^0C) and relative humidity (30-70%) with a 12:12 (light: dark) cycle. The animals were given standard diet and water at libitum. All procedures and protocols were approved by Institutional Animal Ethics Committee(Protocol no. DIPS/IAEC/NO: 09/2018-19).

Experimental Group

Group-I treated as Vehicle. (Water)
Group-II treated as Standard drug (Imipramine 10mg/kg)
Group-III treated as Test drug (200 mg/kg)

Group-IV treated as Test drug (400 mg/kg)

METHODS:

Tail Suspension Test:

Animals are transported from the housing room to the testing area in their own cages and allowed to adapt to the new environment for 1 h before testing. They are brought to the laboratory at least one day before the experiment and are housed separately in cages with free access to food and water.4 Group of 6 animals are treated with by intra-peritoneal injection 30 minutes prior to testing. For the test, the mice were suspended on the edge of a shelf about 58 cm above top of table by adhesive tape and placed approximately 1cm from the tip of the tail. The duration of immobility is recorded for periods of 5 min. Mice are considered immobile when they hang passively and completely motionless for at least 1 min.[15]

Despair Swim Test:

Naive rats are individually forced to swim inside a vertical Plexiglas cylinder (height: 40 cm; diameter: 18 cm, containing 15 cm of water maintained at 25 °C). Animals placed in the cylinders. After placing for the first time, animals were highly active initially and they vigorously swimming in circle then they trying to climb the wall or diving to the bottom. Activity begins to fall down and to be interspersed with phases of immobility or floating of increasing length, after 2–3 min of experiment. After 5–6 min, immobility in rats reaches a plateau where the rats remain immobile for approximately 80% of the time. After 15 min in the water the rats were removed and allowed to dry in a heated enclosure (32 °C) before being returned to their home cages. They are again placed in the cylinder after 24 h later of previous experiment and the total duration of immobility is measured during a 5 min test. Floating behavior during in this 5 min period has been found to be reproducible in different groups of rats. Whenever animals remains floating passively in the water in a slightly hunched but upright position, its nose just above to the surface, an animal is judged to be immobile. 4 Group of 6 animals are treated with by intra-peritoneal injection. Test drugs or standard were administered to animals prior one hour to testing. Since experiments with the standard drug (Imipramine) showed that injections 1, 5 and 24 h prior the test gave the most stable results in reducing floating these times are chosen for the experiment.[16]

Statistical Analysis:

Data were analyzed by Graph Pad Prism version 8.1.1 software and presented as mean and SEM values. The statistical tests used one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test.

RESULT:

Table:1 Percentage yield of fresh weight and dried weight of *Clitoria ternatea* Linn flowers.

Plant	Weight (gm)	Dry weight (gm)	Percentage yield
Clitoria ternatea Linn.	35	6.03	17.22

Table:2 Qualitative analysis of the methanolic extracts of *Clitoria ternatea* to screen for the presence of phytochemicals.

S. No.	Phytochemicals	Plant parts
		Flower
1.	Alkaloids	+ve
2.	Flavonoids	+ve
3.	Tannin	+ve
4.	Glycoside	+ve
5.	Resin	+ve
6.	Steroids	-ve
7.	Saponin	-ve
8.	Phenol	-ve

+ Presence of the compound.
- Absence of the compound.

For the Qualitative analysis, we analyzed the methanolic extract of *Clitoria ternatea* flower. We analyzed that, this extract confirmed the presence of flavonoids, tannins, alkaloids, glycosides, resin. Then we have done the Quantitative analysis.

Table. 3 Quantitative analysis of the methanolic extracts of *Clitoria ternatea* for estimation of phytochemicals.

Phytochemicals	Plant parts	Average Estimated Value (mg/gm)
Flavonoids	Flowers	42±0

When we go for the Quantitative analysis for estimation of phytochemicals, we found that, flower extract content 42 mg/gm of flavonoids.

Pharmacological Analysis:

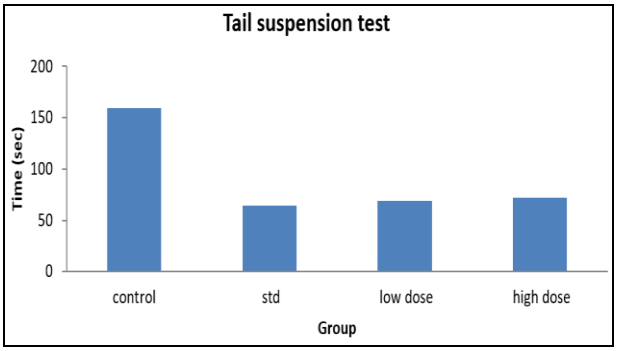
The antidepressant effects of *Clitoria ternatea* (200 and 400 mg/kg) and Imipramine were studied by observing the changes in the duration of immobility in the two models, Forced swim test (FST) and Tail suspension test (TST).

Tail suspension test:

Table: 4 Effect of methanolic flower extract of *Clitoria ternatea* on duration of immobility time in the Tail suspension test

S. no	Groups	Treatment	Tail suspension test Duration of immobility(in sec)
1.	Control group	Vehicle (water)	160.2±2.45
2.	Test group	*Clitoria ternatea*(200 mg/kg)	69.3±1.6**
3.	Test group	*Clitoria ternatea* (400 mg/kg)	74.2±1.26**
4.	Standard group	Imipramine (10 mg/kg)	62.1±1.07***

Values represented as Mean ± SEM **P< 0.05 vs control group for test solutions and for standard group represent the significant value***P<0.001vs control group.

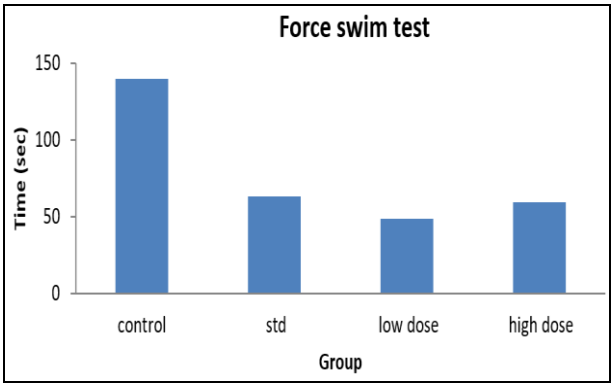


Forced swim test-

Table:5 Effect of methanolic flower extract of *Clitoria ternatea* on duration of immobility time in the Forced swim test

S. no	Groups	Treatment	Forced swim test Duration of immobility (in sec)
1.	Control group	Vehicle (water)	140±1.11
2.	Test group	*Clitoria ternatea* (200 mg/kg)	51.8±1.59**
3.	Test group	*Clitoria ternatea* (400 mg/kg)	59.2±0.76**
4.	Standard group	Imipramine (10 mg/kg)	61.6±0.58***

Values represented as Mean ± SEM **P< 0.05 vs control group for test solutions and for standard group represent the significant value***P<0.001vs control group.



DISCUSSION:

A number of side effects like liver damage, cardiac problems and mutagenesis are associated with the use of antianxiety drugs obtained from synthetic sources. Thus, researchers are exploring natural resources to find out newer and safer natural anxiolytic agents.

We have collected about 50 gm of fresh flower of *Clitoria ternatea*, after shaded drying, we got 35 gm of flowers. Then, flowers subjected for the extraction process by Soxhlet extraction method by using methanol as a solvent. After extraction, we got 6.03 gm of methanolic extract and got % yield of methanolic extract 17.22 %. Preliminary phytochemical studies showed presence of flavonoids and triterpenoids in methanol extract of C. ternatea. The results of present investigations are in agreement with the available literature where flavonoids – kaempferol[17] and apigenin[18] and triterpenoids – α, β-amyrin have been reported to exhibit antianxiety activity. Active compounds found in various pieces of plants have been appeared to have remedial qualities. Utilizing entire plant as a medication would thus be able to be utilized to receive greatest helpful rewards.[19] Flavonoids found in various medicinal plants have been appeared to have antianxiety and depression reducing impact, for example, Hypericum perforatum.[20]

Anxiolytic activity of methanol extract of blue variety of C. ternatea flower was evaluated employing widely used model, i.e., FST and TSM. The FST model was chosen since these are effective, cheap, simple, less time consuming and do not cause much discomfort to the animals while handling[18, 21]. The models are principally based on the observations that exposure of animals to approach–avoidance conflict which is manifested as an exploratory-cum-fear drive.

In TST, *Clitoria ternatea* 200 and 400 mg/kg, (intra peritoneal) produced significant reduction ($p<0.05$ respectively) in the immobility period when compared with that of control group animals that received only the vehicle. The extract (400 mg/kg) was found to be effective and it exhibited activity similar to that of the standard drug Imipramine ($p<0.001$). In FST, *Clitoria ternatea* 200 and 400 mg/kg, (intra peritoneal) produced significant reduction ($p<0.05$ respectively) in the immobility period when compared with that of control group animals that received only the vehicle. The extract (400 mg/kg) was found to be effective and it exhibited activity similar to that of the standard drug Imipramine ($p<0.001$).The above results indicated that methanolic extract of *C. ternatea* possess anti anxiety activities. The CNS depressant activity was also observed at higher dose levels. The traditional claim of *C.ternatea* being used as anxiolytic has been scientifically validated.

It is suggested that methanol extract of C. ternatea may be act via binding to benzodiazepine receptors as agonist[22] increase ascorbic acid level in brain[23], inhibition of γ-amino butyric acid transmission[24] and monoamine oxidase inhibition. Further, it is concluded that triterpenoids or flavonoids may be responsible for antianxiety activity of both varieties of C. ternatea. It can be finally concluded that these phytoconstituent (s) may be isolated using column chromatography to develop anxiolytic agent.

Since ancient times, people have been using a no. of plants in various ways as a source of medicine. From the above preclinical study, we can conclude that

methanolic flower extracts of *Clitoria ternatea* show a significant antidepressant activity in TST and FST models of depression. We believe that *Clitoria ternatea* has the potential to be used as an adjuvant in the treatment of depression and other mood disorders. To gain closer insights into the exact mechanism of its action, further research is required.

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CONFLICT OF INTEREST STATEMENT:

We declare that we have no conflict of interest.

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RESEARCH ARTICLE

Acid Catalyzed Synthesis of substituted-n-((1, 3-diphenyl-1h-pyrazol-4-yl) methyl) benzenamine

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ABSTRACT:

A new derivative synthesis of Substituted-N-((1, 3-diphenyl-1h-pyrazol-4-yl) methyl) benzenamine has attempted by using commonly available key starting materials Acetophenone (1) and substituted phenyl hydrazine in presence of sulfuric acid catalyst and alcohol solvent to afford potential antiviral active analogues. The synthesis proceeds through Vilsmeier-Haack reaction followed by reductive amination by using sodium borohydride.

KEYWORDS: (1,3-diphenyl-1H-pyrazol-4-yl) methyl) benzenamine, Vilsmeier-Haack reaction, reductive amination with sodium borohydride.

INTRODUCTION:

The author proposed 1,3-diphenyl-1H-pyrazol-4-yl-methylbenzenamine synthesis methodologies were reported in few journals[1-4], but none of the synthetic procedure having adequate commercial or scalable process information with respect to the cost of production, process safety and environmental requirements.

Heterocyclic compounds have attracted considerable attention in the design of biologically active molecules and advanced organic materials[5.] Hence a practical method for the preparation of such compounds is of great interest in synthetic organic chemistry.

Pyrazoles and their derivatives, a class of well-known nitrogen containing heterocyclic compounds, occupy an important position in medicinal and pesticide chemistry having a wide range of bioactivities such as anti-microbial,[6] anti-cancer,[7] anti-inflammatories,[8] antidepressants,[9] anti-convulsant[10], [11] anti-hyperglycemic,[12] anti-pyretic,[13] anti-bacterial,[14] antifungal activities,[15] CNS regulants,[16] and selective enzyme inhibitory activities[17].

It has been found that pyrazoles are also known as inhibitors and deactivators of liver alcohol dehydrogenase and oxidoreductases. It has been shown *in vivo* that some of the pyrazole derivatives have appreciable anti-hypertensive activity. These compounds also exhibit properties such as cannabinoid hCB1 and hCB2 receptor, inhibitors of p38 Kinase, and CB1 receptor antagonists [18, 19, 20]. As early as in 1884, Knorr discovered the anti-pyretic action of a pyrazole derivative in man, he named the compound antipyrine. This stimulated the interest in pyrazole chemistry[21, 22, 23].

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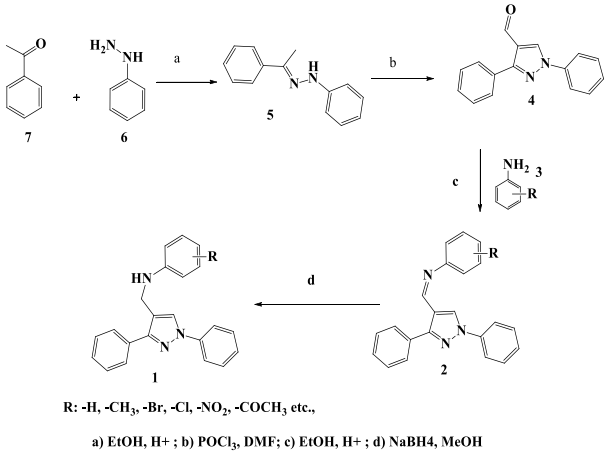
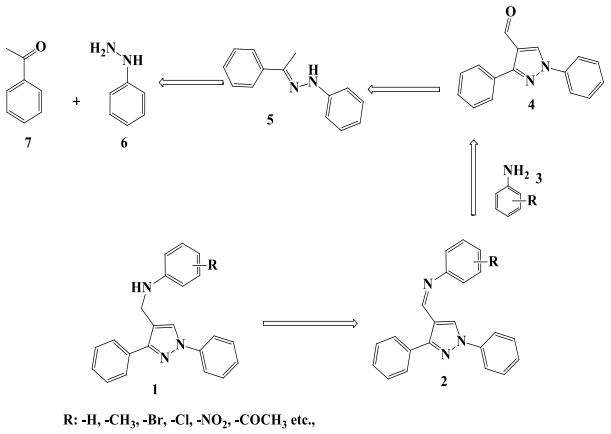


Scheme 1: Proposed route of synthesis



Scheme 2: retro synthetic approach of proposed route

RESULTS AND DISCUSSION:

MATERIALS AND METHODS:

Synthesized materials melting points were determined using a manual POLMON electro thermal apparatus (Range 0–300°C) in glass capillary tubes and are uncorrected. IR spectra were recorded on a Perkin-Elmer FT IR 1600. 1H & 13CNMR spectra were recorded on a Bruker-400 MHz spectrometer and are expressed in a using TMS as internal reference. All the reactions were monitored by thin layer chromatography over pre-coated silica gel plates, using UV lamp, iodine vapors or KMnO4 spray as developing agents. The purification was carried out by using 60-120 mesh silica gel by column chromatography (obtained from Merck) with a suitable eluting system with various polarity mobile phase ratio's such as n-Hexane, Ethyl acetate, Chloroform, Chloroform: Methanol.

The biological activity of chemically synthesized molecules was studied by Inhibition zone diameter calculation method against *Candida Albicam, Aspergillus Niger cell cultures* with reference drug standard Fluconazole.

Inoculum Preparation:

Potato Dextrose Broth PDB (commercially available).

Procedure for Antifungal activity:

Weigh 80mg of Potato Dextrose Broth powder. Add to a 1 Liter Erlenmeyer flask containing 800mL of water**,** stir to dissolve into solution. Transfer the solution to a 1 Liter volumetric flask and dilute to 1 Liter. Transfer the 1 Liter media solution to a 1 Liter media storage bottle and label with appropriate information, Autoclave the solution at 121°C for 15 mines to ensure that the LB is sterilized of all foreign matter and contaminants. After cooling of the broth, using a sterile pipette tip, select a single spore from a pure culture plate. Drop the tip into the liquid PDB and swirl. Incubate fungal culture at 25°C for 5-7 days in shaking incubator at 80rpm.

Preparation of Compound Stock Solution:

1mg/ml stock solution of each extract was prepared in a suitable solvent.

Preparation of Potato Dextrose Agar PDA: (Agar Well Diffusion):

Weigh PD Agar as per requirement and dissolve in distilled water as per the guidelines given by manufacturer. Autoclave the media at 121°C for 15 minutes, after autoclaving, pour the media in sterile Petri plates and keep for solidification.

Procedure:

Sterilize a cork borer by autoclaving or disinfect it by rinsing in alcohol followed by sterile water. On a PD agar plate aseptically punch 5 holes using a cork borer, using a marker, mark the underside of the Petri to label the wells. Aseptically spread 20µl of the indicator organism onto the MH agar plate. Let the plate stand for 5 minutes, Place 10µl, 25µl, 50µl, 100µl and 150µl of the extract in the appropriate wells. Incubate the plate at 25°C for 72-96 hrs. Measure the zones of inhibition in millimeters, using a ruler on the underside of the plate. Same Procedure is repeated with Fluconazole as a standard with concentrations of 10µg, 25µg, 50µg, 100 µg, 150µg

EXPERIMENTAL:

Experimental procedure:

Step-I: General Procedure for Substituted (E)-1-phenyl-2-(1-phenylethylidene) hydrazine derivatives (5):

In a 250ml round bottom flask taken a mixture of Acetohenone (1.0mmol), substituted phenyl hydrazine (1.2mmol), and 1% Sulfuric acid used as catalyst in absolute ethanol (5V) at room temperature. Stir the reaction mass for 4-5 hours, after completion of TLC, the solvent removed under reduced pressure and then add two volumes of water extract the aqueous layer with

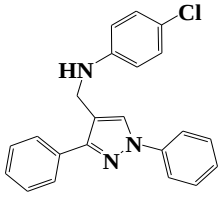
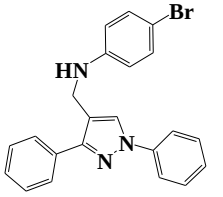
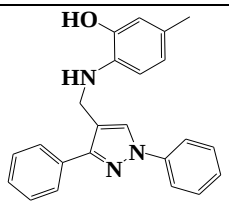
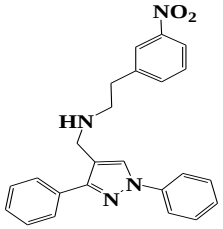
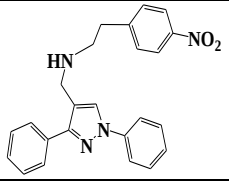
methylene dichloride (3X2 volumes) and then separate the layers, organic layer washed with brine solution, finally dried over anhydrous sodium sulphate. The obtained solid after distillation of dried organic layer was taken for purification in hexanes (3-4 volumes) to give desired substituted (E)-1-phenyl-2-(1-phenylethylidene) hydrazine derivatives (5p-5x) yield range: 70-90%

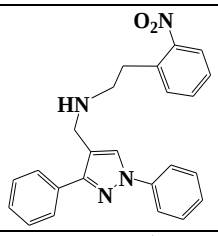
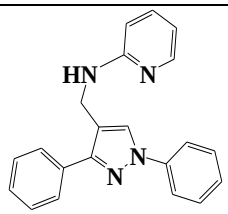
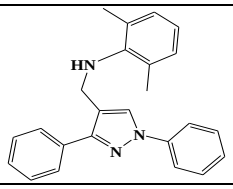
Step-II: General procedure for alpha bromination of 1,3-diphenyl-1H-pyrazole-4-carbaldehyde derivatives (4):

To a cooled solution of substituted (E)-1-phenyl-2-(1-phenylethylidene) hydrazine derivative (1.0mmol) individual compounds were taken in Phosphorous oxy chloride ($POCl_3$ 3.0 equivalents), added anhydrous Dimethyl Formamide (1.02mmol) portion wise to above solution at 0 to 5^0C (it took 20 min) and then slowly rose the temperature up to 60 to 70^0C, and Main titan the reaction mass under gentle stirring for 3-5 hours.

Remove the solvent under reduced pressure after TLC complies, the obtained residue taken in aqueous Sodium bicarbonate solution and extract with Methylene dichloride (3 X 5V). The combined organic layers was washed with brine and dried over anhydrous sodium sulphate, the solid obtained end products of substitutes 1, 3-diphenyl-1H-pyrazole-4-carbaldehyde yield range 65-85%

Table-1: Derived analogs details and their individual characterization data

S. No.	Chemical Name	Chemical structure	Mol. Wt.	Code	Spectral characterization Data
1	4-Chloro-N-((1,3-diphenyl-1H-pyrazol-4-yl)methyl)benzenamine		359.85	Sp	1H NMR (DMSO-D6): δ 8.56(S, 1H), δ 7.85(d, 2H), δ 7.50(t, 3H), δ 7.45(m, 2H), δ 7.37(t, 1H), δ 7.30(t, 1H), δ 7.09(d, 2H), δ 6.68 (d, 2H), δ 6.18 (t,1H),δ 4.22(d, 2H), ^{13}C NMR : 153.3, 152.8, 150.7,147.4, 139.4, 138.8, 132.8,131.8, 129.9, 127.9, 126.1, 122.6, 119.6, 118.5,113.6, 38.1 MASS: 359.85 (m+), 358.29(m-H)+
2	4-Bromo-N-((1,3-diphenyl-1H-pyrazol-4-yl)methyl)benzenamine		404.30	Sq	1H NMR (DMSO-D6): δ 8.56(S, 1H), δ 7.85(d, 2H), δ 7.77(d, 2H), δ 7.50(t, 2H), δ 7.45(t, 2H), δ 7.37(t, 1H), δ 7.30(t, 1H), δ 7.22(d, 2H), δ 6.64 (d, 2H), δ 6.20 (t,1H),δ 4.22(d, 2H), ^{13}C NMR :150.3, 147.7, 139.4, 132.7, 131.3, 129.5, 128.5, 127.7, 126.1, 122.4, 118.5, 114.1,106.7, 38.0 MASS: 404.30 (m+), 404.22(m-H)+
3	2-((1,3-diphenyl-1H-pyrazol-4-yl) methyl amino)-5-methylphenol		355.43	Sr	1H NMR (DMSO-D6): δ 7.98 (S, 1H), δ 7.82 (d, 2H), δ 7.80 (d, 2H), δ 7.44 (t, 4H), δ 7.36 (t, 1H), δ 7.27 (d, 1H), δ 6.62 (t, 1H), δ 6.52 (s, 1H), δ 6.46 (t, 1H), δ δ 4.37 (s, 2H), 2.24 (S,3H), MASS: 355.43 (m+), 356.03 (m-H)+
4	2-(3-nitrophenyl)-N-((1,3-diphenyl-1H-pyrazol-4-yl)methyl)ethanamine		398.60	St	1H NMR (DMSO-D6): δ 8.45 (S, 1H), δ 7.82 (d, 2H), δ 7.94 (d, 1H), δ 7.49 (t, 3H), δ 7.25 (m, 5H), δ 3.77 (s, 2H), δ 2.92 (t, 2H), 2.83 (t, 2H), MASS: 398.46 (m+), 397.2(m-H)+
5	2-(4-nitrophenyl)-N-((1,3-diphenyl-1H-pyrazol-4-yl)methyl)ethanamine		398.60	Su	1H NMR (DMSO-D6): δ 8.61 (S, 1H), δ 7.90(d, 2H), δ 7.80(d, 2H), δ 7.53(m, 6H), δ 7.39(t, 2H), δ 7.31(t, 2H), ,δ 4.22(d, 2H), δ 3.3 (t,4H) MASS: 398.46 (m+), 397.3(m-H)+

6	2-(2-nitrophenyl)-N-((1,3-diphenyl-1H-pyrazol-4-yl)methyl)ethanamine		398.60	Sv	1H NMR (DMSO-D6): δ 8.66(S, 1H), δ 7.87(d, 2H), δ 7.81(d, 2H), δ 7.51(t, 2H), δ 7.35(m, 6H), δ 7.30(t, 1H), δ 4.63 (s, 2H), MASS: 398.46 (m+), 397.1 (m-H)+
7	N-((1,3-diphenyl-1H-pyrazol-4-yl)methyl)pyridin-2-amine		419.32	Sw	1H NMR (DMSO-D6): δ 9.22(S, 1H), δ 8.66(s, 1H), δ 7.88(dd, 2H), δ 7.81(d, 2H), δ 7.66 (d, 1H), δ 7.51(m, 5H), δ 7.37 (m, 4H), δ 4.63(d, 2H), δ 2.27 (s, 1H), δ 6.20 (t,1H),δ 4.22(d, 2H), MASS: 326.39 (m+), 327.2 (m-H)+
8	2,6-dimethyl-N-((1,3-diphenyl-1H-pyrazol-4-yl)methyl)benzenamine		353.46	Sx	1H NMR (DMSO-D6): δ 8.65 (S, 1H), δ 7.44 (d, 1H), δ 7.84 (m, 1H), δ 7.66 (d, 1H), δ 7.53(m, 3H), δ 7.37(m, 1H), δ 6.78(d, 4H), δ 6.40 (t, 2H), δ 4.45 (s, 2H), ,δ 2.05(S, 6H), MASS: 353.19 (m+), 352.3 (m-H)+

Step-III: General Procedure for Substituted (Z)-N-((1, 3-diphenyl-1H-pyrazol-4-yl) methylene) aniline (2):

Refluxed a mixture of substituted 1, 3-diphenyl-1H-pyrazole-4-carbaldehyde (1.0 mmol) compounds (4a-4x) and substitutes anilines (3a-3x) in absolute ethanol (5 volumes) in presence of acid catalyst for 8 hours. Remove the solvent under vacuum once TLC get complies, the crude obtained was purified in n-hexane to afford desired products (2a- 2x). Yield range: 40-60%.

Step-IV: General Procedure for Substituted N-((1, 3-diphenyl-1H-pyrazol-4-yl) methyl) aniline (1) (Sp-Sx):

In a 500mL round bottom flask individual compounds of 2a-2x (1.o mol) was taken in dry methanol (6Volumes), Sodium borohydride (0.5 moles) and catalytic amount of sodium hydroxide. The reaction mixture was stirred for 45 minutes at room temperature to complete the reduction reaction. Once reaction gets complies quench with moisture sodium sulfate and then remove the excess methanol under reduced pressure, further extracted with Methylene dichloride (3 X 5V). The combined organic layers was washed with brine and dried over anhydrous sodium sulphate, the resulting solid materials were purified by n-heptanes to afford targeted compounds (Sp-Sx).

Table-2: In-vitro biological activity test results of synthesized new chemical entities against selected antifungal cultures

S. No	Compound Code	*Candida Albicam*					*Aspergillus Niger*				
		Zone Inhibition diameter in µg/mm					Zone Inhibition diameter in µg/mm				
		10	**25**	**50**	**100**	**150**	**10**	**25**	**50**	**100**	**150**
1.	Sp	-	2	-	-	-	-	-	-	-	**2**
2.	Sq	-	-	-	-	-	-	-	1	-	-
3.	Sr	-	-	1	-	-	-	-	-	-	-
4.	Ss	3	-	-	-	-	-	5	-	-	-
5.	St	-	-	-	-	-					
6.	Su	-	-	-	2	-	-	-	-	-	-
7.	Sv	-	-	6	-	-	-	-	-		
8.	Sw	-	-	-	-	-	-	-	-	3	-
9.	Sx	4	8	10*	12*	12*	-	-	-	-	-
10.	Fluconazole(**Std**)*	-	-	**12**	**18**	**18**	-	-	**6**	**10**	**15**

CONCLUSION:

The synthetic methodology which is described in this research and biology (in-vitro) of derived substituted biphenyl aminothaizoles analogues such as Sp, Sq, Sr, Ss, St, Su, Sv, Sw and Sx were successful. The Anti-fungal activity of these compounds has been studied against cell cultures like Candida *Albicam, Aspergillus Niger*. Among all synthesized new molecular entities S_X is said to be promising molecule which exhibits more potentiality towards anti-fungal activity and equivalent

to the standard drug substance Fluconazole. Hence, it is believed that these molecules having more scope to study further stage of biology studies to understand the routing to a new drug moiety for future medical applications.

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RESEARCH ARTICLE

Chemical Composition and Antiproliferative and Antioxidant Activities of Methanolic Extract of *Alcea setosa* A. Malvaceae

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ABSTRACT:

*Alcea setosa A. (*Malvaceae) is a wild plant that grows in Jordan and have several traditional medicinal uses. This study aims to collect and chemically analyze the methanolic extract from *Alcea setosa* A. from Jordan and to evaluate its cytotoxic and antioxidant activity against human breast cancer cells (T47D), colorectal adenocarcinoma cells (CACO2), and normal human fibroblasts (MRC5). The extract was extracted by methanol solvent and analyzed by liquid chromatography coupled with a mass spectrometer. Cell viability was assessed using trypan blue, neutral red, and MTT assays, and antioxidant activity was evaluated using DPPH scavenging activity assay. A total of 290 compounds, 12 among which were identified when compared to available standards, the extract contained six flavons derivatives, Two fatty acids, one ketone derivative, one flavonol derivative, one organic acid, and one coumarin derivative. The results also revealed that the IC50 values of the viability assays were higher among normal cells compared to the human cancer cell lines, and the viability inhibition was significant at higher concentrations compared to untreated cells. Nevertheless, moderate antioxidant activity was observed for the extract in the DPPH scavenging activity test. To sum up, this study indicates that samples of *A. setosa* collected from Jordan is likely to be an effective antioxidant, is optimistically potential to be utilized in breast and colon cancers treatment due to its preferential cytotoxicity against cancer cells.

KEYWORDS: *Alcea setosa*, Methanolic extract, Cell viability, Antioxidant activity.

INTRODUCTION:

The various phytogeographical areas of Jordan are rich in a variety of wild plants[1]. Twenty percent of all Jordan's plant species are medicinal plants that are used by local inhabitants and in pharmaceutical industry[2]. There are more than 2500 plant species, 868 genera, and 142 families[1,3-5]. Medicinal plants that are rich in bioactive compounds are abundant in Jordan's geographic regions. In Jordan, more than 485 species of medicinal plants and 99 families have been identified as therapeutic agents[6].

Medicinal plants in Jordan can be exemplified by *Varthemia iphinoides*, *Bongardia chrysogonum*, *Ajuga chia*, *Salvia palaestina*, *Micromeria nervosa* and *Rubus sanguineus* and many others [2,3,7,8].

There has been increasing interest in the extracts of medicinal plants because they have potential of numerous benefits as a source of drugs to all mankind. Nowadays, the discovery of natural and plant -based products that can prevent the growth of cancer cells with minimum side effects, is urgent and essential[53]. The medicinal value of these plants lies on bioactive phytochemical constituents that produce definite physiological action on the human body. Examples on the most important bioactive phytochemical constituents are alkaloids, essential oils, flavonoids, tannins, terpenoids, saponins, phenolic compounds and many more[9]. A wide variety of free radical scavenging

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molecules are found in plants, such as flavonoids, anthocyanins, carotenoids, vitamins, dietary glutathione, and endogenous metabolites are rich in antioxidant activities[46].

A variety of plant families in Jordan has been investigated for their anti-tumoral effect[10-14]. In Jordan, cancer is the second leading cause of death after heart disease, and breast and colon cancers are among the most prevalent types[15]. The genus *Alcea* includes more than 40 subspecies[16]. Some of them are not studied for their health-related properties, and the investigated ones were not given enough attention. *A. setosa* is prevalent in east Mediterranean area[16]. It is a perennial herb that is rich of pollen and nectar and characterized with pink flowers and 1–2 m tall stalks with a diameter of 8–13 cm[17,41].

The plant is traditionally orally consumed in some countries as a diuretic, expectorant, and emollient, to treat inflammation and asthma, and to relieve stomach and intestine pain[18,19]. The roots are used for cough and tooth inflammation[3]. Methanolic extract from flowers of *A. setosa* was found to be compromised mainly of polyphenols and flavonoids[20]. Leaves methanolic extract from *A. setosa* has been studied for its anti-tumor potential against mouse fibrosarcoma cells, and no cytotoxic activity was found[21]. *A. setosa* has been evaluated for its antioxidant activity only twice earlier. In either study, it did not show any significant effect[20,22]. With regards to the phytochemical analysis of essential oil and methanolic extract, no previous studies in the available literature were found to be performed on *A. setosa*.

MATERIAL AND METHODS:

Plant material:

A. setosa flowers were collected from Mahis and Wadi Al- Seer (31°57'26.2"N 35°50'46.1"E 31°57'35.4"N+ 35°50'17.6"E) in North western Jordan in May, 2020. The plant was taxonomically and authentically identified by Professor Sawsan Oran; a plant taxonomist, (Department of Biological Sciences, the University of Jordan). Voucher specimens have been deposited at the herbarium of the Department of Biological Sciences-University of Jordan [specimen 4].

Plant Extraction:

The flowers of *Alcea setosa* were dried at room temperature (23-25 °C) for (2-3) weeks at botany research lab at the department of biological sciences at the University of Jordan; dried plants were powdered using an electrical blender. The powdered plant then exposed to methanolic extraction. 100 gm of each powdered plant was dissolved in 950 ml methanol and stirred using hotplate magnetic stirrer. The solvent left for seven days then the extraction filtered by Whitman no.1 filtered paper. The filtrated solvents were then evaporated using rotary evaporator at 60 °C. The evaporated extract was kept in refrigerator for further study (stock extracts)[23].

Identification of extract components:

For stock solution preparation, an appropriate amount of *Alcea setosa* methanolic extract was dissolved with 2.0 ml of Dimethyl sulfoxide-DMSO (analytical grade), then completed to 50 ml by Acetonitrile. The sample was centrifuged at 4000 rpm for 2.0 min, 1.0 ml was taken and transferred to auto-sampler and 3.0 µl was injected. for identification of exact MS and retention time. All the other reagents, Acetonitrile, methanol, water, and formic acid used were LC/MS grade. All standards used for identification of ms/z and the retention time.

A Bruker Daltonik (Bremen, Germany) Impact II ESI-Q-TOF System equipped with Bruker Dalotonik Elute UPLC system (Bremen, Germany) was used for screening compounds of interest. Standards for identification of m/z with high resolution Bruker TOF MS and exact retention time of each analyte after chromatographic separation were used.

Cell culture:

Human colorectal adenocarcinoma cells (CACO2), breast cancer cells (T47D), and normal human fibroblasts (MRC5) were purchased from American Type Culture Collection Company (ATCC). The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) mixed with 10% fetal bovine serum (FBS), 1% L-glutamine and penicillin (100 U/ml) and incubated in a humidified atmosphere of 5% CO_2 at 37°C. After three days of incubation the cells were harvested by trypsin-EDTA solution and incubated for 10 minutes. 3 ml of culture media was added to the flask, and then the cells were transferred to a tube and vortexed. Finally, the cells were counted, and viability test was performed by trypan blue dye test. The cells were seeded in multi-well plates at the appropriate density recommended for each test[35,37].

Cytotoxicity Assays

Trypan exclusion assay:

Trypan blue assay is one of the earliest and most common methods for counting cells and determining increase or decrease in number of viable cells to indicate proliferation activity[38]. Viable cells with intact plasma membranes that are not destructed by the extract remain clear and exclude the blue dye, while nonviable cells with ruptured membrane, regardless of death mechanism, are stained blue under the light microscope. Cells were seeded at high concentration (1.0 x10^4

cells/ml) in 96-well plates and incubated at 37°C to allow for cell attachment. Then 100 µl of drug mixed with media was added to each well and allowed to incubate for 72 hours. After 72 hours, the contents of the wells were emptied by trypsinization and 25 µl of the cell suspension and 25 µl of trypan blue dye (sigma) were mixed in a mixing well. After 30 seconds, 25 µl of the mixture was added to a hemocytometer and observed under microscope. The number of stained cells and the total number of cells were counted. The IC50 indicating a net loss of cells following treatment was calculated from:

[(*Ti*-*Tz*)/*Tz*] x 100 = – 50, where *Ti* is the initial cell count and *Tz* is the final cell count[38].

Neutral Red Assay:

Neutral red assay is one the most common employed tests for the detection of cell viability following exposure to anticancer substances. The assay is based on detecting the accumulation of the neutral red dye in the lysosomes of viable, uninjured cells[39]. According to previous procedure[40], about 10,000 cells from each cell line were seeded in each well of 96-well plates and incubated overnight at 37°C and 5% CO_2. The extract of *A. setosa* was prepared by mixing 16 µl of the extracts stock (25 mg/ml) with 984 µl media, and using the equation: C1*V1 = C2*V2. A serial dilution of (800, 400, 200, 100, 50) µg/mL were prepared, and incubated again for 24 hours. Doxorubicin was used as positive control. Then, the cells were washed with phosphate-buffered saline PBS (1X) and the supernatant was discarded. A total of 100 µl neutral red (NR) solution (50 µg/ml) was added and incubated at 37°C for 2 hours. NR then was removed, and wells were washed with PBS, and after 10 minutes, absorbance was detected by a dual-wavelength UV spectrometer at 520 nm with a 650 nm reference wavelength. The percentage of antiproliferative activity compared to the untreated cells was determined as:

% Antiproliferative activity = [100 × (Absorbance of untreated group−Absorbance of treated group)]/Absorbance of untreated group.

MTT cytotoxicity assay:

The diluted extract of *A. setosa* (25 mg/ml) was added to about 10,000 cells from each cell line (T47D, CACO2, and MRC5) at final concentrations of 800, 400, 200, 100, 50, 25, 12.5, 6.25 and 3.125 µg/ml, and the cells were incubated for 72hrs. After exposure, 3-(4,5-dimethylthiazol-2w-yl)-2,5-diphenyl tetrazolium bromide (MTT) was added to the wells and incubated for another 4 hours. 100 ul of DMSO solution was then added to solubilize the MTT crystals before reading optical density (OD) by multi-well plate reader (Bio-Tek Instrument, USA) at 570 nm using a reference wavelength of 630 nm[35,37,50,51]. Additionally, doxorubicin at final concentrations of 0.1, 0.5, 1 ,5, 10, 25, 50, 100, 200 µg/mL was used as positive control. The percentage of viability was calculated as the absorbance ratio between treated and untreated (control) wells; hence the absorbance of untreated cells was considered as 100%. Percentage of growth inhibition of cells was calculated as follows: % Inhibition = 100 − (Treated OD/Non-treated OD) × 100)[21]. IC50 values were calculated as concentrations that exhibited 50% inhibition of proliferation on the tested cell line.

Antioxidant activity:

Following a published protocol[33], the 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity assay was employed to measure the antioxidant activity. DPPH is useful to assess antioxidant activity of specific compounds of extracts, and is a stable free radical reactive with substance able to donate a hydrogen atom[49]. Briefly, a volume of 200 mL of 400, 600, 800, and 1000 µg/ml of the extract or 100, 50, 25, 12.5, 6.25, 3.125, and 1.56 ug/ml ascorbic acid (standard) were prepared in methanol and added to 2 mL DPPH (0.21 mM in 95% ethanol). The mixture was shaken, left for 60 min at room temperature in the dark, and the absorbance was detected at 517 nm in a spectrophotometer. The percentage of DPPH inhibition were calculated using the following equation: percentage of inhibition = [(*Ac* -*As*)/(*Ac*)] x 100, where *Ac* is the absorbance of the control reaction and *As* is the absorbance of the sample reaction. The sample concentration (in 1 mL reaction mixture) providing 50% inhibition (IC50) was estimated by plotting percentages of inhibition against concentrations of sample.

Statistical analysis:

All assays were conducted in triplicate. The results were expressed as mean ± SD, and the differences between the means were tested for significance in the statistical analysis software IBM SPSS Statistics Version 23 (Armonk, New York, USA) using one-way analysis of variance (ANOVA) followed by Tukey post-hoc test. The significance level was set at $P < 0.05$.

RESULTS AND DISCUSSION:

***A. setosa* composition:**

The methanolic extract of *A. setosa* flowers gave an amount of (~ 5g) of paste. The extract was analyzed by means of Liquid Chromatography–Mass Spectrometry which combined the physical separation capabilities of liquid chromatography with the mass analysis capabilities of mass spectrometry. The separated compounds were successfully identified by applying MS data library matching coupled with comparison standards for identification of m/z with high resolution and exact retention time of each analyte after chromatographic separation. Results shown in table 1 represents the components of methanolic extract from *A. setosa*.

Table 1: Chemical composition of *Alcea setosa* methanolic extract analyzed by LC-MS.

No.	Name	m/z meas.	RT [min]	Molecular Formula	Area of peaks
1	3,5-Dimethoxy-4-hydroxyacetophenone (ketone)	195.0613	4.92	C10H12O4	25302
2	3-Rha-7-Rha Quercetin (NMR)(flavonol derivative)	593.1463	5.55	C27H30O15	1570084
3	Apigenin-7-O-glucoside (Apigetrin)(Flavone derivatives)	431.0941	7.54	C21H20O10	41321
4	FAHFA 18:1; FAHFA 2:0/16:1; [M-H]- (Fatty acids)	311.2183	15.94	C18H32O4	175079
5	FAHFA 18:2; FAHFA 2:0/16:2; [M-H]- (fatty acids)	309.203	14.78	C18H30O4	125602
6	Genistein (isoflavones derivative)	269.0425	9.94	C15H10O5	413195
7	Hispidulin (flavone)	299.0527	10.79	C16H12O6	146875
8	Isorhoifolin (flavone)	577.1516	6.33	C27H30O14	2035325
9	Kaempferol-3-O-glucoside (Flavone derivatives)	447.0889	6.56	C21H20O11	744671
10	Salicylic acid (organic acid)	137.0224	5.75	C7H6O3	60954
11	Scopoletin (coumarin derivative)	191.0327	5.07	C10H8O4	22262
12	Tiliroside (flavone derivative)	593.1254	9.06	C30H26O13	339072

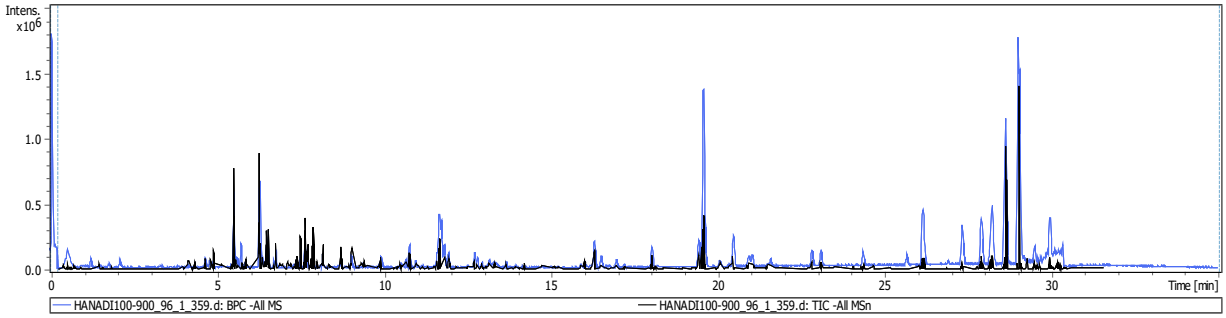

Fig. 1: LC-MS chromatogram of *Alcea setosa* methanolic extract

Table 2: IC_{50} values (mean ± SD µg/ml) of *A. setosa extract* from three cytotoxicity assays.

Cytotoxicity assay	Treatment	Cell line		
		Caco II	T47D	Fibroblasts (MRC5)
Trypan blue	ME	74.14 ± 0.42	110.3 ± 0.36	162.4 ± 0.26
Neutral Red	ME	134.4 ± 0.26	164 ± 0.21	200 ± 0.36
MTT assay	ME	138.4 ± 0.32	142.6 ± 0.23	446.3 ± 0.42
	Doxorubicin	4.5 ± 0.36	2.66 ± 0.44	6.3 ± 0.09

A total of 290 compounds, 12 among which were identified when compared to available standards, belonging to various metabolite classes and their derivatives, were characterized in *A. setosa* extracts. Fig. 1 shows the base peak chromatograms (BPCs) of the extract analyzed. Six flavons derivatives, Two fatty acids, one ketone derivative, one flavonol derivative, one organic acid, and one coumarin derivative.

There is only one study related to genus *Alcea* reporting chemical content by HPLC. In this study, the major component in the quantitative analysis of methanol extracts of *A. pallida* and *A. apterocarpa* species by HPLC was determined as salicylic acid. There are very few studies related to the genus and no studies related to this species[24]. Samples of *A. setosa* from Palestine have been tested and methanolic extract was determined to have a total Phenolic content (GAE mg/g) of 2.01 ± 0.16 and a total flavonoid content (QE mg/g) of 0.52± 0.03[20]. No previous studies in the available literature were found to be performed on *A. setosa*, with regards to the phytochemical analysis of methanolic extract using LC/MS.

Cytotoxicity Assay:

The cytotoxicity of the methanolic extract of *Alcea setosa* on human colon carcinoma cell line and human breast cancer cell line compared to normal human fibroblast cell line was determined using three *in vitro* cytotoxicity/ cell viability assays; one dye exclusion test (trypan blue) and two colorimetric methods (MTT and neutral red). *Alcea setosa* extract exhibited a significant inhibitory action on all 3 cell lines. However, the IC50 was significantly lower for Caco-II and T47D cell lines when compared to fibroblasts. In contrast to MTT and Neutral red assays, Trypan blue exclusion test is based on the fact that live cells possess intact cell membranes that exclude certain dyes, such as trypan blue or Eosin, whereas dead cells do not[48].

Table 2 illustrates the IC50 values calculated for the tested methanolic extract against the three cell lines. Also, doxorubicin's IC50 values were calculated from MTT test and they ranged between 2.66 and 6.3 µg/ml, which are consistent with the normal values reported in the literature for this cytotoxic drug[44,45,36], and that confirms the accuracy of the experiments.

The results obtained from trypan blue assay confirm the preferential cytotoxicity of the extract on cancer cells compared to normal fibroblasts; IC50 was 74.14 ± 0.42 µg/ml for Caco II cells and 110.3 ± 0.36 µg/ml for T47D cells, compared to 162.4 ± 0.26 µg/ml in the case of the Fibroblasts (MRC5). With regards to the neutral red and MTT assays, the table also shows that the extract demonstrated higher inhibitory effect on the growth of the cancer cell lines, compared to the normal cells; neutral red values of IC50 were 134.4 ± 0.26, 164 ± 0.21, and 200 ± 0.36 µg/ml for Caco II, T47D and MRC5 cell lines, respectively, and the values from MTT assay were 138.4 ± 0.32, 142.6 ± 0.23, and 446.3 ± 0.42 µg/ml. Our results showed decreased cytotoxic effect on fibroblast normal cell line with IC50 6.3 ± 0.09, which agrees with the findings that Doxorubicin lacked selective cytotoxicity in fibroblasts cells[43].

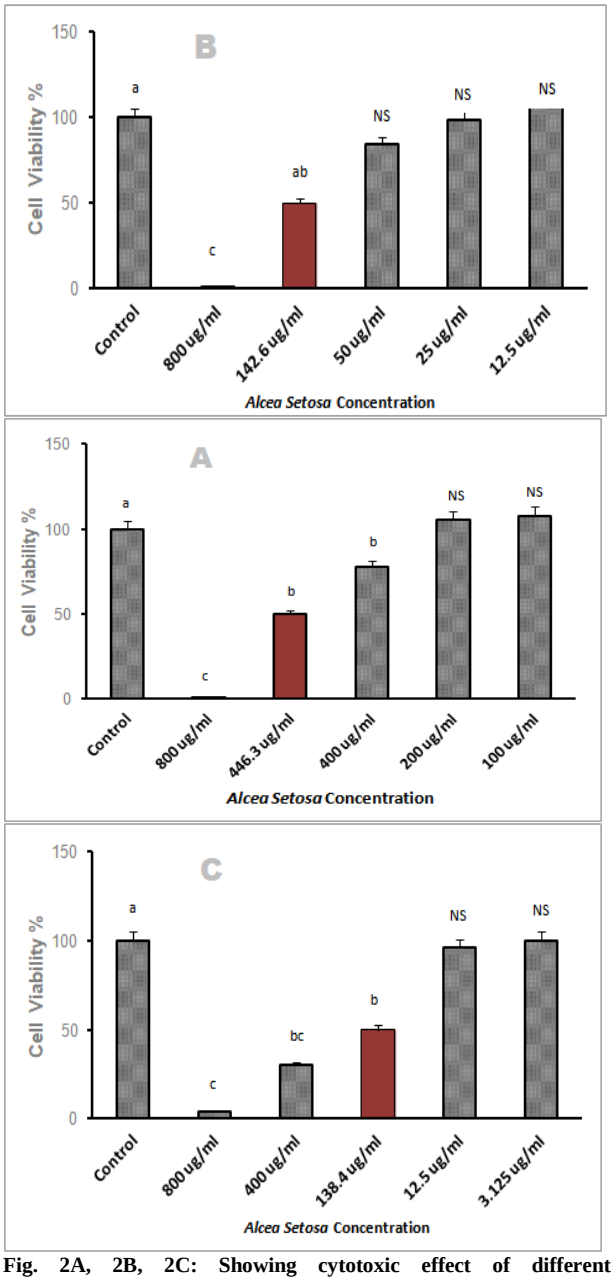

Fig. 2A, 2B, 2C: Showing cytotoxic effect of different concentrations of *Alcea setosa extract* on fibroblast, T47D and Caco cell lines, respectively by MTT assay. IC50 is denoted in red. Data represents at least 3 independent experiments. The error bars represent SD. Different letters represent Tukey's posthoc test to show significant effect compared to control, NS; non-significant.

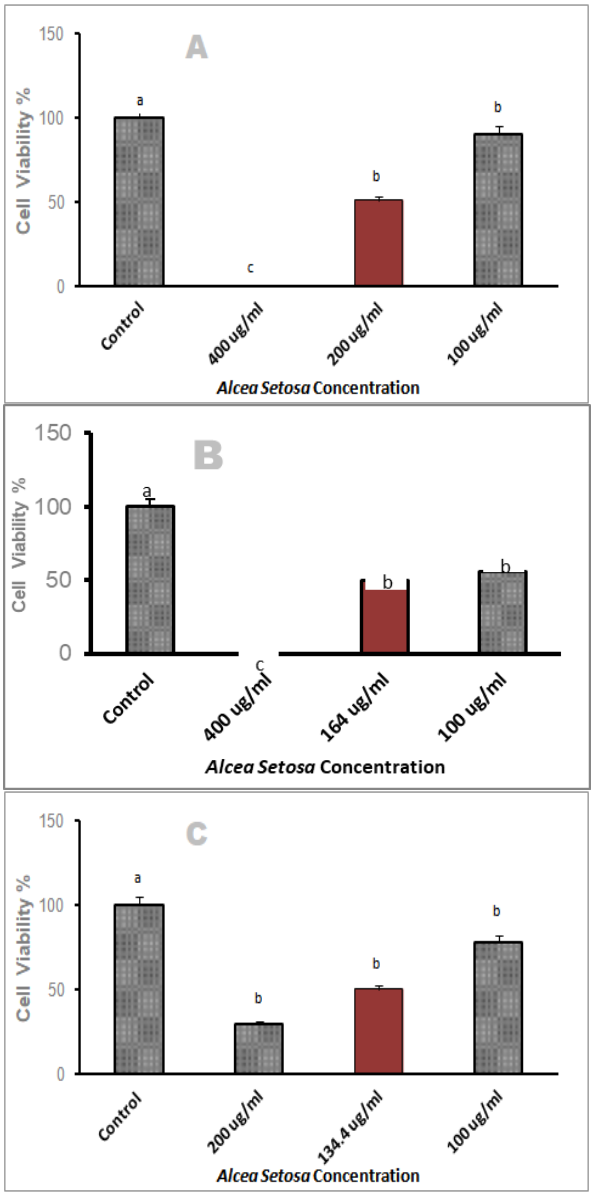

Fig. 3A, 3B, 3C: Showing cytotoxic effect of different concentrations *Alcea setosa* extract on fibroblast, T47D and Caco cell lines, respectively by NR assay. IC50 is denoted in red. Data is representative of at least 3 independent experiments. Data is presented as mean ± SD. Different letters represent homogenous means according Tukey's posthoc test to show significant effect compared to control, NS; non-significant

The difference in IC50 values between tumoral and normal cells suggests the potential of extract to be used in treatment of cancer. Moreover, according to the classification of natural constituents cytotoxicity, that describes ingredients with IC50 values of 100 – 1000 µg/ml as potentially harmful to cancerous cells[34], our cytotoxicity results indicate the possibility of considering *A. setosa* methanolic extract as a therapeutic suggestion in cancer treatment. The significance of the differences between the mean percentages of the viability according to extract concentration is illustrated in Fig. 2 and Fig. 3 for the results of MTT and neutral red assays, respectively. For all the studied cell lines, Fig. 2 indicates significantly lower viability mean values recorded for the highest concentration of extract. Similarly, as shown in Fig. 3, neutral red results illustrate that the extract inhibited the growth of the three cell lines on a dose dependent manner; compared to the control group, treated cells showed significant decrease in viability as the dose of extract increased.

The cytotoxicity of *A. setosa* could be explained by the high content of flavon derivatives such as Kaempferol-3-O-glucoside as explained by Al-Qudah and his team[25,26]. Interestingly, Tin and his colleagues have reported cytotoxic activities by Isorhoifolin of *A. setosa* crude ethanol extraction against HepG2 cancer cell with IC50 of 47.03 µg/mL[27].

In addition, the presence of bioactive phenolics such as Apigenin, Kaempferol-3-O-rutinoside and Quercetin 3-O-glucoside supports the belief in Kurdistan folk medicine that the root of *Euphorbia condylocarpa* plant is an effective treatment for cancer as suggested by Hassan and his team[28]. Cytotoxicity of *A. setosa* extract maybe attributed to the presence of Genistein; an Isoflavon derivative, and is a natural tyrosine kinase inhibitor which exhibited protection from cancers. Genistein was shown to be anti- proliferative and an inducer of cell cycle arrest at the G_2-M phase in prostate, breast, and jurkat T cell leukemia cell lines[29,32].

Another study has investigated the polyphenol extract of *Hibiscus sabdariffa* on some cancer cell lines, one was estrogen receptor-expressing breast cancer cell line (T47D); results have showed 50% growth inhibition in a dose dependent manner[52]. Those findings suggest that *A. setosa* phenolic compounds played an important role in inducing cytotoxicity in our experiments. According to Azab's discussion of the modern research reports of the biological activities of the various species of *Alcea* genus, *Alcea rosea* is the most investigated subspecies, whereas, the very common subspecies *A. setosa*, have not been studied yet[16]. With regards to *A. setosa* in Jordan no cytotoxicity data were found for the comparison purpose.

Antioxidant activity:

The antioxidant activity of *A. setosa* extract was assessed using DPPH assay. The concentrations ranged from 200 ug/ml to 1000 µg/ml. The scavenging ability of different compounds is concentration dependent and increases with concentration gradient. Ascorbic acid (100 µg/ml) was used as a positive control. The IC50 of ascorbic acid and *A. setosa* extract was 4.1ug/ml, 30.0 µg/ml respectively. The antioxidant activity of *A.setosa* extract is expressed in Table 3 in terms of IC50 values. The IC50 value for the DPPH radical scavenging activity was 30.0 ± 0.26 µg/ml.

Table 3: Antioxidant activity of *J. phoenicea* EO (Mean ± SD µg/ml).

Sample	IC50
A.setosa	30.0 ± 0.26
Ascorbic acid	4.1 ± 0.15

Several studies have described the antioxidant activity of *A. setosa extract*; a study concerning *A. setosa*, revealed the studied extracts exhibited very weak antioxidant activity. The leaf extract was more potent which displayed an inhibition of 72% of the radical at 1 mg/mL [22]. Our results indicated that *A. setosa* flower extract exhibited a moderate scavenging activity with IC50 of 30 µg/ml.

In another study, the IC50 for the DPPH radical scavenging activity was above 1000 µg/ml[20].

The moderate antioxidant activity shown in this study is potentially attributed to the high content of Salicylic acid in the extract[30]; Peak area for Salicylic acid in the extract was 60954. High phenolic content, such as flavons and flavanols in the *A. setosa* extract may be the contributor to the moderate scavenging activity exhibited[29]. A similar study on ethanolic extract of *Euphorbia hirta* showed a dose dependent antioxidant activity due to the presence of phenol and flavonoid which exhibit high radicle scavenging ability[47]. These phytochemicals can scavenge the reactive oxygen species (ROS) in the body, such as hydroxyl radical, hydrogen peroxide superoxide anion, and singlet oxygen. The oxidative damage initiated by these species may lead to DNA damage[55].

Phenolic and flavonoid compounds are essential secondary metabolites that contain an aromatic ring with at least one hydroxyl group. Phenolic compounds donate electrons and mainly is the hydroxyl groups which contribute to antioxidant activity. Alkaloids, flavanoids, tannins, and steroids are common phytochemicals present in plant methanolic extracts[54]. Phenolic compounds prevent oxidative stress, inhibit free radicles, decompose peroxide, and scavenge oxygen species in biological systems. Flavonoids are polyphenolic compounds with low molecular weight,

and are good antioxidant source that increase the overall antioxidant power of an organism and protect it against lipid peroxidation[42]. In fact, the isoflavones, have exhibited to inhibit NF-κB activation stimulated by ROS suggesting their ability to scavenge free radicles. Isoflavones could have inhibited cancer growth by inducing apoptosis and the modulating expression of the genes related to the cell growth and apoptotic processes[31].

CONCLUSION:

To our knowledge, this is the first study for the chemical analysis for the extract contents of *A. setosa* and the evaluation the antiproliferative of antioxidant potential for the Jordanian species *A. setosa*. The methanolic extract of *A. setosa* from Jordan was found to be rich in flavons, fatty acids flavons derivatives, ketone derivative, flavonol derivative, organic acid, and coumarin derivative. The biological active compounds explain preferential *in-vitro* antiproliferative and antioxidant potential of the extract on the tested cancer cell lines. *A. setosa* is recommended for further studies and identification of the main phytochemical components and their anti-proliferative mechanism.

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CONFLICT OF INTEREST:

The authors declare no conflict of interest.

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RESEARCH ARTICLE

Optimization of phenolics and antioxidants extraction from *Centaurium erythraea* using response surface methodology

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ABSTRACT:

In recent years, *Centaurium erythraea* extracts have attracted much research attention in the context of prevention or treatment of many diseases due to its bioactive compounds content and antioxidant activity. The antioxidants of *C. erythraea* are very effective as they possess excellent antioxidant activity. Thus, it can be used as a safe and natural food preservative. The aim of this study is to make extracts more effective by optimizing the extraction conditions of the phenolics and antioxidants from *C. erythraea* using response surface methodology (RSM) based on a central composite design (CCD). Two process variables (Methanol volume fraction and solid - solvent ratio) were evaluated at five levels (13 experimental designs). Multiple regression analyses were performed to obtain quadratic polynomial equations using RSM; each response was fitted by a quadratic model. The adequacy of the models was proven using the analysis of variance (ANOVA). The significant effects of the factors and their interactions on the extraction efficiency were investigated at 95% confidence interval. RSM indicated that the optimal extraction conditions were 71% methanol volume fraction and 2.2:10 solid:solvent ratio. Predicted values thus obtained were close to the experimental values indicating suitability of the model.

KEYWORDS: CCD, *Centaurium erythraea*, DPPH radicals, Polyphenols, RSM

1. INTRODUCTION:

Oxidative stress can damage tissues and play a critical role in many diseases progression[1], due to free radicals production, which attack important macromolecules such as nucleic acids, lipids and proteins leading to cell damage and contributing to aging and degenerative disease.[2] Hence, antioxidants have a basic role in the prevention of free radicals related diseases.[3] There are two major categories of antioxidants, synthetic and natural.[1] However, Natural antioxidants are more desirable than those of synthetic ones which have many adverse effects on health.[4]

Many medicinal and aromatic plants are rich sources of biologically active compounds that play an essential role in the food industry as flavorings and preservatives, and are also used in the pharmaceutical industry for their biological activities in the human body. [5]

Centaurium erythraea (Common Centaury) is one of the most important pharmacological species belongs to the *Gentianaceae* family.[6] It is a widespread plant in various regions of the world. It is growing in Europe, North Africa and Southwest Asia. [7] It has long been used in traditional medicine, and listed in pharmacopoeias of many countries.[8,9] In food industries, *C. erythraea* extracts are utilized as natural sources of food flavoring due to their bitter flavor and also recommended as potent food preservative.[10] Phytochemical investigations of *C. erythraea* extracts resulted into isolation and identification of variety of secondary metabolites including terpenoids [11,12], phenolic acids [13], flavones [14],

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flavonols [14,15] and xanthones [16] which are thought to be associated with antioxidant activities. Secoiridoids are another important group of metabolites, which are bitter glycosides present in *C. erythraea*. [17]

Phenolic compounds are a very important class of metabolites present in *C. erythraea* identified by the attachment of one or more hydroxyl groups to one or more aromatic rings. [18] They provide many benefits of a human health, and help to protect the body from oxidative stress. [19]

Extraction process is an important step in separating the phytochemicals of plant tissues from the inactive components. [20] The good extraction process is the one that provides the maximum yield of the target compounds and the greatest anti-oxidation power of the extracts. [21] There are many parameters influence the extraction of the plant's bioactive compounds such as time, pH, temperature, reagents concentration, irradiation time, flow rate, and solvent to solid ratio. [22, 23] So, it is very important to optimize the extraction process by controlling these parameters.

Response surface methodology (RSM) is an economic, widely used method to optimize the conditions of different food industry processes, such as extraction, drying, blanching, enzymatic hydrolysis and clarification, production of microbial metabolites, formulation and many chemical and biochemical processes.[22] There are many scientific researches focused on the utilization of Response Surface Methodology in optimization of chemical and pharmaceutical processes.[24-32] Central composite design (CCD) is the major experimental design for optimization of the extraction processes and analytical procedures by RSM. [33]

Thus, the objectives of this study were to optimize the extraction conditions of *C. erythraea*, to obtain maximum yield of phenolic compounds and higher antioxidant activity, by employing Response surface methodology (RSM) and using central composite design (CCD) to predict the optimal conditions of independent variables (i.e., solvent volume fraction and solid- to-solvent ratio).

2. MATERIALS AND METHODS:

2.1. Samples collection:

Samples were collected from the wild *C. erythraea* plants growing in the coastal mountains of Syria, in Eastern Mediterranean region, at flowering stage (May, 2019), and identified as *Centaurium erythraea* by professors of the department of Pharmacognosy, the Faculty of Pharmacy, Tartous University.

2.2. Sample Preparation:

The collected parts of plants were dried in the dark for 72 h, and then were stored in paper bags at room temperature in a dry place prior to use. Before performing the analyses, dried plants were grounded in a grinder to obtain the powder. The moisture content of the dried plant materials powder was determined and it was 6.91%.

2.3. Chemicals:

Folin-Ciocalteau (2N) reagent (Sigma-Aldrich, Switzerland), 2,2-diphenyl-1-picryl-hydrazyl (DPPH) (Sigma-Aldrich, USA), Sodium carbonate (Himedia, India), Gallic acid (Sigma-Aldrich, China). Methanol 99.5% pure pharma grade (Sigma-Aldrich, USA).

2.4. Extraction procedures:

A series of extraction experiments were carried out, each one was performed using the following steps: the weighed powdered samples were added to specific volume of methanol in the ratio mentioned in the experiment plan and allowed to macerate for 72h in the dark at room temperature. After the decantation, the crude extracts preparation process was finished.

2.5. Determination total phenolic content (Folin–Ciocalteu assay):

Total phenolic compounds content in the methanolic extracts was quantified using Folin–Ciocalteau colorimetric method [34], with slight modifications.

The crude extracts obtained from extraction were diluted before use (1 mL from each extract diluted to 100 mL with distilled water). 0.5 mL of Folin–Ciocalteu (2N) reagent and 4.5 mL of distilled water were added to 1 mL of diluted extracts. The mixture was left at room temperature for 5 min, and followed by the addition of 4mL of Na_2CO_3 (7.5%) and incubated in water bath at 45 °C for 30min. The absorbance was measured at 734 nm using spectrophotometer against a blank. The measurements were carried out in duplicate. Gallic acid was used as calibration standard and the results were expressed as Gallic acid equivalents (GAE) in milligrams per 100 grams of dry-material.

2.6. Radical scavenging activity assay (DPPH assay):

The antioxidant activity of the extracts was determined using the DPPH assay, based on the evaluation of the DPPH radical scavenging activity according to the slightly modified procedure described by Brand-Wiliams *et al*..[35]

1 mL from each extract diluted to 100 ml with distilled water. Then, 200 µL of diluted samples were added to 2 mL of DPPH (100 µM/L methanol), and the mixture was shacked. The absorbance was measured at 520 nm after

60 min incubation of reaction mixtures in the dark at room temperature. The radical scavenging activity was calculated by the following Eq.(1):

DPPH radical scavenging activity (%) =
[(Abs control – Abs sample)]/ (Abs control)]*100 (1)

Where Abs control, is the absorbance of DPPH radical+ solvent; Abs sample, is the absorbance of DPPH radical + sample extract**.**

2.7. Screening study and experimental design with RSM:

Optimization of the extraction conditions for the phenolic compounds yields and the antioxidant activity of the extracts were carried out using Response Surface Methodology (RSM), which was performed using Minitab 19 software.

Based on the literature review, two independent variables (methanol volume fraction (X_1) and solid to solvent ratio (X_2)) were chosen as the most influential factors of the dependent variables, and a preliminary experiment was conducted to determine narrower, more effective variation limits of independent variables (the data was shown in Table 1).

Table 1: Levels of variations of independent variables.

	-α	-1	0	1	+α
X_1: Methanol volume fraction (%)	71	75	85	95	99
X_2: Solid/liquid Ratio (g/mL)	0.793/10	01-Oct	1.5/10	02-Oct	2.207/10
α=1.414					

The effect of two independent variables (X_1, X_2) on the extraction process was investigated, and a central composite design (CCD) was adopted, where the response variable and the experiment matrix designs were shown in Table 2. Using CCD design 13 experiments are required according to Eq.(2): [33]

$N=k^2 + 2k+cp$ ...(2)

where (k) is the number of factors and (cp) is the number of replicate of the central point;(k=2, cp=5).

In this model, 13 experiments (4 factorial points, 4 axial points and 5 replicates of the center point) with two replicate of each one to test the five levels (−α, − 1, 0, + 1, +α) of the two independent variables (X_1, X_2) with full-factorial CCD were employed and the response values were determined and shown in Table 2.

Table 2: The experimental plan with two independent variables (x_1, x_2) and the obtained results.

Run	Coded value	Actual value	Coded value	Actual value	Observed Y_1	Observed Y_2
	X_1	X_1 (%)	X_2	X_2 (g/mL)	(%)	(mg GAE /g DW)
1	+	95	+	0.2	60.2	24
2	-	75	+	0.2	70.1	25.9
3	+	95	-	0.1	40.2	26.8
4	-	75	-	0.1	42.2	26.9
5	1.414	99	0	0.15	58.6	28.9
6	-1.414	71	0	0.15	60.8	26.2
7	0	85	1.414	0.22	55.8	21
8	0	85	-1.414	0.079	35.4	26.9
9	0	85	0	0.15	37	18.8
10	0	85	0	0.15	38.7	19.7
11	0	85	0	0.15	39.4	20.6
12	0	85	0	0.15	41.6	20.7
13	0	85	0	0.15	42.9	20.8

The data obtained from the CCD design was modeled by using a second order polynomial equation:

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i X_i + \sum_{i=1}^{n-1} \sum_{j=2}^{n} \beta_{ij} X_i X_j + \sum_{i=1}^{n} \beta_{ii} X_i^2 .$$

Where, Y is the predicted response; β_0 is the constant coefficient; β_i is the linear coefficient; β_{ii} is the quadratic coefficient, β_{ij} is the interaction coefficient; and X_i and X_j are independent variables; n is the number of independent variables (factors).

2.8. Optimization of the extraction process:

Desirability approach was used to find the best operative conditions capable to get simultaneously a maximum yield for TPC and DPPHsc. To achieve that, the obtained data were converted (y_i) into desirability function (d_i), which can be optimized by univariate techniques(3) [36]

2.9. Validation and Statistical analysis:

The experimental data and the adequacy of the model were verified statistically, and statistical significance of the terms in the two regression equations was examined using ANOVA analysis for each response. Minitab 19 software and R were used in statistical optimization and analysis.

3. RESULTS AND DISCUSSION:

3.1. Screening of the extraction variables effects on antioxidant capacity:

CCD was used for the optimization of extraction with 13 runs of experiments. The antioxidant capacity of the samples ranged from 35.4% to 70.1 %. Run 2 (methanol volume fraction 75%; Solid:liquid Ratio 2:10) showed highest DPPHsc while run 8 (methanol volume fraction 85%; Solid:liquid Ratio 1.5:10) showed the lowest DPPHsc. Based on the experimental values obtained as stated in Table 2, the regression equation was determined as following:

$$y_1 = 7.37 - 16.84x_1 + 1.65x_2 - 3.85x_1x_2 + 10.14x_1^2 + 11.85x_2^2 . \quad \ldots (4)$$

This equation describes antioxidant capacity predicted as a function of the coded variables (methanol volume fraction (X_1) and solid to solvent ratio (X_2).

The contour plot for DPPHsc as a function of methanol volume fraction (X_1) and solid to solvent ratio (X_2) are given in Figure 1. Results show that higher solid to solvent ratio(X_2) with low methanol volume fraction (X_1) (<75%) would give the highest DPPHsc value (>80%).

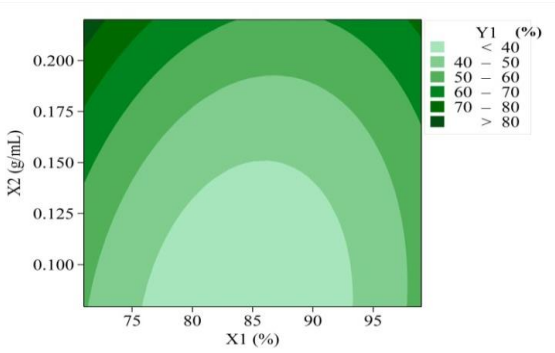

Figure 1: Contour plot of the DPPHsc as a function of methanol volume fraction (x_1) and solid to solvent ratio (x_2)

In order to find parameters which significantly affect the antioxidant capacity response, ANOVA analysis of the model Eq.(4) generated and summarized in Table 3.

Table 3: ANOVA of the predicted polynomial model y_1 (DPPHsc).

	DF	regression coefficients	T-Value	F-Value	P-Value	Signif.
X_1	1	-16.84	-1.6	2.57	0.153	
X_2	1	1.65	8.24	67.86	p≤0.001	***
$X_1.X_2$	1	-3.85	-1.17	1.36	0.281	
X_1^2	1	10.15	7.99	63.81	p≤0.001	***
X_2^2	1	11.85	2.35	5.54	0.051	.
Model	5			27.58	p≤0.001	***
Lack-of-Fit	3			3.28	0.140	

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

The significance of regression coefficients can be evaluated using the p-value. Low p-value (<0.05) indicates that the variable is statistically significant. [37] Based on ANOVA results in Table 3, the most significant terms of the polynomial are the input factor x_2 (p≤0.001) and the factor $x_1$in the square term (p≤0.001), and the interaction effects are not significant.

A positive linear coefficient of the significant factor x_2 indicates that there is a positive correlation between the independent variable x_2 and the dependent variable y_1. The main effects plots display that y1 value increases with $x_2$value increasing, and the slope of the x_2 effect line is steeper (Figure 2).

The study by Bouyahya *et al*.[38] agrees with our results, they have evaluated the antiradical activity using DPPH scavenging assay and found that the ability to scavenge DPPH radical increased significantly with extract concentration increasing from 0.03 g/mL to 0.5 g/mL. Đorđević *et al*.[39] also determined the antioxidant activity of *C. erythraea* methanol extract and found that an increase in extract concentration would led to increase DPPH radical scavenging activity, and our results are in consensus with this study.

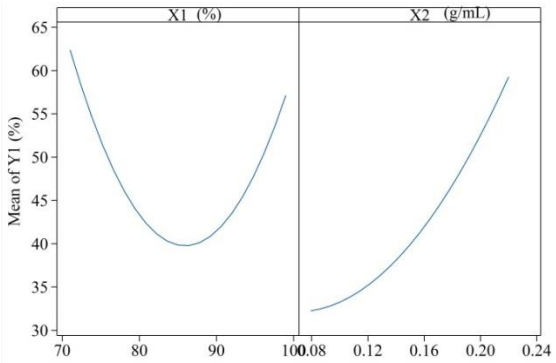

Figure 2: Main effects plots for the antioxidant capacity response (y_1).

Methanol volume fraction factor effects on the antioxidant capacity in the quadratic term are probably due to the effects of solvent polarity on the extraction and purification of phytochemical and antioxidant compounds from the plant materials. [40,20] The methanol–water mixtures are efficient and most widely used to

extract phenolic and antioxidant compounds because of their high dielectric constant and high polarity. [41] Similarly, Majeed *et al.*[42] reported that the maximum antioxidant of *Origanum vulgare* leaves extract is obtained using methanol (70%).

3.2. Screening of the extraction variables effect on total polyphenol content (TPC):

The total phenolic content ranged from 18.86 to 28.94 mg Gallic acid equivalents/g DW). Run 5 (methanol volume fraction 99%; Solid:liquid Ratio 1.5:10) showed highest TPC while run 9 (methanol volume fraction 85%; Solid:liquid Ratio 1.5:10) showed the lowest TPC. The experimental values were fitted using Minitab 19 software into the quadratic regression model, and its obtained second-order polynomial equation was as following:

$$y_2 = 0.29 - 0.63x_1 - 0.18x_2 - 0.09x_1x_2 + 0.38x_1^2 + 0.78x_2^2 . \quad \ldots(5)$$

Where y_2 is the predicted value of the total polyphenol content; and the coded variables are: x_1, the methanol volume fraction; and x_2, the solid to solvent ratio.

Figure 3 represents the contour plot showing the effect of methanol and solvent/sample ratio on the total polyphenol content. It demonstrates the changes in TPC values (y_2) as a function of methanol volume fraction (X_1) and solid to solvent ratio (X_2). Results showed that the highest phenolic content (TPC > 32.5 mg GAE /g DW) would be obtained by using a high methanol volume fraction with low solid to solvent ratio. However, lower methanol volume fraction (<75%) with lower solid to solvent ratio (<0.1 g/mL) would give a high yield of TPC (30 – 32.5 mg GAE /g DW).

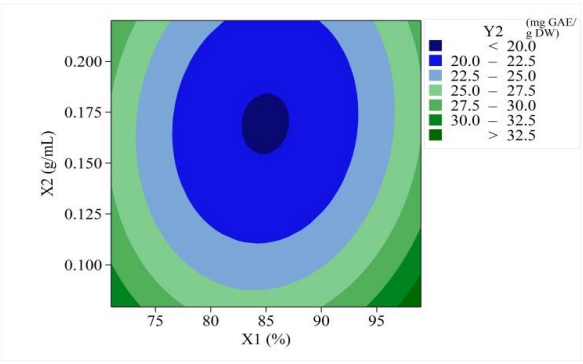

Figure 3: Contour plot of the TPC as a function of methanol volume fraction (x_1) and solid to solvent ratio (x_2).

Based on ANOVA results in Table 4, it can be seen that the variable with the largest effect on the extraction yield was the quadratic term of x_1^2 (factor x_1 in the square term, p≤0.001), followed by x_2^2(factor $x_2$in the square term, p=0.003) , and the linear term of x_2 (p=0.008) respectively.

Table 4: ANOVA of the predicted polynomial model y_2 (TPC).

	DF	regression coefficients	T-Value	F-Value	P-Value	Signif.
X_1	1	-0.63	0.54	0.3	0.603	
X_2	1	-0.18	-3.68	13.53	0.008	***
$X_1.X_2$	1	0.09	-0.76	0.58	0.469	
X_1^2	1	0.38	8.41	70.69	p≤0.001	***
X_2^2	1	0.78	4.34	18.82	0.003	.
Model	5			19.19	0.001	***
Lack-of-Fit	3			3.05	0.155	

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

The interaction effects were not significant, that is in agreement with both previous studies reported that the interaction between solvent volume fraction factor (%) and solid to liquid ratio factor (g/mL) had insignificant effects (p>0.05) on the extraction yield of TPC from *Asparagus officinalis* L.[43] and flavonoids from *Phyllanthus emblica* L..[44]

The main effects plots display the negative correlation between the independent variable x_2 and the dependent variable y_2 (Figure 4). These results are consistent with a previous study, which showed that the TPC increased when the methanol to sample ratio increasing [45], which is probably due to the fact that during mass transfer of extraction process, when a lower solid-to-solvent ratio was used, concentration gradient between the solid and the bulk of the liquid would be greater, resulting in an enhancement intermolecular driving force and increase of the diffusion rate, which means more solvent could enter plant cells while more phenolic compounds could permeate into the solvent thus increase total phenolic yield. [46,47]

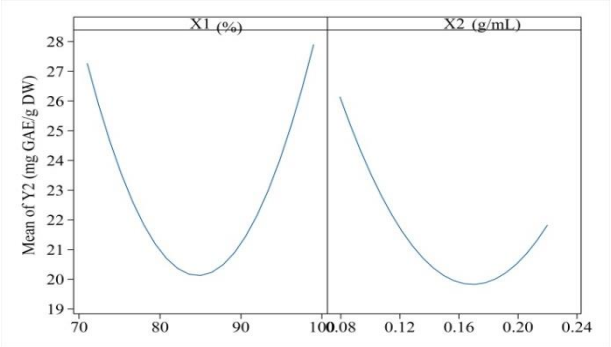

Figure 4: Main effects plots for the TPC response (y_2).

Results showed that a high yield of TPC could be achieved when methanol volume fraction was lower than 75%. This is similar to the study conducted by Lovrić *et al.*[48] which proved that 70% aqueous solutions of alcohol (both ethanol and methanol) were efficient for the extraction of total phenolic content (TPC), total flavonoids (TF), total hydroxycinnamic acids (THC) and total flavonols.

Tawaha *et al*.[49] reported that a higher TPC than 20 mg GAE/g plant extract refers to a very high level of flavonoids and polyphenols. So, *C. erythraea* can be considered as a rich source of phenolic compounds.

Đorđević *et al.*[39] reported that total phenolic content of *C. erythraea* extract was 25.13 ± 0.45 mg GAE/g when using 96% methanol as solvent with 2:10(w:v) solid:liquid Ratio. This study is in agreement with our study; under the same conditions of 95% methanol volume fraction and 2:10 solid:liquid ratio, we found that the TPC of *C. erythraea* extract was 24 mg GAE/g DW.

3.3. Optimization of the extraction process using desirability function:

A multiple response method was applied for numerical optimization of two goals combination using Desirability function which is an objective function that ranges from zero outside of the limits, to one at the goal.[50]

In this work, the basis for the RSM optimization is based on the maximization of both polyphenols and antioxidant activity. Minitab 19 statistical analysis software was used to determine optimal settings for the variables, x_1 and x_2, by maximizing the composite desirability. The optimal solution was chosen based on the closest D values of 1. The optimal X_1 and X_2 were obtained to be 71% and 0.22 g/mL, respectively. The predicted values of Y_1 and Y_2 under optimal conditions were 85.7% and 29.8 mg GAE/g DW, respectively. With desirability value equal to one (D=100%), which indicates the ideal condition.

3.4. Verification of predictive models and Validation of optimized extraction conditions:

Analysis of variance (ANOVA) is used to verify if the mathematical model satisfactorily describes the experimental domain studied.[51] A significant regression model and a non-significant lack of fit indicate to the mathematical model well fits the experimental data.[52] To evaluate the statistical significance of the model, ANOVA was used. Analysis of variance (ANOVA) for the quadratic model for antioxidant capacity (y_1) and total phenolic content (y_2) are summarized in Table 3 and Table 4, respectively. The obtained values of the determination coefficients (R^2) for y_1 and y_2 were 95.17% and 93.2%, respectively. R^2 Coefficients were greater than 0.8, which indicates a good fit.[53] The model F-value of 27.58 for Y_1 and 19.19 for Y_2 confirmed that the model was significant. While, the lack of fit (LOF) did not result in a significant F value in all responses, LOF was not significant. The results above confirmed the adequacy of the model.

To validate the model, Conditions with highest value of desirability (D=1) was selected as optimum conditions values and were validated experimentally. Individual experiment was carried out in three replicates, resulted 79.8% ± 0.28 and 30.7±0.024 for y_1 and y_2, respectively. The prediction intervals PI 95% were (71.97; 99.44) and (24.93; 34.78) for y1 and y2, respectively. Experimental results under optimal conditions were close to predicted values by the models, and contained within PI 95%. Therefore, the mathematical models can be successfully used to predict the DPPHsc and TPC of *C. erythraea* extracts within the range of the performed experimentation.

The correlation coefficients between the antioxidant activity and total phenolic content were evaluated. Moderate positive relationship was obtained between total phenolic content and the antioxidant activity evaluated by DPPH (0.39 for experimented data). This result is in agreement with the study by Šiler *et al.*[10] which reported that although strong antioxidant activity of plant extracts relays on phenolic, including flavonoids and xanthones, *C. Erythraea* extracts might contain other non-phenolic antioxidant compounds that could contribute to their potential overall antioxidant capacity.

4. CONCLUSION:

Our results showed that *C. erythraea* extract is a rich source of pharmaceutically valuable phenolic compounds, and potent antioxidants with high capacity of scavenging DPPH free radicals. So, it can be used in the food and pharmaceutical industries as functional nutrients. RSM was successfully applied and it can be recommended the models obtained is used to describe the experimental space and optimize the phenolics and antioxidants extraction of *C. erythraea* using Methanol solvent. Methanol volume fraction of 71% and solid:solvent ratio of 2.2:10 were found to be optimal conditions for the extraction process.

5. CONFLICT OF INTEREST:

The authors declare that there is no conflict of interests regarding the publication of this paper.

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RESEARCH ARTICLE

TLC-contact bioautography and disc diffusion method for investigation of the antibacterial activity of *Melastoma malabathricum* L. leaves

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ABSTRACT:

The emergence of multi-resistant strains of bacteria reinforces the need to discover new antibacterial agents that are able to combat resistant microorganisms. Medicinal plants are a valuable natural source of bioactive substances against various infectious diseases. *Melastoma malabathricum* L. is an important herb that is traditionally used to treat several ailments associated with microbial infection diseases such as wounds, diarrhea, dysentery, and toothache. This study investigated extracts of *M. malabathricum* L. for antibacterial properties against *Staphylococcus aureus* and Methicillin-resistant *Staphylococcus aureus* (MRSA). Disc diffusion and TLC-contact bioautography techniques were employed to examine antibacterial properties of n-hexane, ethyl acetate, and ethanol extracts with observations of diameter inhibition zones and Rf values. Investigation of active compounds in TLC-bioautography used several reagents including citroboric, cerium (IV) sulfate and 2,2-diphenyl-1-picrylhydrazyl (DPPH), continued by identification of chromatogram profiles through densitometry analysis. The three extracts showed good inhibition against bacterial strains with diameter inhibition zones in the range of 8.0 – 14.0 mm with a number of active spots on TLC-contact bioautography for each extract. This plant may serve as useful source of antibacterial agents for resistant microorganisms and further investigation is needed of its bioactive pure compounds as well as their particular therapeutic potentials and applications.

KEYWORDS: Densitometry, disc diffusion, *Melastoma malabathricum* L., Methicillin-resistant *Staphylococcus aureus*, TLC-contact bioautography.

INTRODUCTION:

Infectious diseases are a major global health concern that have high morbidity and mortality caused by pathogenic bacteria[1]. The discovery of antimicrobial agents was one of the major inventions of the twentieth century. However, over years of applications, the antibiotics have led to the development of antibiotic resistance of several bacterial pathogens. Antibiotic resistance is emerging alarmingly to harmfully high levels in all parts of the world. A growing number of infections such as pneumonia, tuberculosis, and salmonellosis are becoming difficult to treat by the antibiotics typically used to treat them.

Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the resistant bacteria that have increased dramatically during the recent decades in both hospital and community settings[2]. The increased bacterial resistance to different classes of antibacterial agents is a serious and dangerous problem that threatens human health[3]. The emergence of drug resistance has made management of infectious diseases precarious and unpredictable, and there is an urgent need for new bioactive antimicrobial compounds[4]. In the efforts to discover new lead compounds for antibacterial activities, numerous studies have screened plant extracts to identify secondary metabolites with relevant biological activities[5–8].

Plant-derived substances have gained attention due to their significant biological and pharmacological activities. Since ancient times, people have utilized plants for preventing pathogenic diseases and as food preservatives[9]. It has long been established that

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medicinal plants have naturally occurring substances with versatile applications. These plants have advantages in drug discovery due to their significant antimicrobial activities and less toxicity[10]. Many plants have been utilized because they are the richest bio-source of drugs for traditional medicine, food supplements, folk medicines, pharmaceutical intermediates and chemical entities for synthetic drugs[11,12]. It has been estimated that about 28% of higher plant species are used medicinally and that 74% of pharmacologically active plant derived substances were discovered after following up on ethnomedicinal use of the plants. A number of interesting outcomes have been found with the use of mixtures of natural products for healing ailments, including most notably the synergistic effects and non-toxic pharmacological applications of plant extracts[13].

Melastoma malabathricum L. belonging to the family Melastomataceae is a shrub plant used as an alternative medicine due to its numerous therapeutic properties which include antibacterial, antioxidant, antidiabetic, anticytotoxic, anti-inflammatory, antiulcer and immunomodulatory properties[14–17]. The plant is known to be grow widely and abundantly throughout the tropics including Ocean Island, South and South East Asia, China, Taiwan, Australia, and South Pacific Ocean[18]. As a traditional medicinal plant, the leaves of *M. malabathricum* L. are chewed up and pounded to form a poultice applied in topical therapy to stop bleeding and accelerate the dryness of the wounds [18,19]. Young leaves are also useful for healing of ulcers, gastric ulcers, scar, and black spots on the skin [20]. Combinations of the leaves and roots are applied to wounds and pox scars, while combinations of the leaves and flowers are used in the treatment of cholera, prolonged fever, dysentery and leucorrhea[18,21,22].

The discovery of bioactive compounds for antimicrobial agents takes considerable effort to isolate and purify them from plant extracts[23]. To overcome this problem, the approach called bioactivity-guided fractionation was developed[24]. Thin layer chromatography (TLC) is a powerful technique for separating the mixture of samples. The TLC combined with the biological detection method is known as TLC bioautography, and is an economical alternative with high efficiency and strong specificity in the selection of a chromatographic process and biological detection system[25]. This method can be used to reduce the time and labor involved in the process to identify biologically active components of plant extracts. Contact-TLC bioautography connects the steps of separation on the adsorbent layer with biological assay performed directly on it[24].

Although there are several studies focusing on the biological activity of *M. malabathricum*, there are varying outcomes concerning the constituents or bioactive compounds of the plants implying where they grow (habitat) plays a significant role in the phytochemistry of medicinal plants. Therefore, we aimed to assess the antimicrobial activity of three extracts of *M. malabathricum* L. leaves through TLC-contact bioautography and disc diffusion method.

MATERIALS AND METHODS:

Chemical and reagents:

All solvents: *n*-hexane, ethanol, ethyl acetate, formic acid, methanol, chloroform were of analytical grade supplied from Merck (Merck, Darmstadt, Germany). Cerium (IV) sulfate and citroboric acid reagent. Extracts were monitored by TLC which was done on pre-coated silica gel 60 F_{254} plates (Merck). All chemical solvents used were of analytical grades. In addition, Mueller Hinton Agar, Nutrient Agar, and Nutrient Broth were purchased from Oxoid, UK.

Plant material:

The leaves of *M. malabathricum* were collected in June 2018 from the Kuantan Singingi region of Riau province, Sumatera Island, Indonesia with coordinates: 0º33'2"S and 101º32'11"E. *Melastoma malabathricum* L. that was used in this study was authenticated by a botanist (Dr. Djoko Santosa) and a voucher specimen was deposited at the Department of Pharmaceutical Biology, Universitas Gadjah Mada, Yogyakarta, Indonesia. Once dried at room temperature (after a week), the leaves were ground and store in cool dry conditions before use.

Preparation of extracts:

The dried powdered leaf of *M. malabathricum* L. was extracted by the Soxhlet Apparatus by n-hexane for two weeks. After separation of the n-hexane extracts, the residue was macerated by ethyl acetate and finally was macerated by ethanol for three days as well as regularly stirred. The extracts were filtered and concentrated under vacuum in a rotary evaporator. All these extracts and fractions were stored at 4°C in the refrigerator for further use.

Bacterial strains:

The antibacterial activities of the *M. malabathricum* L. extract were tested with bacterial samples from American Type Culture Collection (ATCC) including *Staphylococcus aureus* (ATCC 25923) and Methicillin-Resistant *Staphylococcus aureus* (MRSA) (ATCC 33591). These bacterial samples were supplied from the Microbiology Laboratory, Department of Pharmaceutical Biology, Faculty of Pharmacy, Universitas Gadjah Mada.

Thin layer chromatography (TLC):
A 10 µL or 20 µL aliquots of the n-hexane, ethyl acetate, ethanol extract were applied onto TLC silica gel plate 60- F_{254}. The plates were developed in the mobile phase containing: chloroform 100% for n-hexane extract, chloroform: methanol: ethyl acetate: formic acid (50: 15: 30:5 v/v/v/v) for ethyl acetate extract and n-hexane: ethyl acetate (8: 2 v/v) for ethanol extract in chamber over a distance 6 cm. After development, the plates were dried in a stream of warm air. The plates were scanned subsequently at two scanning wavelengths, 254 nm and 366 nm by the TLC Visualizer (CAMAG). Profile chromatogram was performed via Rf values separated into a wide range of Rf values from 0.00 to 1.00. All data obtained were processed with the software winCATS version 11.4.7.2018 (CAMAG). The plates were used for bioautography assay and other sets for derivatization with several sprayers.

TLC-contact bioautography technique:
Contact or contact bioautography was done according to the protocols explained by[26] with slight modifications. The inocula of representative bacterial strains with 10^8CFU/mL concentration namely MRSA and *S. aureus* were swabbed onto the Mueller-Hinton agar plates for use in TLC contact bioautography technique. The dried developed TLC plate was then place aseptically onto the seeded Mueller-Hinton agar medium for ± 1 hour to allow diffusion of bioactive compounds. After that, the TLC plate was removed and the inoculated agar plate was further incubated at 37°C for 24 hours in aerobic condition. The spots that exhibited antibacterial activity were located by comparing to the TLC plate previously removed. The bioautographic assay allows the detection of active components in a crude plant extract on the TLC plate by recording of Rf values.

Post chromatographic derivatization:
After development, the plates were dried in room temperature for 30 min. The plates were derivatized with sprayer for preliminary identification the class of compounds. The plates were sprayed citroboric and cerium (IV) sulfate reagent. On the basis of Rf values, the substances of the extract samples could be determined by sprayed analysis. The determination of free radical scavenging activity of separation spots on TLC plates was performed by qualitative DPPH test by preparing 0.2% freshly methanolic solution of DPPH. After spraying the DPPH solution on TLC plates, these were stored in a dark room for 30 min. The yellow spots on purple background revealed the zones where substances with the highest free radical scavenging activity were present.

Agar diffusion method:
The antibacterial activity assay was examined using the disc diffusion method. Petri dishes (diameter size 9 cm) contained 10 mL of Mueller Hinton Agar medium (Oxoid, UK) seeded with 100 µL of the culture suspension of the microorganisms in Broth Heart Infusion (BHI, Oxfoid) using the spread plate technique. The inoculum size was adjusted to bacterial cells 10^6 colony forming units (CFU/mL) estimated by equivalent of 0.5 McFarland standard or can be accurately measured using a spectrophotometer with a 1-cm light path at 600 nm which corresponding to an absorbance reading of 0.1[27]. Following this step, a sterile paper disk (Whatmann No. 1; 6 mm in diameter) was impregnated with test materials (30 µL to give the final concentration 150 µg/mL) and the disc was placed on the agar medium. The plates were left to dry and after that, the petri dishes were incubated at 37°C for 24 h under aerobic condition. Chloramphenicol (30 µg/mL, Oxoid) and solvent (n-hexane, ethyl acetate, ethanol) were used as positive and negative control, respectively. All disc diffusion tests were performed in triplicate and antimicrobial activity was expressed as the mean diameter of clear zone of growth inhibition (diameter expressed in millimeters) around the disc.

RESULTS AND DISCUSSION:

TLC-contact bioautography:
To perform a rapid screening study of potential antibacterial activity of n-hexane, ethyl acetate and ethanol extracts of *M. malabathricum* L. leaves against *S. aureus* and MRSA, TLC-bioautography following a disc diffusion method was applied as the combination method to identify bioactive compounds. The successful TLC bioautography method depends on the mobile phase used to separate a composition of matrix samples in TLC plates of plant extracts. Several mobile phases were examined in optimization of the separation of extracts in TLC plates with the following mixtures of solvents: chloroform: methanol: ethyl acetate: formic acid (50: 10: 35: 5 v/v/v/v); n-hexane: acetone (8: 2 v/v); ethyl acetate: methanol: water: formic acid (100: 13: 10: 2 v/v/v/v); dichloromethane: ethyl acetate: formic acid (50: 45: 5 v/v/v); chloroform (100%); n-hexane: ethanol (8:2 v/v). Starting with TLC, several spots with broad range of polarities were obtained. Therefore, the use of the proper solvent system enables the separation of *M. malabathricum* L. extract into spots with a wider range of polarities.

TLC-bioautography assay allows target-directed isolation of bioactive components for further examination, hence preventing isolation of inactive compounds[28]. The results clearly demonstrated that the plant extracts exhibited significant antimicrobial activity with the chromatogram profiles of TLC (Figure 1) and the active spots on TLC plate of three extracts showed in their Rf values (Table 1).

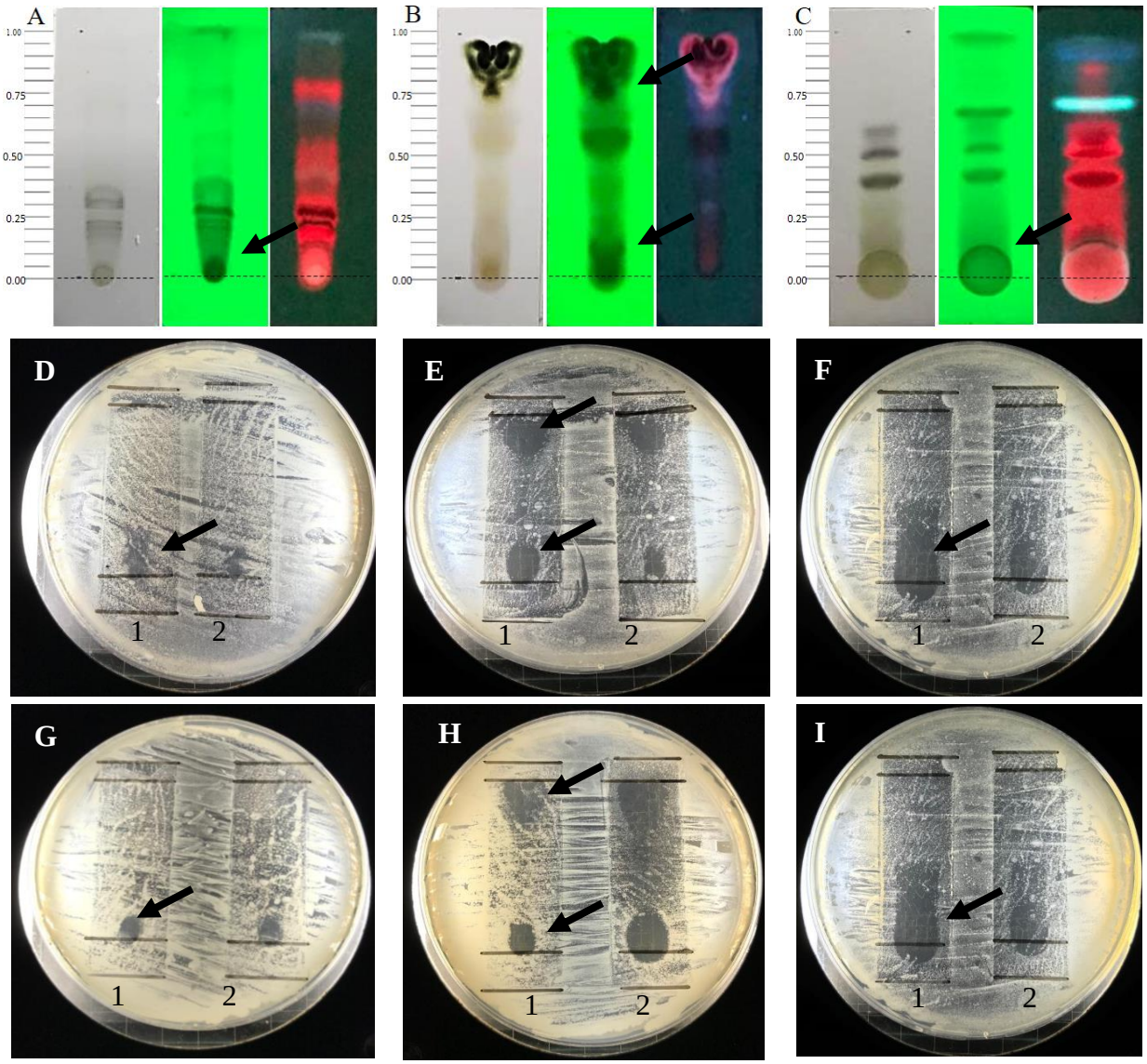

Figure 1. TLC chromatogram profiles of n-hexane (A), ethyl acetate (B) and ethanol (C) extract. TLC-contact bioautography of n-hexane (D), ethyl acetate (E) and ethanol (F) extract against *S. aureus* while G, H and I showed the activity of n-hexane, ethyl acetate, ethanol extract against MRSA with loading mass of samples: 100µg (1) and 50µg (2). Mobile phase used on the separation TLC: chloroform 100% for n-hexane extract, ethyl acetate extract: chloroform: methanol: ethyl acetate: formic acid (50: 15: 30: 5 v/v/v/v) for ethyl acetate extract and n:-hexane: ethyl acetate (8:2 v/v) for ethanol extract.

Table 1. Rf value of bioactive compounds of n-hexane, ethyl acetate and ethanol extract against MRSA and *S. aureus*

Extract	Concentration	*Methicillin-resistant Staphylococcus aureus*	*Staphylococcus aureus*	Active spot
n-hexane	100µg	0.00 – 0.16	0.00 – 0.25	1
	50µg	0.00 – 0.15	0.00 – 0.25	
ethyl acetate	100µg	0.00 – 0.24 0.72 – 1.00	0.00 – 0.25 0.75 – 1.00	2
	50µg	0.00 – 0.17 0.75 – 1.00	0.00 – 0.22 0.80 – 1.00	
ethanol	100µg	0.00 – 0.28 0.29 – 0.50	0.00 – 0.29 0.30 – 0.51	2
	50µg	0.00 – 0.27 0.28 – 0.48	0.00 – 0.27 0.28 – 0.50	

This study employed the bioautographic method to examine bacterial growth inhibition by visually observing a clear zone and Rf values. The samples were showing the zone of inhibition confirming that they possess good inhibitory activity against test organisms[29]. Ethyl acetate and ethanol showed excellent antimicrobial activity with two active spots in Rf values of 0.00 to 0.24 and 0.72 to 1.00 for ethyl acetate and 0.00 to 0.28 and 0.29 to 0.50 for ethanol extracts in concentration 100µg against MRSA. However, n-hexane showed moderate antimicrobial activity with one active spot in Rf value of 0 to 0.16 and 0.00 to 0.25 against MRSA and *S. aureus*, respectively. There is different significance of antibacterial properties of the three extracts against MRSA and *S. aureus*. Inhibition of MRSA growth had a broad range of Rf values compared to the *S. aureus* particularly in n-hexane extract.

The most significant advantage of the TLC bioautography technique is the antibacterial active spots can be located and separated by planar chromatography for investigation of the antimicrobial effects in complex samples. The plates are placed on the surface of nutrient agar plates inoculated with microorganism so antimicrobial compounds are transferred from the TLC plate to an inoculated agar plate by direct contact. It is based on the diffusion of compounds, already separated by TLC, from adsorbent or paper to the agar medium[30]. This is in contrast with work using agar dishes, which does not distinguish between active and inactive components found together in zones of inhibitions. In this case, only the bioactive sum of a sample is indicated, not the activities of single compounds. There are some examples of the application of this methodology for screening antibacterial, antifungal, xanthine oxidase, or free radical scavenging activities[31].

TLC is generally employed as a fast, efficient and inexpensive tool for a screening method in different stages of monitoring processes including synthesis, isolation, and other biological studies[32]. Several studies have documented the application of TLC for the phytochemical profiling, fingerprint analyses of plant extracts[33]. TLC spots can be analyzed both qualitatively and quantitatively by densitometry in ultraviolet (UV) absorbance as shown in Figure 2. For non-UV absorbing or non-fluorescent compounds, densitometry can be applied after derivatization using chromogenic agents[34]. The results of densitometry analysis are computerized by chromatogram profiles and area under peak[35] following detection under UV lamp of 254 nm and 366 nm.

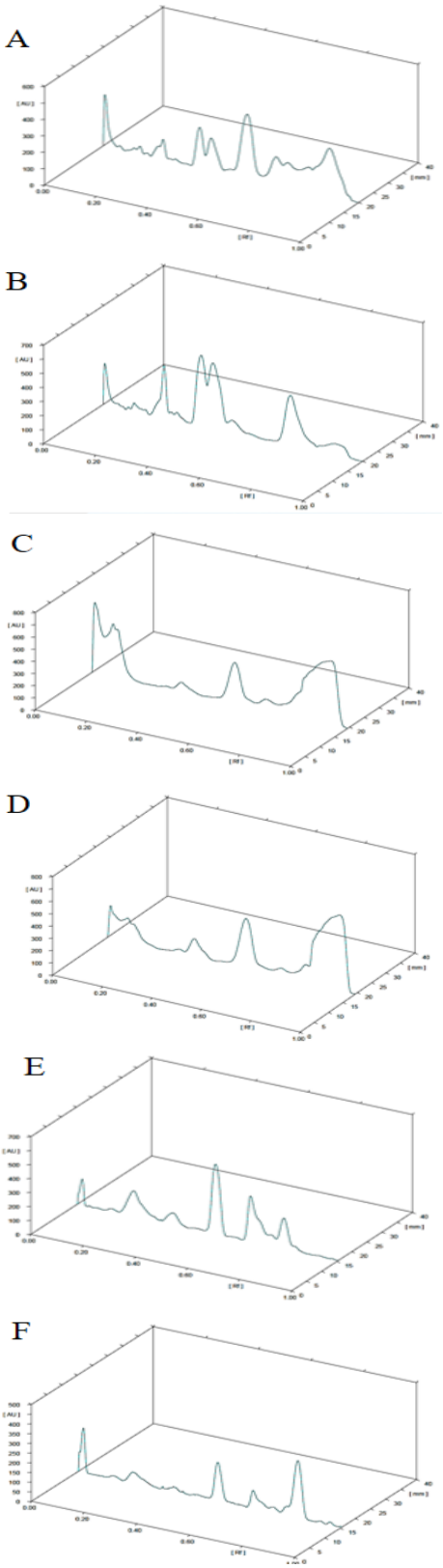

Figure 2. TLC 3D-profile densitograms of n-hexane, ethyl acetate, and ethanol extract under UV 254 nm light (A, C, E) and n-hexane, ethyl acetate, and ethanol extract under UV 366 nm light (B, D, F), respectively.

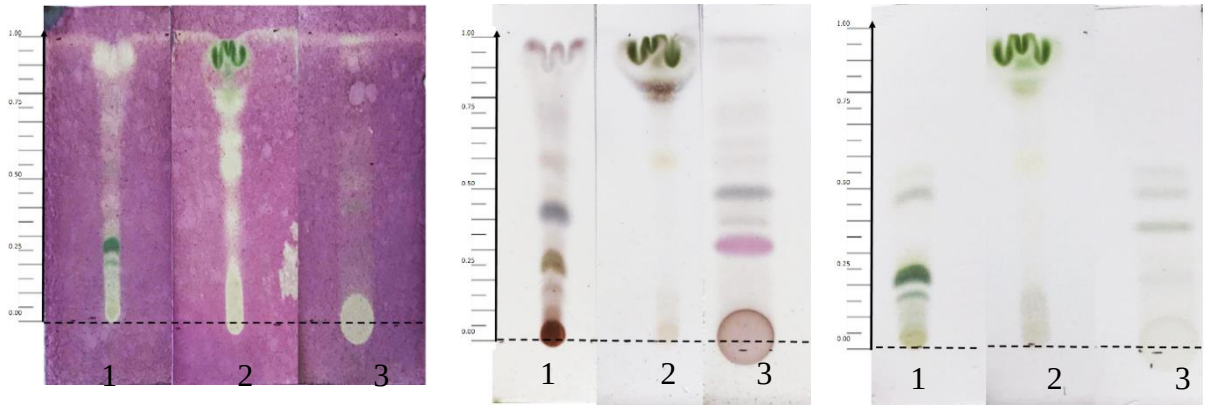

Figure 3. TLC plates of n-hexane (1), ethyl acetate (2), and ethanol (3) extracts after spraying with 2,2-diphenylpicryl hydrazyl (DPPH) (A), cerium sulfate ($CeSO_4$) (B), and citroboric acid reagent (C).

Important steps of thin layer chromatography analysis include the detection of investigated substances of the plant extracts. Besides providing information about separated compounds by retardation factors, TLC can also identify the class of compounds by traditional visualization. Figure 3 summarizes the derivatization of TLC plates represented by color descriptions of the separated spots on the TLC visualized under visible light after sprayed with DPPH, citroboric, and cerium (IV) sulfate. The separated components by TLC can be detected with individual colors of substances or fluorescence of substances in UV light and color reaction of separated compounds of TLC with visualizing reactions. These extracts showed activity in the range of UV light and can be directly detected and determined on the chromatographic plate, and hence by densitometric analysis. The methods were found to be equivalent on the basis of repeatability when the results from densitometric measurements were compared with those obtained. The TLC separation was followed by derivatization of DPPH in methanol (0.02% w/v). The compounds possessing radical scavenging activity were detected as bright yellow bands against a purple backgrounds.

Color reaction on the reagent spray cerium (IV) sulfate mechanism showed the cerium (IV) sulfate consisting of concentrated sulfuric acid and acetic anhydride, where sulfuric acid has a destructive and oxidative effect. Cerium (IV) sulfate reagent is used for detecting components of organic compounds[36]. Citroboric acid is one of the specific sprayers for identification of the presence of flavonoid compounds. The samples that contained flavonoid group exhibit yellow spots on TLC plate[37]. Based on chromatogram in Figure 3, TLC profiles showed some spots that have Rf values of 0.55 in ethyl acetate extract with brownish yellow in visible light after sprayed by citroboric reagent. For DPPH analysis, the active compound on TLC plates exhibits yellow bands against a purple background. It indicated the presence of phenolic compounds as well as flavonoid compounds. In the present study, ethyl acetate extract showed high content of phenolic compounds. Flavonoids is one of the phenolic compound class that have antioxidant and antibacterial activity. The antimicrobial activity of the *M. malabathricum* L. is suggested to be due to the presence of phenolic, flavonoid and other semipolar compounds of the mixture samples extracts.

Disc diffusion method:

The results of the disc diffusion method of *M. malabathricum* L. extracts are in line with the data obtained from TLC-bioautography. The anti-MRSA activity of chloramphenicol was more significant than the ethanolic extract. Ethanol extract showed the greater antimicrobial activity than n-hexane and ethyl acetate extracts. The antimicrobial activities for the three extracts using the disc diffusion method are shown in Table 2.

Table 2. Disc diffusion method of antibacterial activity of extracts against MRSA and *S. aureus*

Extract	Diameter of inhibition zone (mm)	
	Methicillin-Resistant *Staphylococcus aureus*	*Staphylococcus aureus*
n-Hexane	11.0 ± 0.2	8.0 ± 0.5
Ethyl acetate	10.0 ± 0.5	12.2 ± 0.4
Ethanol	12.5 ± 0.4	14.0 ± 0.8
Chloramphenicol	16.2 ± 0.1	25.0 ± 1.5

The disc diffusion assay showed that ethanol extract had strong growth inhibition activity against both *S. aureus* and MRSA with diameter 14 ± 0.8 and 12.5 ± 0.4, respectively. Ethanol extract showed the highest antibacterial activity against *S. aureus* and MRSA with the big clear zone. Previous study revealed that ethanol extract was the most effective solvent for extracting a broad spectrum of antimicrobial[38,39] and polymicrobial biofilm[40] substances derived from plants. However methanol extract exhibited moderate antimicrobial

activity against six out of seven bacterial microorgabisms[41]. Ethyl acetate extract exhibited the moderate activity against *S. aureus* and MRSA with the diameters of the inhibition zone of 12.2 ± 0.4 and 10.0 ± 0.5, respectively. Extract of n-hexane also had moderate inhibition activity against *S. aureus* and MRSA with diameters 8.00 ± 0.5 and 11.0 ± 0.2, respectively. The clear zone of positive control chloramphenicol was 25.0 ± 1.5 and 12.2 ± 0.4 against *S. aureus* and MRSA, respectively.

The presence of the clear zone indicates the inhibitory activity of the examined compounds against the bacterial growth. According to previous report, extracts with inhibition zone diameter over >11 mm are assumed to have strong antimicrobial activity, 6-11 mm categorized as moderate activity with < 6 mm as weak activity. The antibiotic chloramphenicol was used as the reference drug (positive control) in this study. The antibacterial potential of the extracts can be assumed as good and shows they have potential activity as antibacterial agents although the zone diameters are much lower than that of antibiotics[42]. In the disc diffusion technique, the disc was used as reservoirs containing the solutions of the examined samples and solutions with a low activity needed a large concentration or volume[43].

Antibacterial properties *M. malabathricum* L. extract might be due to the presence of bioactive compounds that have been reported in the plants. The bioactive constituents included the phenolic compounds such as tannins: malabathrin A, pedunculagin, strictinin, nobotanin, flavonoid including quercetin, kaempferol, quercitrin, rutin, kaempferol glycosides, phenolic acids such as p-hydroxyl benzoate, gallic acid, and few terpenoids and alkaloids[14,15,18,44]. In fact, the presence of phenolic compounds has generally contributed to the antimicrobial activity. Polyphenols are well documented to have antimicrobial activity against a huge number of pathogenic bacteria. In addition, oxidized phenols also have an inhibitory effect against bacterial growth[45,46]. The hydroxylation process increased with the increasing number of hydroxyl group, which in turn increased the antimicrobial activity. The mechanisms involved in polyphenols as antimicrobial agents include envelope transport proteins, inhibition of hydrolytic enzymes and non-specific interactions with the carbohydrates. In addition, flavonoids and tannins could be able to bind or form precipitates with various proteins[47].

CONCLUSIONS:

The results of this study confirmed the presence of the various bioactive compounds in the *M. malabathricum* L. leaves responsible for their therapeutic activities. We have identified several active spots on TLC plate in TLC-contact bioautography technique against *S. aureus* and MRSA. The results of disc diffusion and TLC-contact bioautography showed excellent activity of the three *M. malabathricum* L. extracts and supports the potential development of these novel therapeutic antimicrobial agents from *M. malabathricum* L. attributed to its traditionally use as anti-infection treatment of diseases. Future studies are directed towards the development of purified bioactive substances to improve existing drugs or to create new agents of antibacterial activities. Additionally, considering all of the inhibitory effects of the plant extracts, it may have potential for further development as a natural agent in prevention of infectious diseases. Therefore, it could serve as potential sources of industrial drugs useful in some therapy against bacterial infections.

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CONFLICT OF INTEREST:

The authors declare that there is no conflict of interest in this article.

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RESEARCH ARTICLE

Isolation and Structural Elucidation of Impurity in Sulfonamide Intermediate of Glyburide

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ABSTRACT:
A reproducible isolation method by Reverse Phase (RP) preparative HPLC technique for the isolation of one crucial impurity at 1.65 RRT (Relative Retention Time) in sulfonamide stage of Glyburide API (Active Pharmaceuticals Ingredient) was developed. Preparative chromatography was done on Luna C8, 10µm (250 mm x 21.2mm) preparative HPLC column with acetonitrile: water in 70:30 % v/v proportion as a mobile phase and 8 ml/min as a flow rate. This impurity was detected at 300 nm UV-wavelength maximum. This impurity was isolated from synthesized crude impurity of sulfonamide stage of Glyburide substance by preparative HPLC by injecting 50 mg/ml concentration over 5 ml fixed loop. Isolated impurity was elucidated as N-methyl impurity of sulfonamide intermediate of Glyburide API by means of chromatographic and spectral data. Structural elucidation carried out by spectral data was reviewed. This impurity was analyzed by reverse phase HPLC for purity analysis. A Inertsil C8 (250 x 4.6) mm, 5µ particle size was employed for separation. The mobile phase consisted of Water: Acetonitrile: Methanol in the ratio of 60:15:25 % v/v. The flow rate was set at 1 ml/min. Detection was carried out at 300 nm. 10µL of 2 mg/ml concentration of sample in methanol was injected. The column oven temperature was at 25°C.

KEYWORDS: Isolation, Preparative HPLC, Glyburide, Sulfonamide, Structure elucidation.

INTRODUCTION:
When pancreas does not able to produce enough insulin or when the body cannot effectively use the insulin it produces a chronic disease named Diabetes occurs. Millions of people in world are suffering from Diabetes mellitus with type-1 or type-2.[1-3] This disease is the disease that precludes body from appropriately consuming energy from the food eaten. It has marked as long lasting disease in world with disturbing value of life of the patients severely which employees a major fiscal burden on the world.[4-5]

Treatment and prevention of diabetes and its complications is decisive. Glyburide (also known as Glibenclamide: IUPAC Name: (A) 1-[4-[2-(chloro-2-methoxybenzamide) ethyl]-benzene sulfonyl]-cyclohexylurea, (B)5-chloro-N-[2-(4-[[(cyclohexylcarbamoyl)amino] sulfonyl] phenyl) ethyl]-2-methoxybenzamide is known for anti-diabetic drug which belongs to sulfonyl urea group and used to control diabetes mellitus with mild to soberly severe type II which does not require insulin and can be controlled sufficiently by diet alone.[6] In recent years HPLC is developed as a sensitive tool to estimate and identify API or drug molecule.[7-8] Some method other then HPLC are also developed to validate and estimate glibenclamide.[9] High performance Liquid Chromatograph (HPLC) analysis of sulfonamide intermediate of Glyburide drug saw an impurity content at 1.65 RRT with more than 0.1% level. Impurities are unexpected contents present in the Active Pharmaceuticals Ingredient (API) coming from normal synthesis process during manufacture, degradation on stability and trace (forced) degradation study.[10-12]

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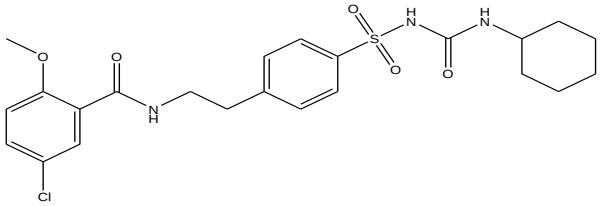
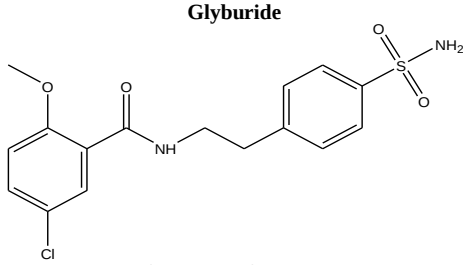
Impurities have no beneficial importance and are actively and potentially harmful to the body. Therefore, they need to be controlled. As per the International Conference on Harmonisation of technical requirement for registration of pharmaceuticals for human use (ICH) guideline and regulatory bodies[13-15] from the world the threshold for impurity in drug products is ≤ 0.15% for known impurities and ≤ 0.10 for unknown impurities per maximum daily dose of drug product is ≤ 1gm and is ≤ 0.05% per maximum, daily dose of drug product is > 1gm. To meet these stringent regulatory requirements, the impurity profile study has to be carried out for final API to identify and characterize the impurity that is present at > 0.1%. This paper demonstrates the isolation and structure elucidation of impurity present in the sulfonamide intermediate of Glyburide API using preparative HPLC.[16-18]



Glyburide



Sulfonamide of Glyburide

Figure 1 Chemical structure of Glyburide and Sulfonamide of Glyburide

MATERIALS AND METHODS:

Chemicals and reagents:

HPLC grade Acetonitrile, Methanol and Water were purchased from Merck (Mumbai, India). Crude sample of Isomeric impurity at 1.65 RRT for impurity isolation and Sulphonamide of Glyburide Reference standard were obtained as gratis sample from Cadila Pharmaceuticals Ltd. Research Laboratories (Ahmedabad, India).

High performance Liquid Chromatography (Conventional):

This impurity was analyzed by RP-HPLC for purity analysis. A Waters alliance quaternary e2695 chromatography system equipped with Waters 2998 PDA detector was used (Waters Corporation, USA). A Inertsil C8 (250 x 4.6)mm, 5µ particle size (GL Sciences Inc., Japan) was employed for separation. The mobile phase consisted of Water: Acetonitrile: Methanol in the ratio of 60:15:25 (v/v). The flow rate was set at 1 ml/min. Detection was carried out at 300nm. 10µL of 2 mg/mL concentration of sample in methanol was injected. The column oven temperature was at 25°C. Empower3 software was used to record data.

High performance Liquid Chromatography (Preparative):

A Waters quaternary 2535Q preparative chromatography system equipped with Waters 2998 PDA detector (Waters Corporation, USA), and Rheodyne Injector Model 7725i (IDEX Health & Science, USA) with 5.0ml fixed loop was used. A Luna C8 (250 x 21.2) mm, (Phenomenex, USA) was employed for separation. The mobile phase consisted of Water: Acetonitrile in the ratio of 70:30 (v/v). The flow rate was set at 8.0ml/min. Detection was carried out at 300nm. Empower3 software was used to record data.

NMR spectroscopy:

The 1H NMR[19] spectra were recorded on Bruker 400 Ultra shield spectrometer. The 1H (400 MHz) was recorded using TMS and DMSO-d6 as internal standards and diluent, respectively. Topspin 3.1 was used to record data.

Mass spectrometry:

Mass spectrometer Waters Xevo TQD was used with ESI (72eV) and The ion spray voltage (V), Curtain energy (CE), entrance potential and declustering potential were kept as 3500V, 42V, 19V and 117V, respectively to record mass spectra. The sample was introduced to mass spectrometer with particle beam interface using LC. The source manifold and quadrupole temperatures were maintained at 230°C and 90°C respectively. Nitrogen was used as a reagent gas for chemical ionization (CI) mode.[20-22] The ESI mass spectra were recorded on Waters Xevo mass spectrometer with mass-lynx software.

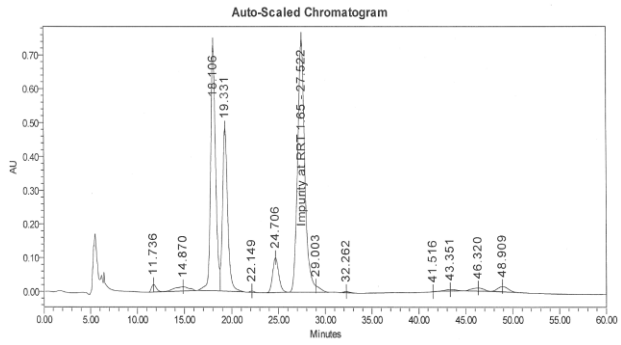
FT-IR spectroscopy:

FT-IR[23-24] spectra were recorded on Shimadzu 8400 series FT-IR as KBr powder.

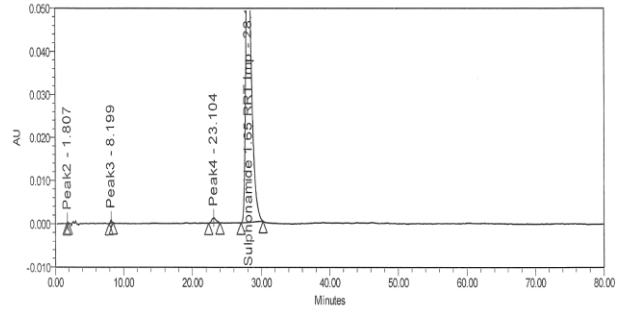
RESULT AND DISCUSSION:

Detection of Impurity:

During a conventional related substance RP-HPLC analysis of sulfonamide intermediate of Glyburide API, a consistence impurity at 1.65 RRT was recorded. This synthesized impurity shows itself impure which need to make pure by isolation process using preparative HPLC. This objective impurity under study was manifested as impurity of sulfonamide intermediate of Glyburide at 1.65 RRT. Analytical chromatogram of 1.65 RRT impurity of sulfonamide intermediate of Glyburide was recorded using related substance HPLC method.



a. Crude 1.65 RRT Impurity of Sulphonamide in Glyburide



b. Isolated impurity from crude 1.65 RRT Impurity of Sulphonamide in Glyburide

Fig. 2. Conventional HPLC chromatogram of impurity of sulfonamide intermediate of Glyburide

Isolation of impurity by preparative HPLC:

A reverse phase isocratic solvent delivery system was developed (High performance Liquid Chromatography - Preparative) and used for the isolation of this crude impurity of sulfonamide intermediate of Glyburide that was mixture of two or more different content. All segments of impurity were collected separately, concentrated and solidify by evaporation of solvent using rotavapour. These isolated solids received from different fractions were then analyzed by related substance HPLC method to check its purity and to use for further analytical exercise for structural elucidation.

Structure elucidation:

The ESI mass spectra of 1.65 RRT impurity of sulfonamide intermediate of Glyburide was displayed the molecular ion peak at m/z 383.12. The ES^+ mass spectra further confirmed this with the presence of protonated molecular ion peak as base peak at m/z 383.12 amu (i.e. M+H), which is equivalent mass unit 382.08 amu to that of N-methyl sulfonamide intermediate of Glyburide. This can be recognized as an impurity of parent substance sulfonamide intermediate of Glyburide. The IR spectra of this isolated impurity shows the presence of all wave number equal to sulfonamide intermediate of Glyburide a parent drug which also further confirms with 1H NMR spectra presented below.

Mass spectra:

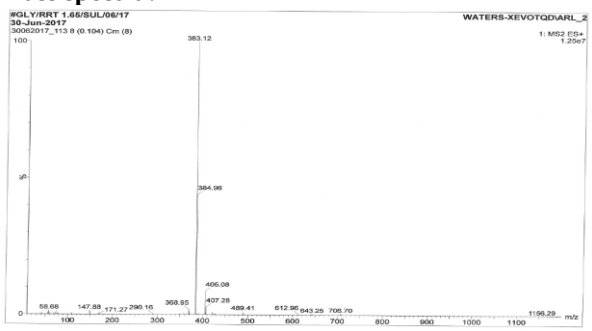


Fig. 3. Mass spectra of 1.65 RRT impurity of sulfonamide intermediate of Glyburide

Mass Spectra	
	1.65 RRT Impurity of Sulfonamide of Glyburide
Exact Mass	382.08
Molecular Ion (m/z)	383.12

1H NMR Spectra

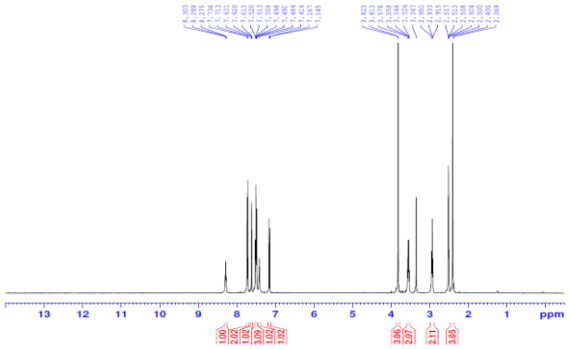
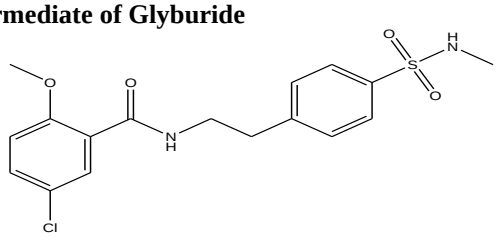


Fig. 3. 1H NMR spectra of 1.65 RRT impurity of sulfonamide intermediate of Glyburide

Assignment of Proton by 1H NMR:

1H-NMR (DMSO-d6, 400 MHz): δH. (ppm): 2.369-2.400 (3H, d, CH3), 2.915-2.950 (2H, t, CH2), 3.526-3.576 (2H, q, CH2), 3.813 (3H, s, CH3), 7.145-7.167 (1H, d, CH aromatic), 7.414 (1H, s, NH), 7.484-7.520 (3H, m, CH aromatic), 7.613-7.631 (1H, m, CH aromatic), 7.713-7.734 (2H, m, CH aromatic), 8.275-8.303 (1H, t, NH).

Structure of 1.65 RRT impurity of sulfonamide intermediate of Glyburide



5-Chloro-2-methoxy-N-[2-(4-methylsulfamoyl-phenyl)-ethyl]-benzamide

Empirical Formula : $C_{17}H_{19}ClN_2O_4S$

Mol. Wt. : 382.86

Exact Mass : 382.08

CONCLUSION:

In conclusion, this process related impurity of sulfonamide intermediate of Glyburide formed during process of sulfonamide intermediate of Glyburide bulk drug, was isolated by preparative HPLC and characterized as N-methyl impurity of sulfonamide intermediate of Glyburide API. Structural elucidations of this content was carried out by using 1H NMR and Mass spectral data along with HPLC analysis. The regulatory requirement was therefore fulfilled by characterizing this impurity and prepared impurity standard. This impurity standard was further used for analytical method validation studies. This work also supported the process development optimization of sulfonamide stage of Glyburide and facilitated to control forming this impurity during the process.

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RESEARCH ARTICLE

Development of High Performance Liquid Chromatographic Estimation of Domperidone and Lansoprazole

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ABSTRACT:

The combination of Domperidone and Lansoprazole is very useful in Gastro-esophageal disinfection (Dyspepsia). These methods provide means to separate the components characterize and quantify the components. An accurate, precise, specific and simple HPLC method was developed for simultaneous estimation of Domperidone and Lansoprazole. By this method retention time, linearity and accuracy data is respectively found for Domperidone and Lansoprazole. Mobile phase was prepared by mixing 51 volume of Acetonitrile and 49 volume of Ammonium Acetate (51:49 V/V) then 25mg each of Domperidone and Lansoprazole was dissolved in small volume of Acetonitrile: Ammonium Acetate (51:49 V/V) separately. Retention time was recorded 4.330 ± 0.003 minute and 5.820 ± 0.003 minute for Domperidone and Lansoprazole with 1.0 ml/min flow rate. The low value of % R.S.D indicates that this method is precise and accurate. Thus it can be concluded that the proposed method was good approach for obtaining reliable result.

KEYWORDS: Domperidone, Lansoprazole, RP-HPLC, Validation method, Method development.

INTRODUCTION:

Domperidone: The chemical name of Domperidone is 6-chloro-3-[1-[3-(2-oxo-3H-benzoimidazol-1-yl) propyl] piperidin-4-yl]-1H-benzoimidazol-2-one. Domperidone is an anti-dopaminergic drug which is used to stimulated lactation. It is a d_2 antagonist, it gives an Antiemetic & Prokinetic actions. Domperidone crosses the blood-brain barrier poorly. Domperidone act on CTZ, Metoclopramide efficacy is higher than antiemetic Domperidone. Bioavailability is only 15% via a first pass metabolism and plasma half life is 7.5 hours. Domperidone suppress nausea and vomiting. Chemical structure of Domperidone is shown in Figure 1.It is used in Parkinson's disease and effective in treatment of gastro paresis. Domperidone is given in combination with metoclopramide and granisetron in treatment of nausea and vomiting[1,2].

Lansoprazole:

The chemical name of Lansoprazole is 2-{[3-mthyl-4-2,2,2-trifuroethoxy) pyridin-2-yl] methylsulfinly]-1H-benzimidazole. It is a proton pump inhibitor which prevents the stomach from producing acid. Lansoprazole in netural PH is inactive, but the PH ≤ 5 it forms a two cationic charges i.e. a sulphenic acid and a sulphenamide, which react covalently with H^+K^+ATPase (with SH groups) and inactivate it irreversibly, especially two molecule of bind with the enzyme. The H†K† ATPase enzyme have a specific location in apical membrane of the parietal cells. Acid secretion is caused by synthesis of H^+K^+ATPase new molecule. It gets inhibited by gastric mucosal carbonic anhydrates. Lansoprazole is inhibited by the H^+K^+ATPase by partly reversible. Chemical structure of Lansoprazole is shown in Figure 1. Lansoprazole plasma half life is longer than omeprazole[3,4].

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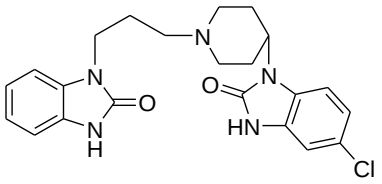
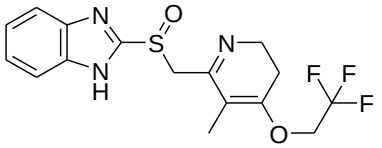


Domperidone



Lansoprazole

Figure 1: Chemical structure of Domperidone and Lansoprazole

MATERIAL AND METHODS:

HPLC system of Shimadzu –SPD 10A with Phenomenex – Luna 5Ω x 250 x 4.6 mm column was used UV-Visible spectrophotometer of Shimadzu with wavelength detector SPD-10A vp. Analytical weighing balance of Shimadzu (Aux 200) was used for weighing. Sonicator (Sonica 2200 MH) was used with Millipore filteration kit for solvents. Domperidone and Lansoprazole samples were received as a gift sample by micro labs, Banglore.

Preparation of Mobile phase:

Mobile phase was prepared by mixing 51 volume of Acetonitrile and 49 volume of Ammonium Acetate in the ratio is 51:49 v/v the mobile phase was sonicated for 10 min. and filtered through 0.45 m nylon membrane filter.

Standard Solution of Domperidone and Lansoprazole:

Accurately weighed 25 mg of Domperidone and Lansoprazole in 25 ml of volumetric flask separately and dissolve in small volume of Acetonitrile: Ammonium Acetate up to the mark with same solvent to obtaining each drug of concentration 1000 mg/ml[5,6].

Chromatographic condition:

Mobile Phase A: Acetonitrile and Mobile phase B: Ammonium Acetate (51:49v/v) wavelength for Domperidone is 200nm and for Lansoprazole is 350 nm by using a 5Ω x 4.6 x 250nm column with a flow rate of 1 ml/min both Domperidone and Lansoprazole showed good response at 289 nm wavelength. Chromatogram for Domperidone is shown in figure 2.

Analysis of laboratory mixture:

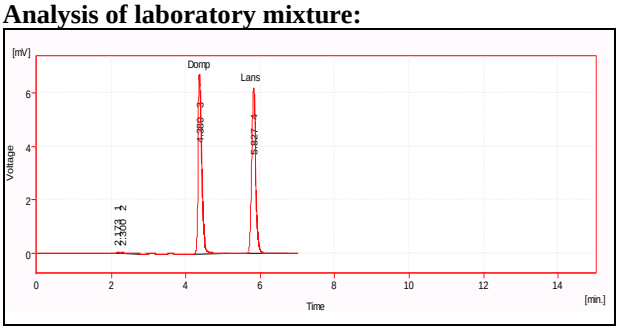


Figure 2: Chromatogram showing Retention Time (R_t) of (a) 16 µg ml^{-1} of Domperidone. (4.380 min) and (b) 12µg ml^{-1} of LANS. (5.827 min) in laboratory-prepared mixture

Method Validation:

Linearity:

The linearity of Domperidone and Lansoprazole was determined by analytical procedure (HPLC) in which nine concentrations of each Domperidone from the range 4-36 mg/ml and Lansoprazole 2-18mg/ml were selected. The graph was plotted between area v/s analyte concentration as shown in figure 3 and 4 of Domperidone and Lansoprazole respectively[7,8].

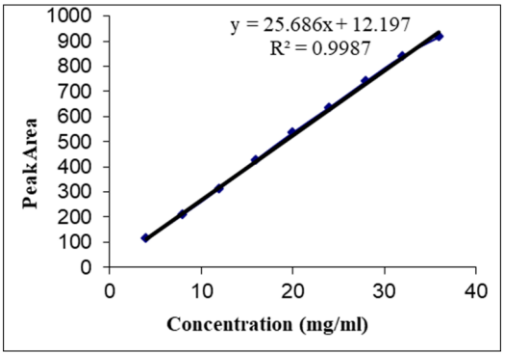


Figure 3: Linearity Curve of Domperidone

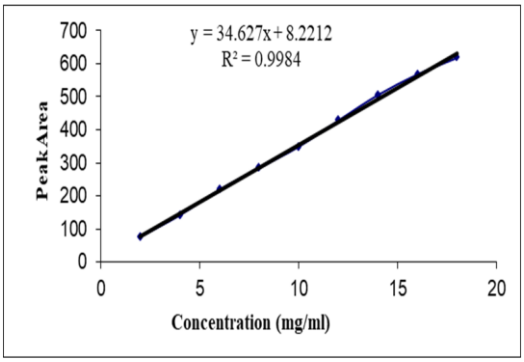


Figure 4: Linearity Curve of Lansoprazole

Precision:

Intermediate precision and repeatability measurement of peak area, retention time for each ingredient were determined by HPLC method (Table 1). The Repeatability, three replicates with in-day for five concentration and interemediate precision, 3 day for five concentrations[9].

Table 1: Precision study results of prepared binary mixture

Validation parameter	HPLC		
	% RSD		
Repeatability[a]	**Peak area**	**Peak asymmetry**	**Retention time**
DOMP.	69.146	0.5183	0.0810
LANS.	69.560	1.277	0.3635
Intermediate precision[b]	Peak area	Peak asymmetry	Retention time
DOMP.	70.256	0.7707	0.0948
LANS.	70.332	1.5508	0.3937

Table 2: Application of standard addiction technique to the analysis of Domperidone and Lansoprazole in Lans.pro-D Capsules

Sr. No.	Domperidone			Lansoprazole		
	Concentration in µg ml^{-1}		% Recovery ± SD[a]	Concentration in µg ml^{-1}		% Recovery ± SD[a]
	Claimed	Added	HPLC	Claimed	Added	HPLC
1	6	0	100.1 ± 0.738	6	0	99.75 ± 0.63
2	6	4	101.03 ± 1.8	6	2	100.475± 0.01
3	6	8	99.96 ± 0.72	6	4	101.02 ± 0.35
4	6	12	100.6 ± 0.91	6	8	99.84± 0.59
5	6	24	99.00 ± 1.1	6	10	100.55 ± 0.97
6	6	28	100.32 ± 0.91	6	12	99.94 ± 0.06

Accuracy:

The proposed HPLC[10] analysed the increasing normal addition of known quantities of studied drugs to an unknown formulation concentration and resulting mixtures. Application of the traditional addiction procedure to Lans.pro-D Capsules for the study of Domperidone and Lansoprazole are given in table 2.

Ruggedness:

The robustness of an analytical method was calculated by aliquots from homogeneous lots using operating and environmental conditions that may vary, but assay parameters are specified (Table 3).

Table 3: Ruggedness report of Domperidone and Lansoprazole

Parameter	Result observed	
	DOMP.	LANS.
Percentage Area	100.406%	99.124%
SD between set of analysis on same date	1.423	1.430
SD between set of analysis on different date	1.651	1.684
RSD between set of analysis on same day	1.417%	1.139%
RSD between set of analysis on different days	1.754%	1.647%

Robustness:

The robustness of the method was determined by making slight changes in the chromatographic conditions.

Apparent PH of the mobile Phase (± 0.3)
Mobile Phase organic content (± 3%)
Mobile Phase flow rate (±0.6 ml/min)
Detection Wave length (±2nm)

RESULT AND DISCUSSION:

Domperidone & Lansoprazole retention times were found to be 4.330 ± 0.003 min and 5.820 ± 0.002 min. Mobile phase acetonitrile: ammonium acetate (54:49 v / v), with a flow rate of 1.0 ml min-1 calibration was linear with a concentration range of 4-36 mg ml-1 and 2-18 mg ml-1, with a regression of 0.996 and 0.9984, intercepts of 10.225 and 8.2212, and slopes of 25.956 and 34.627, respectively, for domperidone and lansoprazole. The low R.S.D percent value suggests that the process is reliable and precise. The mean recoveries were found in the range of 98-102 percent. The percentage of R.S.D reported was found to be less than 2 percent ion performance, showing adequate accuracy , precision, linearity, robustness, which were found to pass all acceptance criteria.

CONCLUSION:

For simultaneous determination of Domperidone and Lansoprazole, a simple RP-HPLC method with UV detection has been developed. For precision , accuracy and linearity, the method was validated. In the assay results of pharmaceutical formulation as well as in laboratory prepared mixtures by the established method in the 4-18 mg ml-1 linearity range, good agreement was seen. It can be concluded that the proposed method was a successful approach to achieving accurate results and was found to be sufficient for routine pharmaceutical formulation estimations of Domperidone and Lansoprazole.

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The Authors are grateful to Micro Labs, Banglore for sending the gift samples of Domperidone and Lansoprazole. The author would like to thank Mr. Sanjeev Govind Rao Q.C Executive {Saar Biotech, Baadi, Solan (H.P)} For Providing guidance and necessary facilities to carry out research work.

CONFLICTS OF INTEREST:

The authors declare no conflict of interest.

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RESEARCH ARTICLE

Adverse Drug Reaction Monitoring of Antidepressant Drugs in a Mental Health Institute in Odisha

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ABSTRACT:

Introduction: Antidepressants are used primarily in the management of depressive and anxiety disorders. The occurrence of adverse drug reactions (ADRs) to antidepressants is a major challenge as it influences patient compliance. **Aim:** The aim of this study was to find out the ADR profile of antidepressant drugs in a mental health institute in Odisha. **Materials and Methods:** This is a cross sectional observational study conducted in Department of Pharmacology in collaboration with Mental Health Institute (Centre of Excellence) S.C.B Medical College and Hospital, Cuttack from September 2017 to September 2019. Patients who received at least one antidepressant drug were included in the study irrespective of age and sex. Data were collected by interviewing the patients or attendants and on detection of ADR, it was recorded on suspected ADR reporting form designed by PvPI. Causality, severity and preventability of ADRs were assessed by, WHO-UMC causality assessment, modified Hartwig-Siegel Scale and modified Schumock-Thornton criteria respectively. **Results:** Out of 180 patients taking antidepressants, ADRs were reported in 24% of patients, with either possible or probable causality. None were labelled as certain. ADRs were observed in 50% of patients who received TCAs and among 34.5% who received polytherapy. Insomnia (27%), fatigue (17%) and agitation (13%) were most common ADRs. Most of the ADRs were of mild severity (91%) and not preventable (84%).

Conclusion: Insomnia, fatigue and agitation were among most common ADRs. There was increased chance of ADRs with polytherapy and use of TCAs. Most ADRs were mild and not preventable.

KEYWORDS: Depression, Antidepressants, Adverse Drug Reactions, SSRI, SNRI, TCA.

INTRODUCTION:

Depressive disorders have a substantial impact on global health. According to a Global Health Estimate the prevalence of such mental health disorders is more than 300 million, being the fourth leading cause of disability and premature death[1].

They constitute an estimated 7.4% of the world's measurable burden of disease[2]. Major Depressive Disorder (MDD) being the second leading cause of years lived with disability (YLDs) globally is the four largest contributor to YLDs in socially diverse regions across the six continents[2]. In India, the prevalence of depressive disorders was 50 million or 4.5% of population in 2015 and total Years Lived with Disability (YLD) was over 10 million years (7.1%).[3,4,5]

As a class of drugs, antidepressants are used primarily in the management of depressive and anxiety disorders. The drugs used in the management of depression include tricyclic antidepressants (TCAs), selective serotonin

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reuptake inhibitor (SSRIs), serotonin norepinephrine reuptake inhibitors (SNRIs), monoamine oxidase inhibitors and atypical antidepressants that primarily effect on the serotonergic or the noradrenergic neurotransmitter system.[6,7,8,9]

Occurrence of adverse drug reactions (ADRs) lead to noncompliance and discontinuation of the therapy.[10,11,12,13,14,15] Updated information on the ADRs of these drugs and the knowledge of risk benefit balance is likely to influence the compliance to treatment.

As there is little data available in terms of safety of antidepressants in the local population, this study was done to analyze the ADR profile of antidepressant drugs in a mental health institute in Odisha.

MATERIAL AND METHODS:

Study Design:
This is a hospital based cross-sectional, observational, descriptive study.

Study Setting:
The study was conducted in Department of Pharmacology in collaboration with Mental Health Institute(Centre of Excellence), SCB Medical College and Hospital, Cuttack.

A study team was constituted comprising of principal investigator and co authors. The study protocol was designed by the team.

Study Period:
The research work was carried over a period of two years from September 2017 to September 2019.

Study Population:
All patients receiving antidepressants, both as outpatient or inpatient, in the Department of Psychiatry, SCB Medical College and Hospital, Cuttack.

Sample Size:
Sample size was calculated to be 180 using the formula

Sample size (n) = $[\{Z_{1-\alpha/2}\}^2 p(1-p)] / d^2$ [16]

Where 'p' is prevalence of antidepressant use and'd' is allowable error.

Ethical Permission:
The study was approved by institutional ethics committee.

Study Subjects:
This study was conducted on patients receiving antidepressants in Department of Psychiatry fulfilling the following criteria:

Inclusion criteria-
All subjects receiving antidepressant medication attending the department of Psychiatry and willing to participate.

Patients of all age groups and both sexes.

Study Procedure:
Demographic details of selected subjects like age, sex, educational, marital and employment status noted in prestructured case record form (CRF). Clinical details like diagnosis, antidepressant used, dosage, route of administration, frequency and duration of treatment noted in CRF. Subject were questioned and assessed for any occurrence of adverse drug reaction (ADR). If ADR present, details of it was noted on suspected ADR reporting form given by PvPI. Causality, severity and preventability of ADRs were assessed by, WHO-UMC causality assessment[17], modified Hartwig-Siegel Scale[18] and modified Schumock-Thornton criteria respectively.[19]

Statistical Analysis:
Results were analyzed using descriptive statistics like mean ± SD, percentages and proportions. Significance, when required, was tested using chi square test at two sided α level at 5%. All the analysis was done by using Microsoft Excel and SPSS v.20.

RESULTS:.
Our study had a total of 180 patients enrolled. Of them majority were females, i.e, 99 (55%) compared to males, i.e, 81(45%). The mean age of the study subjects was 47.5 ± 6.7 years. Most of the subjects, i.e, 70 (38.9%) belonged to the age group of 36-50 years, followed by 51-64 years age group with 55 (30.5%).

Depressive disorders were the primary reason for taking antidepressants (42%) followed by anxiety disorders with 28%.[Fig.-1]

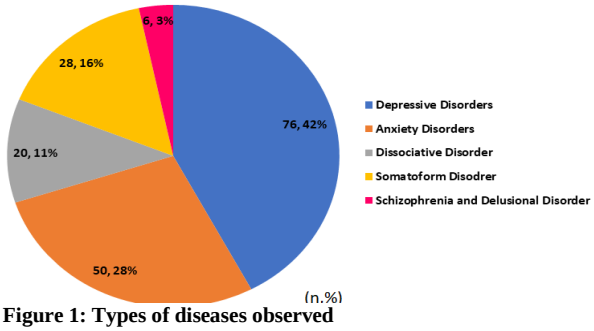

Figure 1: Types of diseases observed

A total of 43 patients were found to have adverse drug reactions. Some of them had more than one adverse event. A total of 52 adverse events were noted. Insomnia

with 14(27%) cases was most common adverse effect. This was followed by fatigue and agitation or nervousness with 9(17.3%) and 7(13.5%) cases respectively. [Table 1]

Table 1: Adverse drug reaction profile (N=52)

ADRs	Frequency (n)	Percentage (%)
Insomnia	14	26.9
Fatigue	9	17.3
Agitation/ Nervousness	7	13.5
Sexual Dysfunction	4	7.7
Increased Sweating	4	7.7
Headache	4	7.7
Nausea	3	5.8
Dry Mouth	3	5.8
Loss of Appetite	2	3.8
Tachycardia	2	3.8
Total	52	100

In our study 24.7% males showed adverse drug reactions in comparison to 23.2% of all females. This difference in incidence was not significant statistically. (Chi Square Test, p=0.84)

Table 2: Distribution of ADRs by gender (N=180)

Gender		ADR		Total
		Present	Absent	
Male	Count	20	61	81
	% Within Gender	24.7	75.3	100
	% of Total	11.1	33.9	45
Female	Count	23	76	99
	% Within Gender	23.2	76.8	100
	% of Total	12.8	42.2	55
Total	Count	43	137	180
	% of Total	23.9	76.1	100

Most number of the patients with ADRs belonged to the 6 to 9 months of antidepressant therapy group with 23 cases. The incidence of ADRs increased with increase in duration of therapy with highest seen in more than 9 months of treatment (44%). [Fig-2]

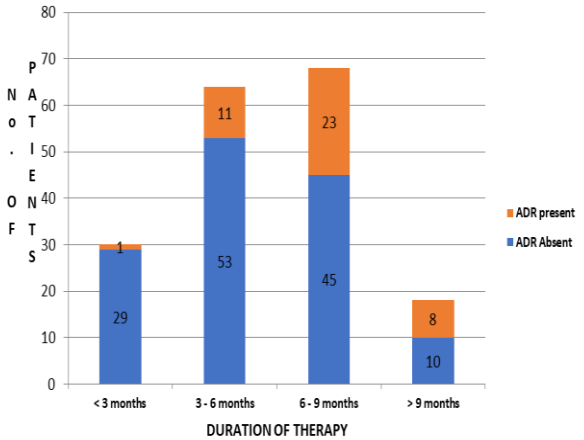

Figure 2: ADRs in relation to duration of therapy

The incidence of ADRs was higher in polytherapy group (34.4%) compared to monotherapy (21.6%). This was not a statistically significant difference. (Chi Square Test, p=0.18) [Table 3]

Table 3: ADR in Relation to Type of Therapy (N=180)

Type of Therapy		ADR		Total
		Present	Absent	
Monotherapy	Count	32	116	148
	% In Type of Therapy	21.6	78.4	100
Polytherapy	Count	11	21	32
	% In Type of Therapy	34.4	65.6	100
Total	Count	43	137	180
	% of Total	23.9	76.1	100

In our study most number of patients with ADRs, i.e, 15 cases were receiving only SSRIs. This was followed by patients with polytherapy (SSRI plus some other drug) with 11 cases and SNRIs only group with 10 cases. But incidence wise TCAs were most unsafe with 50% of patients showing ADRs followed by polytherapy with 34.4% patients showing ADRs. TCAs showed statistically significant higher incidence of ADRs compared to SSRIs (p= 0.04) and SNRIs (p= 0.03). (Chi Square Test) [Fig 3]

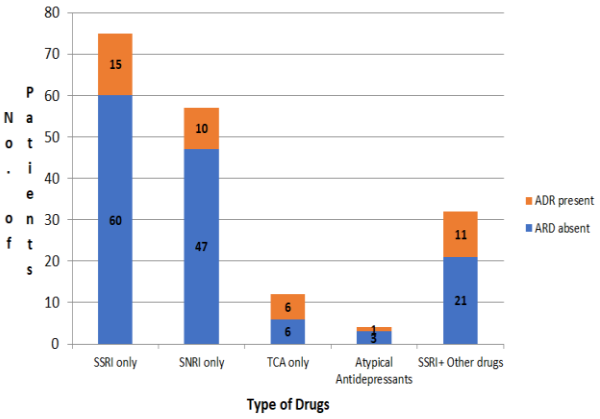

Figure 3: ADRs in relation to group of antidepressant drugs

According to WHO – UMC causality assessment system, 25 patients with ADR were assessed to be grouped in 'Possible' category and 18 patients were assessed to be in 'Probable' category. None of the 43 patients with adverse reactions belonged to any of the other categories. Most of the ADRs, i.e., 39 (90.7%) were 'Mild' and 4 (9.3%) were 'Moderate' based on Hartwig Siegel Scale. None of the study subjects showed any severe adverse drug reaction. Based on modified Schumock – Thornton criteria, 36 (83.7%) patients had ADRs which were 'Not Preventable' and 7 (16.3%) had ADRs which were 'Probably Preventable'. None of the patient had any 'Definitely Preventable' adverse drug reaction.

Table 4: Causality assessment of ADRs (N=43)

WHO-UMC Category	Frequency (n)	Percentage (%)
Certain	Nil	Nil
Probable	18	41.9
Possible	25	58.1
Unlikely	Nil	Nil
Conditional	Nil	Nil
Unclassifiable	Nil	Nil
Total	43	100

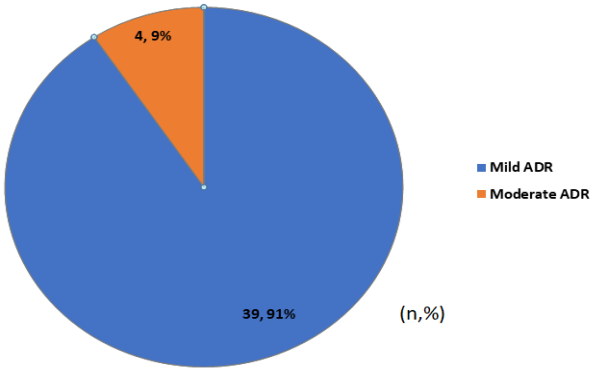

Figure 4: Severity assessment of ADRs

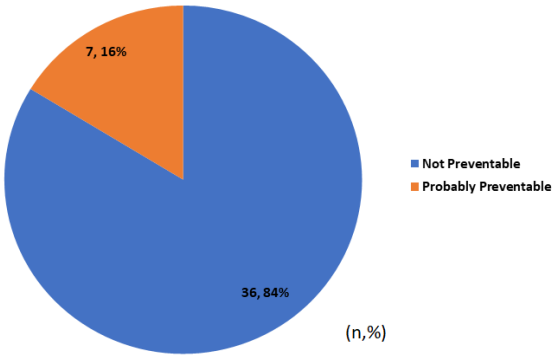

Figure 5: Preventability assessment of ADRs

DISCUSSION:

We have conducted this study with an aim to assess the incidence and profile of suspected ADRs associated with antidepressant medications in a mental health institute in Odisha. Demographic analysis did not confer any significant higher incidence of adverse drug reactions to female compared to males (p = 0.18), a congruent finding to Mishra *et al* and Mukherjee *et al*.[20,21]

Analysis of the diagnostic pattern suggested that depressive disorders were the most common psychiatric illness encountered followed by anxiety disorders. This finding is similar to Mukherjee *et al* and Sridhar *et al*.[21,22]

In our study incidence of ADRs was about 24% with insomnia (8%), fatigue (5%) and nervousness/agitation (4%) being the most common among them. These findings are somewhat similar to that by Mishra *et al* where insomnia and agitation were top most ADRs[20] and Mamatha *et al* where insomnia was most common.[23]

In our study the incidence rate of ADRs gradually higher with the increase in duration of therapy, with highest incidence (44%) in more than 9 months of treatment group. This difference in incidence rate of ADRs between groups was statistically significant, clearly suggesting that with increased duration of therapy there is definitely increase in development of adverse drug reactions,

The incidence of ADRs was higher in patients who received more than one antidepressants compared to those who received just one. This difference however wasn't significant statistically. With a higher sample size may be a significant difference can be elucidated.

Most numbers of ADRs were seen in patients receiving SSRIs, a finding similar to almost all published studies. This could be due to the higher rate of usage of SSRIs compared to other groups. When classes of antidepressants were compared with each other TCAs had higher incidence of ADRs compared to SSRIs and SNRIs, which was statistically significant. This again is a similar finding to almost all the published studies. There was no statistically significant difference in ADR incidence rate of SSRIs compared to SNRIs. This is similar to findings by Mukherjee *et al*, Sharma *et al* and Shah *et al*.[21,24,25]

In causality assessment by WHO-UMC system 52% ADRs were 'Possible' and 48% were 'Probable' in our study. None of the ADRs were assessed to be 'Definite' as no discontinuation or rechallenge was done. These results are in congruence to findings by Mukherjee *et al* and Mishra *et al.* (21) (20) Lucca *et al* found a higher incidence of Probable (61%) ADRs compared to Possible (39%).[26]

Severity assessment of ADRs done by using modified Hartwig – Siegel scale found that in our study majority (91%) of ADRs were of 'Minor' severity and few (9%) of 'Moderate' severity. There was no hospitalization or any permanent damage or any fatality due to the ADRs. So none of the ADRs can be classified as 'Severe'. This finding is similar to that of findings of Mishra *et al*, Mukherjee *et al*, Lucca *et al* and Sengupta *et al*.[20,21,26,27]

In our study none of the ADRs were 'definitely preventable' while a very few (16%) were 'probably preventable'. Most of the ADRs were 'not preventable' indicating towards the inevitable nature of ADRs with treatment by antidepressants. These findings are similar to the findings by Lucca et al, Mukherjee *et al*, Sharma *et al* and Shah *et al*.[21,24,25,26] One study by Thomas *et al* found the rate of definitely preventable ADRs to be at 20%.[28] The lower rate of preventable ADRs might indicate towards proper diligence by the treating psychiatrists at our centre in prescribing suitable antidepressant based on patient's clinical profile.

The current study focuses mostly on the prevalence of ADRs and their nature among antidepressant users, but is limited by its cross sectional nature and single setup. Multicentric prospective studies will bring about even better understanding about ADRs in this specific population.

CONCLUSION:

A moderate incidence of ADRs in patients receiving antidepressant drugs was found in the current study among which insomnia was the most common. SSRIs were the most common group to have ADRs but TCAs were most unsafe. Nature of majority of the ADRs reported in the study was mild and they were of not preventable type. But with better scrutiny the probably preventable adverse drug reactions can be avoided paving way for better use of antidepressants.

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RESEARCH ARTICLE

Hypoglycemic, Hypolipidemic, Renal Protective and Antioxidant Activity of *Annona muricata* in Streptozotocin-Induced Diabetic Rats

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ABSTRACT:

Annona muricata, an herbal plant commonly used in traditional medicine to manage numerous diseases, diabetes as other diseases could be managed with herbal medicine. This study was designed to be investigated the antidiabetic, hypolipidemic, renal protective, and antioxidant effects of aqueous extracts of *Annona muricata* as used alone or combined with metformin in streptozotocin (STZ)-induced diabetic rats. Methods: the study was involved twenty adult Wister albino rats in four groups (five rats in each) and designated as groups, control group (1), and experimental groups (2, 3, 4). Diabetes was induced in experimental groups by 60 mg/kg intravenous streptozotocin injection. Group 2: serves as a diabetic control group, Group 3: diabetic rats treated with oral administration of 100 mg/kg of *Annona muricata* aqueous extract, Group 4: diabetic rats treated with combination (100 mg/kg aqueous extract of *Annona muricata* + 50 mg/kg metformin). The treatment continuous daily for 4 weeks to determine the levels of blood glucose and biochemical analysis. Result: aqueous extract of *Annona muricata* was reduced the serum glucose level effectively in streptozotocin-induced diabetic rats, by 48% and 55% after 28 consecutive days of treatment when used alone and with metformin, respectively. These compared to the preliminary values and the reduction was statistically significant compared to a diabetic control group. Daily oral administration of 100 mg/kg aqueous extract of *Annona muricata* for 4 weeks to streptozotocin-induced diabetic rats significantly reduced the level of total cholesterol, urea, creatinine, and MDA, whereas the reduction was non-significant in triglyceride and VLDL-cholesterol levels as compared to the non-treated diabetic group. However, the reduction is more significant in streptozotocin-induced diabetes rats that were treated with a combination of *Annona muricata* and metformin when compared to the diabetic control group. Conclusion: Aqueous extracts of *Annona muricata* have anti-diabetic action through their hypoglycemic, hypolipidemic, renal protective, and antioxidant effects in streptozotocin-induced diabetic rats. Thus, can be used alone or with anti-hyperglycemic drugs as metformin in the management of DM. The combination is preferred in severe hyperglycemic cases with more hypoglycemic effect requirements.

KEYWORDS: Diabetes Mellitus, Aqueous extracts of *Annona muricata*, Metformin, Streptozotocin (STZ), blood glucose level.

1. INTRODUCTION:

Diabetes Mellitus (DM), a heterogeneous metabolic syndrome, described as a chronic hyperglycemia condition due to an imbalance of pancreatic β-cells function. It referred to high blood glucose levels and its complications cause organ damage[1-4].

The incidence of DM increased in worldwide to act as a major problem in public health. It's probably expected the percentage will rise to reach about 72% in 2025[2,5]. Nowadays, Type 2 DM is more common and the prevalence increased in past decades. This condition is characterized by hyperglycemia, hypercholesterolemia, and hypertriglyceridemia[3,6]. As well, the evidence of oxidative stress augmented in DM that predominately associated with a high level of lipid peroxidation biomarkers as malondialdehyde (MDA)[7].

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Although oral hypoglycemic drugs as metformin can be controlled early diabetes's manifestation, the complications may appear in some patients related to many side effects such as peripheral edema, abdominal pain, lactic acidosis, and others[8-10]. Hence, the scientific works continue to assess the antidiabetic effects of raw materials and extracts of natural compounds, despite the continuous efforts in the scientific community to the production of new drugs and their validation on scientific principles[2, 6, 11, 12]

One of the natural plants from the Annonaceae family called *Annona muricata* (Ann. Muricata), and well-known as Soursop, the leaves, fruit and other parts of its tree had an outdated use in traditional medicine for a long history [13, 14]. *Annona muricata* has a wide range of ethnopharmacological uses in the management of various conditions involving rheumatism, pain, fever, hypertension, diabetes, vomiting, worm, diarrhea, and heart or liver disease [15, 16]. This also acts as an anti-inflammatory and anti-cancer mediator with high effects on the immune system [16]. additionally, *Annona muricata* possesses antibacterial, antimicrobial [16, 17], and antiviral effects [18].

With regards to previous literature, *Annona muricata* has positive effects in several body systems such as anti-hypertensive, anti-spasm, vasodilatation, cardiovascular depression [19, 20], and Suppress seizure with anxiolytic action [21]. Although, several investigators were observed that *Annona muricata* exhibits toxic effects on both the liver and kidney, the low dose of it has a protective effect [22, 23].

Moreover, the leaves of *Annona muricata* were contained biological activity against plasmodium, parasite, protozoa, and anti-anthelmintic in internal and external worms [13, 16]. Likewise, it has beneficial effects in controlling the diabetic patient as a hypoglycemic, hypolipidemic, and antioxidant effect [7, 20, 24]. The alleviating properties of *Annona muricata* as a result of its content. In fact, the extracts of *Annona muricata* contain more than 200 phytochemical constituents with pharmacological activities such as alkaloids, phenol, acetogenins, tannins, flavonoids, coumarins, trepenoid, ellagic acid, stearic acid, and other components [23, 25]. The present study investigates the anti-diabetic, hypolipidemic, antioxidant, and renal protective effects of aqueous extract of *Annona muricata* on diabetic rats after their induction by streptozotocin (STZ) (STZ-induced diabetic rats) as used alone or combined with hypoglycemic drugs (metformin).

2. MATERIALS AND METHODS:

2.1 Animals:

Twenty healthy adult male Wister albino rats, (2-3) months old were used in this study, with a weight between 160 and 190 gm. These animals were collected in animals' houses in the college of pharmacy, University of Basra, the animals were kept under the appropriate laboratory conditions, in a 12 hr. environmental light/ dark cycle at room temperature (25±1) C and humidity (55±5%). The animals were fed a commercial diet, pellets, and water *ad libitum*. All measures were engaged according to the guidelines (National Institutes for health USA publication, 1985).

This study was approved via the Animal Ethics Committee in the college of pharmacy/ Basra University.

2.2 Plant materials:

The leaves of *Annona muricata* were collected in March 2020; the plant was identified in the Pharmacognosy Department, College of Pharmacy, Basra University, Iraq. It dried in the air at room temperature then grind to powder manually. According to the traditional method obtained aqueous extract of powder through the process in which the powder dissolved in distilled water (D.W) within a ratio of 1:3 (1kg of *Annona muricata* with 3 ml D.W) at room temperature for 48 hrs. The oven at 40 C is used to evaporate the filtrate to achieve the desired dose for therapeutic testing [26].

2.3 The procedure design:

In this experiment, the healthy animals were randomly distributed into four groups with five animals in each one to investigate the effects of aqueous extract of *Annona muricata* on streptozotocin (STZ) induced diabetic rats.

2.4 Experimentally Induction of diabetes mellitus in rats:

Diabetes condition induced in animals during 2-4 days after injected of the three groups of rats with streptozotocin (STZ) intravenously (I.V), the dose was used 60 mg /kg dissolved in 0.9% sodium chloride solution. While the control group was received normal saline intravenously at this time. The animals fasted before streptozotocin administration for 16 hrs.

After 72 hr. of diabetes induction, blood samples were engaged from the tail vein rats to measure Blood Glucose level (BG). the glucose level in each blood was measured by using the Glucose Oxidase Method [27]. If the fasting blood glucose of the animals more than 200 is considered diabetic conditions [28].

After that, the animals were kept under careful observation for 2 weeks without treatment to stabilize the diabetic conditions and re-measuring the parameters on the last day to confirm the rising of blood glucose level after streptozotocin induction [20].

2.5 Oral administration of *Annona muricata*:
After 2 weeks of diabetic stabilizing, the experimental rats were separated into four groups (5 animals in each one) for treatment:

- Group (1) control group: (non-diabetic induction rats) were treated with distilled water (D.W) 1 ml/kg only.
- Group (2) STZ- induced diabetic rats (diabetic control rats) were treated with D.W (1 ml/kg) only.
- Group (3) STZ-induced diabetic rats were treated orally with aqueous extract of *Annona muricata* at a dose of 100mg/kg/day dissolved in D.W
- Group (4) STZ-induced diabetic rats were treated orally by a combination of aqueous extract of *Annona muricata* (100mg/kg) dissolved in D.W and metformin with a dose of 50 mg/kg dissolved in 0.9 sodium chloride solution.

The experimental rat's therapy continued for 28 days sequentially via oral gastric intubation, the blood sample was pulled from the tail vein of the rats to monitor the experimental parameters and blood glucose level.

2.6 Blood samples and Biochemical analysis:
Blood samples were obtained from the animals on various days and all the results are recorded. The readings of blood glucose levels were taken in (day 0 before treatment, day 3, 14, 21, and 28 after treatment) as shown in table 1.

At the beginning and end of the treatment, the blood collected in standard tubes then centrifuged in (Genex, Florida, USA) at 3000 rpm for 15 minutes to obtain serum for biochemical analysis (total cholesterol (TC), triglyceride (TG), serum creatinine (S Cr), blood urea and MDA). The parameters were measured by using a spectrophotometer (Integra machine, Roche Germany) following the instruction of the commercial diagnostic kits.

2.7 Statistical analysis:
The results of the study were analyzed by using the statistical system (spss) version 20. Parameters values were expressed as mean ± standard error of the mean. The statistical significance was determined by using one-way analysis of variance (ANOVA), then Tukey test. P-value < 0.05 was reflected statistically significant.

3. RESULTS:

3.1 Effect on blood glucose level:
The intravenous administration of Streptozotocin results in increased levels of blood glucose significantly during the period of the study in three experimental groups as compared to the normal group, as shown in table 1. The values of blood glucose showed significantly reduced (p-value < 0.05) after the daily administration of aqueous extract of *Annona muricata* alone and with metformin compared with the diabetic non-treated group on day 14. Then on day 21, the blood glucose levels were reduced by 42.1% for the group that received *Annona muricata* extract, and 53.5 % for the group provided with aqueous extract of *Annona muricata* and metformin, and the reduction was statistically significant compared with a diabetic control group. After 4 weeks of management, the reduction in the glucose level in blood was 48% and 55 % for *Annona muricata* extract-treated group and *Annona muricata* extract plus metformin-treated group, respectively, these compared to the preliminary values, and the reduction was statistically significant as compared to the non-treated diabetic group.

3.2. Effect on serum lipid profile.
The serum lipid profile in experimental diabetic rats showed significantly high levels in total cholesterol, triglycerides, and VLDL-cholesterol as compared to the normal control group (p-value < 0.05). After 4 weeks of treatment with an aqueous extract of *Annona muricata*, the total cholesterol level has shown a significant decrease (20%) when compared with the non-treated diabetic rats (p-value < 0.05). As well, the administration of aqueous extract of *Annona muricata* with metformin to STZ-induced diabetic rats reduced the total cholesterol level significantly (14%) as compared to the non-treated diabetic group. Whereas, the reduction in triglycerides and VLDL cholesterol levels did not reach the statistical significance level (p > 0.05), as shown in table 2.

Table 1: Effect of aqueous extract of *Annona muricata* on levels of blood glucose in STZ induced diabetic rats.

Treatment groups	Blood Glucose levels (mg/dl)				
	Day(0)	Day (3)	Day (14)	Day (21)	Day (28)
Normal control	106.2 ±6.1	104.2±7.9	105.2±4.2	105±6.04	103.8±8.7
Diabetic control	231.4 ±3.9*	231.8±2.5*	219.6±3.7*	225.8±2.6*	225.6±6.7*
Annona.muricata 100 mg/kg/day	234.2 ±4.2*	230.6±3.4*	142.2±3.3#*	135.6±3.4#*	121.8±3.6#
Annona muricata + metformin	234.8 ±2.5*	225.4±4.1*	127..4±3.8#*	109.2±2.9#	105.7±2.5#

Data are expressed as mean ± SEM, n=5
* P < 0.05 significantly different as compared to the normal control group.
# P < 0.05 significantly different as compared to the diabetic control group.

Table 2: effect of aqueous extract of *Annona muricata* on serum lipid profile after four weeks of treatment.

Lipid Parameters	Control group	Diabetic control group	*Annona muricata* extract100 mg/kg/day group	*Annona muricata* extract+metformin group
Total cholesterol (mg/dL)	105.4±8.7	139.2±5.1*	111.3± 6#	119.7±4.1#
Triglycerides (mg/dL)	75.1±7.4	110.3±10.7*	94.5±8.7	102.4±6.2*
VLDL- cholesterol (mg/dL)	15.1±1.4	22.04±2.1*	18.8±1.7	20.4±1.3

Data were expressed as mean ± SEM, n=5
* P-value < 0.05 significantly different as compared to the normal control group.
# P-value < 0.05 significantly different as compared to non-treated diabetic group.

Table 3: Effect of aqueous extract of *Annona muricata* on renal function after four weeks of treatment.

Renal function Parameters	Control group	Diabetic control group	*Annona muricata* extract100 mg/kg group	*Annona muricata* extract+metformin group
Creatinine (mg/dL)	1.1±0.1	1.85±0.2*	1.07±0.1#	0.92±0.1#
Urea (mg/dL)	23.4±1.2	52±6.8*	24.4±1.7 #	21.6±2.5#

Data expressed as mean ± SEM, n = 5
* P-value < 0.05 significantly different as compared to the control group.
# P-value < 0.05 significantly different as compared to the diabetic control group.

3.3. Effect of aqueous extract of *Annona muricata* on Renal function in experimental diabetic rats.

Table 3 illustrated the significant increase in Urea and Creatinine levels in STZ induced diabetic group as compared to the normal control group. The treatment for four weeks with100 mg/kg aqueous extract of *Annona muricata* alone and with a combination of Annona. muricata extract and metformin result in a significant reduction in the Urea and Creatinine levels (p-value < 0.05) when compared to the non-treated diabetic group.

3.4. Effect of *Annona muricata* as an antioxidant:

The level of MDA was elevated significantly in experimental diabetic rats when compared with the control group (p< 0.05), the daily treatment with plant extracts decrease the MDA levels significantly (68.6%) as compared with the non-treated diabetic group. Also, the group treated with *Annona muricata* extract and metformin show a significant reduction in the MDA level (52.7%) after 28 days of treatment. Table 4.

Table 4: Effect of aqueous extract of *Annona muricata* on MDA level after four weeks of treatment

Biomarker	Control group	Diabetic control group	*Annona muricata* extract100 mg/kg group	*Annona muricata* extract+metformin group
MDA (nmol)	0.87±0.2	2.83±0.4*	0.89±0.1#	1.34±0.2#

Data expressed as mean ± SEM,n=5
* P-value < 0.05 significantly different as compared to the control group.
#P value < 0.05 significantly different as compared to the diabetic control group

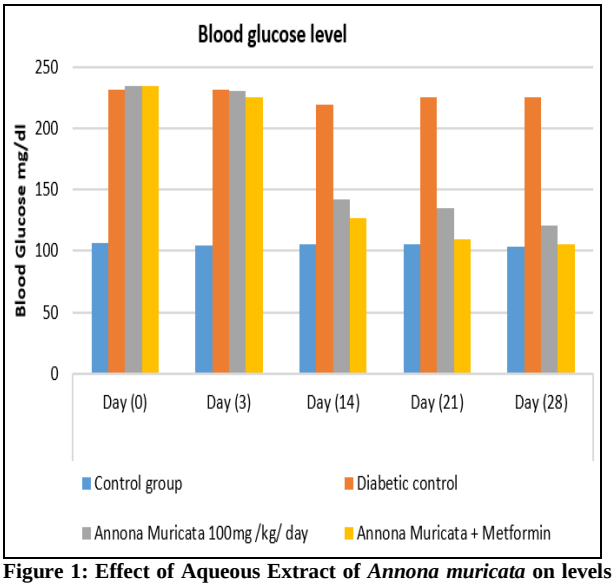

Figure 1: Effect of Aqueous Extract of *Annona muricata* on levels of blood glucose in Streptozotocin-induced diabetic rats.

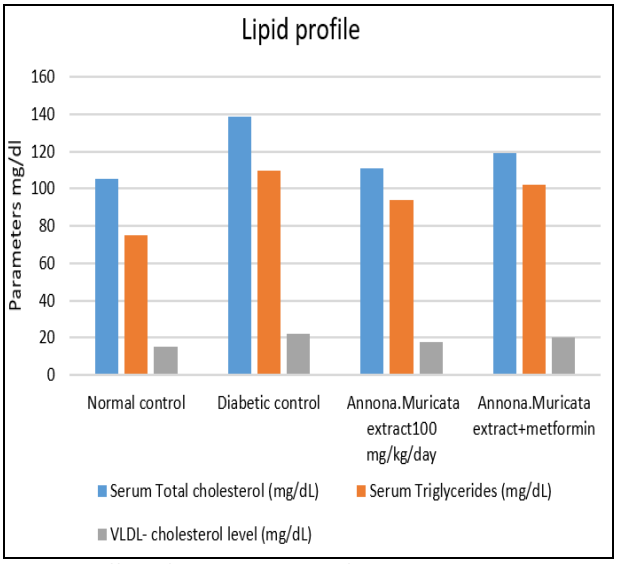

Figure 2: effect of aqueous extract of *Annona muricata* on serum lipid profile after four weeks of treatment.

4. DISCUSSION:

Diabetes Mellitus, one of the main global health problems, which associated with abnormalities in glucose hemostasis and lipid profile [3,29,30]. The high level of blood glucose can result from the impairment in secretion and/or effect of insulin [3,31,32]. Scientists are ongoing to search for plant extracts having a protective role and effectiveness in the management of diabetic animals and humans [33,34]. The necessity to use alternative antidiabetic compounds to overcome the undesirable effects of the current therapy. Thus, the controlling and treatment of DM need exploring of low toxicity, inexpensive, and antioxidant natural compounds with inhibitory enzymatic activity [6, 25, 34-36]. One of these herbal extracts is *Annona muricata*; the present study demonstrates the effects of the aqueous extracts of *Annona muricata* in improving the hyperglycemic, hyperlipidemia, and renal function in streptozotocin-induced diabetic rats.

Generally, streptozotocin could destruct the β- cell in Langerhans islet in the pancreatic tissue that induces experimental DM after 3 days of administration [28, 37]. Our results have been shown; the continuous daily treatment of aqueous extract of *Annona muricata* for 28 days in STZ- induced diabetic groups significantly reduces the blood glucose levels, which consistently manages the diabetes condition after induction by streptozotocin. The levels of blood glucose in experimental diabetic rats dropped within 14 days after treatment and continuously lowered until reach a normal level. This is attributed to the aqueous extracts of *Annona muricata* contain hypoglycemic phytochemical constituents as flavonoid, alkaloid, tannin, triterpenoids, and other secondary metabolites [14, 16, 38]. Additionally, the previous researchers suggested that *Annona muricata* can exert a hypoglycemic effect through its stimulated insulin secretion, enhanced the repairing of β cell, and increased the activity of both insulin and adrenalin [20, 39, 40]. Our findings were similar to the results of previous studies [20, 40], which found that the administration of 100mg/ kg of *Annona muricata* to diabetic rats renormalized the blood glucose level and improved glycemic control. These also in agreement with Gavamukulya et al [41] who identified that *Annona muricata* has the traditional uses and pharmacological properties as the anti-hyperglycemic effect. Besides, The work done by Vidona et al [42] observed that there are beneficial effects of *Annona muricata* aqueous pulp extracts on diabetic animals by reducing body weight, serum glucose levels, and hyperglycemia symptoms. The glucose level in blood is significantly more reduced in the STZ- induced diabetic group treated with a combination of aqueous extract of *Annona muricata* and metformin compared to the STZ- induced diabetic group treated with *Annona muricata* alone as a result of using both compounds with a hypoglycemic effect., this is similar to the finding of Michael et al [43]. While the study of Vidona et al [42] demonstrated that *Annona muricata* has a moderate effect when used alone compared with metformin in diabetic rats. So the combination is recommended in severe cases of hyperglycemia as a synergistic effect and reduces the complication [42].

Induction of diabetes by streptozotocin could be elevated the lipid profile as triglyceride (TG), total cholesterol, atherogenic index, LDL-cholesterol, VLDL-cholesterol, and reduced HDL-cholesterol [29]. The increasing of TG level in DM is attributed to the high production of very-low-density lipoprotein, defect in its catabolism, and attenuating the activity of lipoprotein lipase enzyme which responsible for triglyceride degradation [44]. The results of the present study revealed that the administration of *Annona muricata* aqueous extracts alone or combined with metformin in STZ-induced diabetic for 4 weeks significantly decrease the level of serum total cholesterol as compared to a diabetic control group that ascribed to the anti-hyperlipidemia and anti-hyperglycemic properties of *Annona muricata* [25, 44]. While the non-significant reduction of the TG and LDL-cholesterol levels after treatment probably due to an inadequate dose of plant or required prolonging the duration of treatment. Additionally, increased the circulatory of TG and VLDL- cholesterol may result from defective of their clearance from blood circulation [44, 45]. These results agree with the conclusion of previous studies [20,44] which observed that *Annona muricata* reduced the lipid profile in diabetic conditions. Hence, the aqueous extracts of *Annona muricata* could have possibly decreased the risk of cardiovascular disease in DM and death [44, 46].

On the other hand, both serum creatinine and urea were elevated significantly in experimental diabetes rats, which indicates the end stage of kidney disease and extracellular dehydration [47]. The daily use of the *Annona muricata* extract in treating experimental groups could significantly lower these markers through its protective effects in low dose, thus, may be an improved renal function [22, 48]. These findings were in line with Florence et al [20], who suggested that *Annona muricata* significantly reduced the serum creatinine level.

Based on the previous evidence that streptozotocin influenced pancreatic injury by stimulating the free radical and oxidative stress. Alteration of enzymatic and non-enzymatic antioxidant systems with enhanced lipid peroxidation is the most appearance of oxidative stress in diabetic status [7, 49]. The MDA increased in the diabetic group, which acts as a biomarker of oxidative stress that is raised in hyperglycemia. The administration of aqueous extract of *Annona muricata*

for four weeks significantly reduced the level of MDA, that caused by the *Annona muricata* has protective activity against oxidative stress [25, 41]. The antioxidants activity of *Annona muricata* due to contains flavonoids, which reduce the MDA level either directly through neutralizing the toxicity of free radicals by donating hydrogen ions or indirectly through elevating the endogenous antioxidant gene expression [26]. Furthermore, the protective effect and antioxidant properties of Annona Muricate were observed in previous studies that highly agreeing with the results of the current study [7, 20].

5. CONCLUSION:

Based on the results of this study, the aqueous extracts of *Annona muricata* have anti-diabetic action through their hypoglycemic, hypolipidemic, renal protective, and antioxidant effects in streptozotocin-induced diabetic rats. So this plant is widely presented with low cost in a different geographical area. Thus, can be used alone or with anti-hyperglycemic drugs as metformin in the management of DM. The combination is preferred in severe hyperglycemia cases with more hypoglycemic effect requirements. These results indicate that need further studies on the beneficial effect of a combination of *Annona muricata* with hypoglycemic drugs in controlling DM.

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RESEARCH ARTICLE

Evaluation of an *Ayurvedic* preparation-'*Ekangaveera Rasa'* for possible metal toxicity: Behavioral, Biochemical and Histopathological Analysis

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ABSTRACT:

Ayurveda, an ancient Indian medicinal system, is highly regarded as complementary and alternative medicine in recent years. However, there are reports to show the toxic effects of specific *Ayurvedic* preparations, which use metals as an ingredient in them. One such medicine is '*Ekangaveera Rasa'* This herbo-minero-metallic preparation is used to treat the pain of neurological origin. Therefore, the present study aimed to evaluate the possible alteration caused by *Ekangaveera Rasa* on the brain, liver, and kidneys by histological, biochemical, and behavioral analysis. For this evaluation, Wistar rats were divided into four groups: Group 1- normal control; group 2, 3 and 4 were treated with *Ekangaveera Rasa* at doses of 120, 240 and 600 mg/kg bodyweight for 30 days. Behavioral parameters were assessed on the 15th and 30th day by using the passive avoidance test. At the end of the treatment period, blood was collected and subjected to the basophilic stripling and biochemical analysis. Tissues were processed for histopathological evaluation. The histopathological, biochemical, and behavioral analysis did not show any harmful effect, thereby rendering *Ekangaveera Rasa* nontoxic and further glorifying the healing benefits of *Ayurveda.*

KEYWORDS: *Ekangaveera Rasa, Ayurveda*, Traditional medicine, Toxicity, Organs, Analysis.

INTRODUCTION:

Ayurveda, an Indian system of medicine that is rooted over 5000 years ago, is now considered as complementary and alternative medicine[1]. Traditional medicine is the knowledge, skills, and practices of holistic healthcare recognized and accepted for its role in the maintenance of health and the treatment of diseases. It is based on indigenous theories, beliefs, and experiences that are passed on from generation to generation [2-,4]. Ayurveda is globally recognized and accepted at present under its qualitative strength and its focus on the consistent functioning of life. Despite the constant effort in promoting the therapies of this vibrant ancient medicine, some areas still require further research and scientific approach and extensive evidence.

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Expensive new drugs, increasing side effects, the need for remedial treatment for several chronic diseases, are some of the reasons that have created public interest in adopting and accepting complementary and alternative medicines[5].

Medicinal plants are believed to possess powerful constituents that are life sustaining[6]. It has compelled scientists to examine these plants to determine potential healing benefits. Such plants are used in various medicinal preparations in Ayurveda[7-10].

Although these traditional medicines contain valuable elements necessarily, they also carry certain metals as an ingredient. From the therapeutic point of view, metals are used for the maintenance of health and curing of diseases in Ayurveda. Preparation and use of metallic *bhashma* in the Indian System of Medicine have also been documented previously[11]. However, monitoring the safety of herbal medications become essential, and there is a need for pharmaco-vigilant practices for *Ayurvedic*

medicine[12]. One such herbo-metallic-preparation is '*Ekangaveera Rasa.'* It is used to treat the pain of neurological origin and conditions such as facial palsy, hemiplegia, sciatica, cervical spondylosis, brachial neuralgia, etc. It contains equal parts of pepper, fresh ginger, Indian gooseberry fruit, *Bhringaraja* as herbal component and mercury, sulfur, manganese, tin, lead, copper, mica, and iron as its metal component [13].

However, the metals are used in the *Ayurvedic* medicinal preparations, only after following a meticulous process of purification, i.e., S*hodhana*, thereby extracting a nontoxic compound[14,15]. But, in recent years, specific metallic preparations used in the Indian medicinal system are alleged to cause adverse effects of the hepatic, renal, and nervous system along with various other side effects[12].

The available work in the field of science, which reports the toxic effects of metals in the ayurvedic preparation of *Ekangaveera Rasa*, are few. Hence we aimed to study and evaluate the possible toxicity and the alteration caused by the *Ekangaveera Rasa* on biochemical., behavioral analysis and histopathological of brain, liver, and kidneys.

MATERIAL AND METHODS:

Experimental animals:

Healthy adult male Wistar albino rats weighing about 150-200 g were used for the study. The animals were housed under standard environmental conditions of temperature and humidity (25±0.5°C) with 12 hours light/ dark cycle. They were fed with a diet of standard pellets and water ad *libitum*. The experiments were performed in the Central Animal House Facility, Kasturba Medical College, Manipal, after the approval from the Institutional animal ethics committee (Ref. No: IAEC/KMC/49/2013).

Procurement and preparation of Test Material:

Ekangaveera Rasa was procured from Shree Dhootapapeshwar Ltd. Khetwadi, Mumbai, India. It was then fed to the animals after calculating and measuring the desired dose and dissolving it in distilled water.

Experimental model and Treatment groups:

The animals grouped into four sets with six animals in each group, i.e., Group 1: control (treated only with distilled water), Group 2:120 mg/kg body weight (BW), Group 3: 240mg/kg BW and Group 4: 600 mg/kg BW treated groups. The groups 2, 3, and 4 were treated with the recommended human equivalent dose, double the recommended dose, and five times the human equivalent dose, respectively.

Treatment Plan:

The doses of *Ekangaveera Rasa* (120, 240, and 600 mg/kg) in the rat were calculated by extrapolating the human equivalent dose and were orally administered between 10 and 11 a.m. daily for 30days, in a volume not exceeding one ml/100 g rat weight. Rats were subjected to neurobehavioural analysis. At the end of the experiment, i.e., on the 30th day, blood samples were withdrawn from the retro-orbital sinus and processed for biochemical evaluation. Animals were then euthanized, and the liver, kidney, and brain (hippocampal region) tissues were removed and preserved in 10% formalin for histopathological evaluation.

Behavioral analysis:

On the 28th day, a passive avoidance test was performed to evaluate memory retention[16]. However, to enhance the performance in the test, the animals were deprived of food 12 hours before the test. During the acquisition trial, the rats were placed in a well-lit chamber with an additional source of electric light, and a wooden door that separated the light chamber from the dark chamber was opened. Initial latency (IL) to step into the dark chamber was noted. Time spent in the dark compartment and light compartment were noted for 3 minutes. It was repeated for three trials, and in the third trial, electric foot shock (0.2 mA) was delivered to the floor grids for 3 seconds at the end of 3 minutes with the door closed. Five seconds of post-shock treatment, a rat was transferred from the dark chamber to its home cage. The process was repeated for all the animals in each group. After 24 hours, memory retention time was noted in the same way as in the acquisition trial. The foot shock was, however, not delivered.

Biochemical analysis:

On the 30th day, blood samples were collected from the animals by puncturing the retro-orbital sinus. The samples were used for further biochemical analysis.

Basophilic Stippling:

The blood smear was made immediately after collection to study the basophilic stippling by using Leishman's staining method[17], and later the microscopic structure of RBCs was studied.

Liver Function Tests:

The blood samples were allowed to rest at room temperature for 1 hour—following which the samples were centrifuged for 5 minutes at 1000 rpm for serum separation. ALT (Alanine aminotransferase), AST (Aspartate aminotransferase) and ALP (Alkaline phosphatase) levels were estimated from the serum using standard liver function kits (Aspen laboratories, India).

Histopathological evaluation:

After euthanizing the animals by cervical dislocation, the brain, liver, and kidney were collected and carefully cut into the required size and immediately fixed in 10% formalin. They were then dehydrated using graded alcohol (50%, 70%, 90%, and 100%), xylene, and embedded in paraffin wax. Serial sections of 5µ thick were taken using a rotatory microtome. The de-paraffinized sections were stained with hematoxylin and eosin. Sections were then observed under the microscope for the possible changes in the cytoarchitecture. The areas of interest were noted and photographed and analyzed with the help of a pathologist.

Statistical Analysis:

Results were expressed as mean± SEM. The data was analyzed using Graphpad Prism software (Microsoft, San Diego, CA, USA). One-way ANOVA, followed by Dunnett's post-hoc test, was used to compare the experimental groups. *P*-value ≤0.05 was considered statistically significant.

RESULTS:

Behavioral analysis:

Animals treated with low doses of *Ekangaveera Rasa* (120 and 240mg/kg BW) spent less time in the dark compartment. However, the rats treated with a high dose (600 mg/kg BW) of *Ekangaveera Rasa* spent more time in the dark chamber compared to control; nonetheless, it was statistically insignificant (Figure 1).

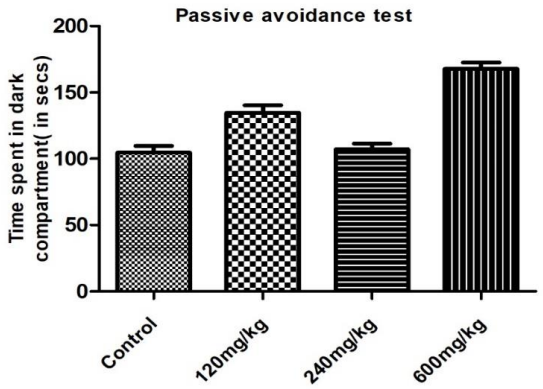

Figure 1: Effect of *Ekangaveera Rasa* on passive avoidance test. 120 mg/kg, 240 mg/kg and 600 mg/kg are the groups treated with the respective doses of the drug.

Biochemical Analysis:

Basophilic stippling of the circulating erythrocytes (BSE):

The BSE represents the spontaneous aggregation of ribosomal RNA due to the poisoning of 5' nucleotidase. The BSE was not visible during routine hematology stains. It indicates that the drug has no poisoning effect within the dose five times as higher than the standard equivalent human dose (Figure 2).

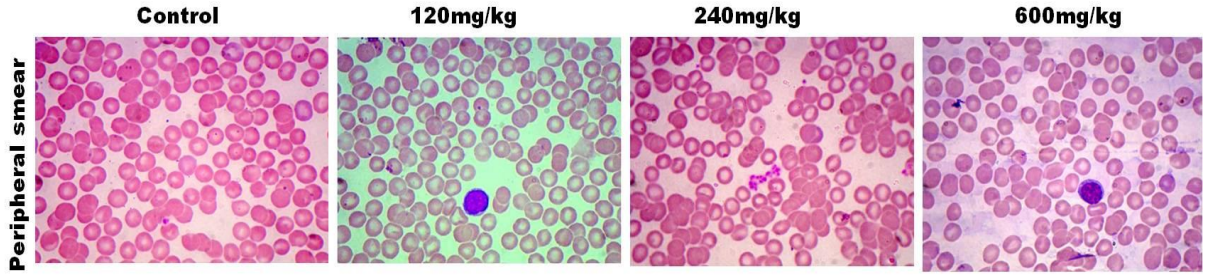

Figure 2: Showing the peripheral blood smear of rats treated with different doses of *Ekangaveera Rasa* for 30 days

Liver function tests:

The serum ALT, AST, and ALP levels were similar in all the *Ekangaveera Rasa* treated groups (120, 240, and 600 mg/kg) and in untreated control (Table 1). A statistically significant difference was not observed in any of the treated groups compared to control. Dunnett's post-hoc analysis further confirmed that the findings were similar among all the treated groups (i.e., all the calculated doses of the drug). This observation further ascertains the fact that '*Ekangavera Rasa'* is non-toxic in all the calculated doses.

Table 1: Liver function test: Groups treated with '*Ekangaveera Rasa'* versus control

Enzymes (IU/L)	NC	120 mg/kg	240 mg/kg	600 mg/kg
ALT	39.857±0.26	36.223±0.03	40.054±0.21	42.321±0.05
AST	70.022±3.32	72.027±4.37	70.009±2.77	76.027±5.64
ALP	81.465±0.55	80.021±0.51	81.402±0.04	80.868±0.22

NC- normal control; 120, 240, and 600 mg/kg body weight is the group treated with the respective doses of *Ekangaveera Rasa*. AST: aspartate aminotransferase; ALT: alanine aminotransferase; ALP: alkaline phosphatase. All values are expressed as mean ± SEM.

Histopathological Observations:
The tissues of the liver, kidney, and brain (hippocampal region) showed normal cytoarchitecture in both control and *Ekangaveera Rasa* (all the calculated doses) treated groups. No degenerative changes were observed in the treated groups. All the calculated doses, i.e. 120, 240, and 600 mg/kg body weight of *Ekangaveera Rasa* were efficient in maintaining the normal structural organisation of the vital organs such as liver, kidney and brain as is observed in the present histological findings (Figure 3).

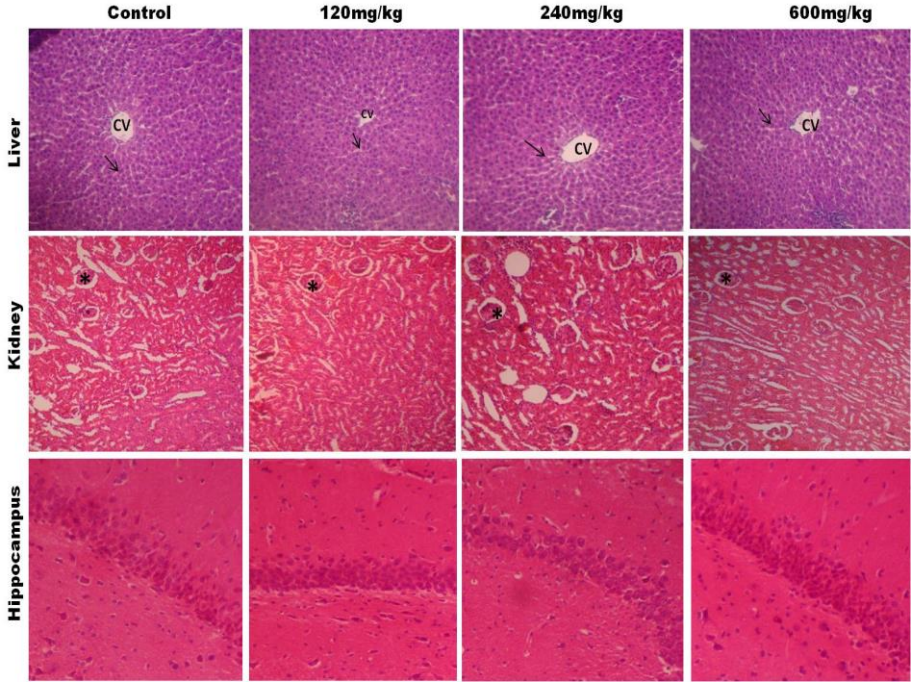

Figure 3. Showing H&E stained images (10X magnification) of the tissue collected from the liver, kidney, and brain (CA1region of the hippocampus) of the rats fed with different doses of *Ekangaveera Rasa*.
CV- a central vein of the liver, arrow represent – normal sinusoid; * represent – glomeruli of the kidney.

DISCUSSION:

Ayurvedic medicines are herb-based traditional medicines. They carry a rich historic background and is one of the highest living traditions [18,19]. In most of the *Ayurvedic* formulations, processed heavy metals, metals, and minerals are the key ingredients. Studies suggest that approximately 35–40% of *Ayurvedic* formulations contain at least one metal [18]. Therefore it becomes imperative that the medicinal plant or plant parts used for different diseases must be checked for heavy metal contaminations in order to make it safe for human consumption[12].

The metals in *Ayurvedic* formulations are frequently subjected to purification, i.e., *Shodhana* (purification and detoxification) and *Marana* (incineration and calcination)[14,15]. Practitioners develop these methods to expel the toxic effects of the raw material by chemical changes and thus modify the properties of materials to improve the therapeutic potential. The *Ayurvedic* formulations, therefore, lose their original elemental form and become nontoxic during the process of purification [18,20,21].

Kumar et al., in their study on *Aarogyavardhini Vati*, an Ayurvedic formulation at a dose of 50, 250, and 500 mg/kg of body weight for 28 rats found no adverse effect on cerebellum, liver, and kidneys. They reported that the drug was safe up to 500 mg/kg body weight [22]. A similar experimental toxicity study on *Makaradhwaja* did not show the signs of toxicity up to a dosage of 480 mg/kg in mice. However, a 28 days' oral toxicity study on *Siddha Makaradhwaja* in rats showed neurodegenerative changes in the brain at a higher dose of 100 mg/kg weight [23]. Aqueous extract of *Coccinea Indica*(roots) was studied for its toxic effects in Swiss albino mice. Herein no significant observations of behavioral changes, toxicity, mortality were observed. However, they suggested that conducting toxicity studies in natural plant products and drugs of the indigenous medicinal system is a must[24].

In the present study, the values of the liver function test were found to be in the normal range. Significant changes were not observed in serum ALT, AST, and ALP levels in all the three doses of *Ekangaveera Rasa*. This suggests that '*Ekangaveera Rasa'* is devoid of any drastic toxic effect in rats even at a dose almost five

times higher than the standard limit.

The behavioral analysis showed no alteration at lower doses in the cognitive functioning in the rats. The histopathological findings in the CA1 region also showed no changes. The CA1 region of the hippocampus is responsible for short term memory. The rats treated with the higher dose of (600 mg/kg) *Ekangaveera Rasa*, showed impaired cognitive function in the behavioral analysis. It was, however, not reflected in the CA1 region of the treated rats as observed histologically.

BSE occurs due to the spontaneous aggregation of ribosomal RNA due to the poisoning of 5' nucleotidase. The finding was adverse in the present study. It indicates that the drug has no poisoning effect within the dose five times as higher than the normal equivalent human dose. The above results were also reaffirmed by the histopathological observations in the liver, kidney, and the brain- hippocampal tissues of the treated groups. The cytoarchitecture of all the three organs was found to be healthy at all the considered doses of *Ekangaveera Rasa*. The non-occurrence of the toxic effect could be a possible indication that the process of *Ayurvedic* detoxification, i.e., *Shodhana*, might have aided in significant changes in the characteristics of the metals. Further, it would have resulted in the elimination of toxicity, yet maintaining its pharmacological characteristics.

CONCLUSION:

"*Ekangaveera Rasa*" has been used clinically for centuries in the traditional Indian Medicinal System, i.e., Ayurveda. The findings of the present study endorse the nontoxic effects of this particular drug and provide valuable evidence supporting the same. The study further supports the idea that "*Ekangaveera Rasa*," when administered at recommended dose and duration to the human population (as is routinely practiced in *Ayurveda*), is harmless. The present study thereby glorifies the healing benefits that our traditional medicinal system has in store for the well-being of humanity without any side effects.

CONFLICT OF INTEREST:

The authors declare no conflict of interest.

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RESEARCH ARTICLE

In-vitro Antioxidant and Antihyperglycemic effect of *Muntingia calabura* L. fruit extracts

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ABSTRACT:

This study investigated the *in-vitro* antioxidant activity and antidiabetic effect of *Muntingia calabura* fruit extract by *in-vitro* α-amylase and α-glucosidase inhibitory activity. *Muntingia calabura* fruit was extracted with aqueous methanol by soxhlet extraction. The total phenols and total flavonoids contents were estimated and evaluated for antioxidant activities (DPPH, ABTS) and *in-vitro* antidiabetic activity by measuring their inhibitory activity on α-amylase and α-glucosidase levels. The findings showed that the fruit extract had high content of total phenol and exhibited moderate free radical scavenging activity. The fruit extract showed inhibitory effect on α-amylase [IC_{50}Value =61.43 µg/mL] and α-glucosidase [IC_{50} Value=140.33 µg/mL] compared to standard acarbose. The fruit extract can be used as a potential source for the development of new hypoglycemic agents may be due to the presence of high phenol content.

KEYWORDS: *Muntingia calabura*, Phytochemicals, Antioxidants, α-amylase and α-glucosidase.

INTRODUCTION:

Diabetes mellitus is a chronic metabolic disorder caused by inherited or acquired deficiency of insulin secretion and by decreased sensitivity of the organ to secrete insulin and its deficiency results in increased blood glucose level than normal blood glucose level, which in turn can damage several functions, including blood vessels and nerves[1]. One of the remedial approaches is to reduce the postprandial hyperglycemia by slowing down the absorption of glucose by inhibition of carbohydrate hydrolyzing enzymes, such as alpha-amylase and alpha-glucosidase[2]. About 90% of diabetic population is affected by type 2 diabetes, shows mild symptoms like fatigue, frequent urination, hunger, and increased thirst, blurred vision, weight loss and slow healing of wounds and can be controlled with a healthy diet and exercise[3].

From this many efforts have been made to look for more effective and safe inhibitors of α-amylase and α-glucosidase from natural materials to develop a physiological system to treat diabetic mellitus[4]. Many traditional plants have reported in India for diabetes, but only a small number of these have received scientific and medical evaluation to assess their efficacy. The medicinal plants are remedial as well as for the curing of human diseases due to the presence of phytochemical constituents. Phytochemicals have defense mechanisms and protect from various human diseases. Primary and secondary metabolites are biologically active compounds. Proteins, common sugars and Chlorophyll, are included in primary constituents and secondary compounds are terpenes, phenolic compounds and nitrogen-containing secondary metabolites such as alkaloids and non-protein amino acids[5]. Alkaloids are second-hand as anesthetic agents and are found in medicinal plants[6]. Phenolic compounds make different vital pharmacological activities i.e., anti-inflammatory, inhibition of cholesterol synthesis, anti-viral, anti-malarial, anti-bacterial activities anti-cancer, anti-diabetes[7] and antioxidant substances to inhibiting the formation of free radicals by scavenging radicals.

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Antioxidants are found in fruits, vegetables and a variety of other foods naturally[8].

Based on tribal information *Muntingia calabura* fruit has been used to treat and prevent diabetes. *Muntingia calabura (M. calabura)* belonging to the family *Muntingiacea* is native to Southern Mexico and tropical South America[9]. It is commonly cultivated and used by many traditional healers in most of the countries like India and Southeast Asia such as Malaysia, Indonesia, and Philippines[10]. In Tamil Nadu, *M. calabura* is commonly seen along the road side trees. The plant is further used traditionally for the treatment as an antiseptic, reduce swelling in lower extremities, reduce gastric ulcer and swelling of the prostate gland, alleviate headache, cold and to treat measles, mouth pimples and stomachache [11-12]. The leaf decoction is drunk as a tea like beverages[13]. *M. calabura* fruit by itself is sweet in taste. Mahmood *et al*. reported that the different parts of *M. calabura* possess bioactive compounds and the pharmacological activities on acute toxicity, cytotoxic activity, antiproliferative, insecticidal activity, hypotensive, antinociceptive, cardioprotective, antipyretic, antiplatelet aggregation, antioxidant, anti-inflammation, anti-diabetic, antiulcer, antimicrobial, anthelmintic activity and hepatoprotective[14].

There has been no report on the *in-vitro* antidiabetic effect on *M. calabura* fruit. So, the present study was aimed to identify the phytochemical constituents and evaluate the *in-vitro* free radical scavenging ability and antidiabetic activity of *M. calabura* extract.

MATERIAL AND METHODS:

Chemicals and reagents:

All the chemicals and solvent were analytical grade and obtained from Hi-media, India.

Collection of plant material:

Muntingia calabura fruits were collected in and around Tirupattur District, Tamil Nadu, India. The fruits were shade dried and properly ground into powder and used for further studies.

Preparation of fruit extract:

Soxhlet extraction method was used to extract 25g of a dried powder sample in 200 mL of solvent (aqueous (20 mL) and (180 mL) methanol). The sample was extracted at ambient temperature for 48 hours and the crude sample was collected and used for further studies.

Quantitative analysis of phytochemical Screening:

Determination of total phenolic content:

Total phenolic content in the extracts were estimated by Folin-Ciocalteu method according to the modified procedure of Tamilventhan *et al*. using gallic acid as a standard [15]. About 500 µg of *M. calabura* fruit extract was mixed with 0.5 mL of water and 0.2 mL of Folin-Ciocalteu's phenol reagent (1:1). About 1 mL of saturated Na_2CO_3 (8 % w/v in water) solution was added after 5 minutes and the volume made up to 5 mL by adding distilled water and kept in dark for 30 min. The absorbance of blue color was measured at 765 nm using UV-vis spectrophotometer. Total phenolic content was expressed in terms of mg/g equivalent to gallic acid (10-320 µg/mL) on the basis of a standard curve. Estimation was repeated thrice and the results were averaged.

Determination of total flavonoid content:

Total flavonoid content in the extracts was determined according to modified method of Ali *et al*. using quercetin as a standard [16]. To 500 µg of *M. calabura* fruit extract, a volume of 0.5 ml of 2% $AlCl_3$ ethanol solution was added and incubated at room temperature for 1 hour. Absorbance was measured at 420 nm using UV-vis spectrophotometer. Total flavonoids were expressed in terms of mg/g equivalent to quercetin (10-320 µg/mL) on the basis of a standard curve. Estimation was repeated thrice and the results were averaged.

In-vitro antioxidant activity:

DPPH radical scavenging activity:

This test was measured as described by modified method of Perumal *et al*[17]. In brief, 0.135 mM DPPH was prepared in methanol. Different Concentration (5, 10, 20, 40, 80, 160 and 320 µg/mL) of *M. calabura* fruit extract was mixed with 2.5 mL of DPPH solution. The reaction mixture was vortexed thoroughly and kept at room temperature for 30 min. The absorbance was measured at 517 nm. The ability of extract to scavenge DPPH radical and control was calculated from the following formula:

% DPPH inhibition = [(OD of control – OD of test sample) / (OD of control)] ×100

The concentration of extract that is required for resulting in 50% of inhibition of enzyme activity (IC_{50}) was determined. The standard inhibitor ascorbic acid was used as a positive control at a same concentration range of 5-320 µg/mL.

ABTS radical scavenging activity:

Antioxidant potential of plant extract was determined against ABTS (2, 2′-azinobis-3-ethylbenzothiazoline-6-sulfonic acid) free radical method. ABTS (7 mM, 25 mL in deionized water) stock solution and potassium persulfate (140 mM, 440 µL) solutions were mixed thoroughly and incubated in dark for the production of ABTS free radical. About different concentrations (5, 10, 20, 40, 80, 160 and 320 µg/mL) of the MCF extract and standard ascorbic acid was mixed with 2.0 mL ABTS working solution and the reaction mixture was incubated for 20 min at room temperature. Finally the absorbance was measured using an UV-vis spectrophotometer at 734 nm. Percentage of ABTS

scavenging potential was calculated using the following formula:

ABTS radical scavenging effect (%) = [(A0 - A1)/A0] ×100

Whereas, A0 is the control; A1 is the test.

Evaluation of *in-vitro* antidiabetic activity:

α-Amylase inhibitory assay:

The inhibition assay of α-amylase starch iodine method was carried out according to the standard method with minor modification according to Unuofin *et al*[18]. About 100 µL of α- Amylase (0.1 mg/mL) and the *M. calabura* fruit extract were mixed at different concentrations (10-320 µg/mL) and incubated at 37°C for 15 min. About 100 µL of soluble starch (1%) was added to each tubes and incubated at 37°C for 60 min and 10 µL of 1M HCl was added to stop the enzymatic reaction, followed by the addition of 100 µL of iodine reagent. The colour change was noted and the absorbance was read at 580 nm in a UV-vis spectrophotometer. A control was prepared using the same method except that the extract was replaced with distilled water. The α-amylase inhibitory activity was calculated as follows:

Percentage of Inhibition (%) = [(Abs Control - Abs Sample) / Abs Control] x 100

The standard inhibitor acarbose was used a positive control at a concentration range of 10-320 µg/mL. The presence of starch indicates formation of dark-blue colour; while no colour complex is developed in the absence of the inhibitor, indicating that starch is totally hydrolysed by *a*-amylase.

α-Glucosidase inhibition assay:

The inhibitory action of α-glucosidase activity was determined using modified procedure of Apostolidis *et al* [19]. A volume of 200 µL of α-glucosidase and the *M. calabura* fruit extract at different concentrations (10-640 µg/mL) was incubated in 100 mM phosphate buffer pH 6.8 at 37°C for 15 min. There after reaction mixture, contained 400 µL of 5mM p-nitrophenyl-α-D-glucopyranoside (pNPG) in 100 mM phosphate buffer pH 6.8 at 37°C for 20 min and the reaction was stopped by adding 1 mL of 0.1 M of Na_2Co_3. The absorbance of the resulting p-nitrophenol was determined at 405 nm using UV–vis spectrophotometer. Acarbose was used as a positive control and the inhibitory activity of α-glucosidase was calculated using the following formula,

Percentage of Inhibition (%) = [(Abs Control - Abs Sample) / Abs Control] x 100

Statistical analysis:

The results were represented as mean ± SD in triplicate experiments. The data were statistically analyzed using GraphPad Prism version 5.

RESULTS:

Total phenol content (TPC) and Total flavonoid content (TFC):

Total phenol content in the *M. calabura* fruit extract using the calibration curve, was found to be 51.84 mg of Gallic acid equivalents/g dry weight and quercetin equivalents/g dry weight of the fruit extract exhibited 23.37 mg of total flavonoid content. The most important phenol content of Gallic acid was found to be higher in aqueous methanolic fruit extract.

***In-vitro* antioxidant activity:**

In the antioxidant activity IC_{50} values are negatively related, as it expresses the total amount of antioxidant needed to reduce its radical concentration by 50%. The lower IC_{50} value represents higher antioxidant potential of the tested sample. As shown in Figures 1 and 2, the dose-response curve confirms the free radical scavenging ability of *M. calabura* fruit extract. In the DPPH method, the scavenging capability of free radicals by the fruit extract was found to be IC_{50} =169.22 µg/mL and higher than ascorbic acid. The percentage of inhibition increased in concentration dependent manner from 5-320 µg/mL compared to standard ascorbic acid which exhibited 10.53% to 99.20% and fruit extract showed 8.37% to 63.72% respectively. *Muntingia calabura* fruit extract was found to be more effective in scavenging the ABTS radical. There was a significant increase in percentage of inhibition on concentration dependent manner (5-320 µg/mL). At the concentration of 20 µg/mL, the inhibition of ascorbic acid was 45.21% and fruit extract was 45.28% respectively. The IC_{50} of ascorbic acid was found to be 24.31 µg/mL while that of fruit extract was 29.58 µg/mL respectively. The findings showed that *M. calabura* fruit extract exhibited good antioxidant activity in the ABTS assay similar to the standard ascorbic acid may be due to its high phenol levels (Table 1).

Table 1: IC_{50} Values (µg/mL) for *M.calabura* and ascorbic acid in Antioxidants assay.

Antioxidants assay	IC_{50} values (µg/ml)	
	Ascorbic acid	*M. calabura* fruit extract
DPPH	19.06	169.22
ABTS	24.31	29.58

*Values are represented as mean±SD (n = 3); statistical significant level at (P<0.05).

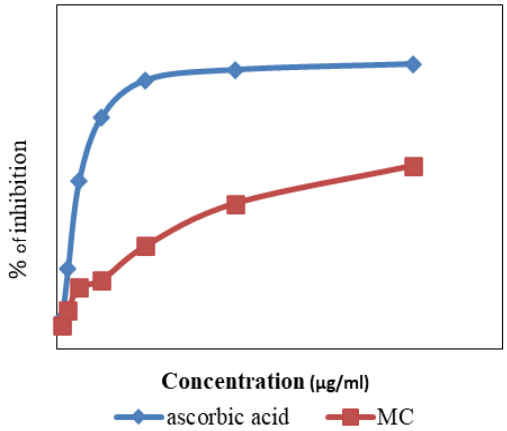

Fig 1: DPPH free radical Scavenging ability of aqueous methanolic extract of *M. calabura* fruit.

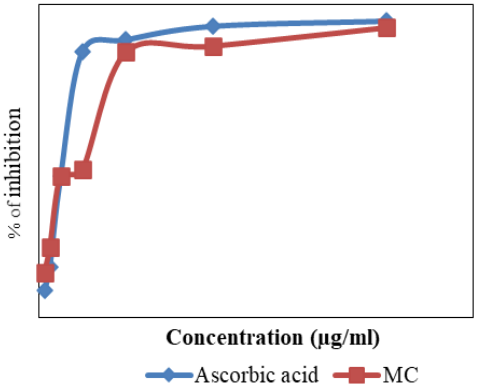

Fig 2: ABTS free radical Scavenging ability of aqueous methanolic extract of *M. calabura* fruit

In-vitro antidiabetic activity:

Figures 3 and 4 showed a significant increase in the percentage of inhibitory activity was noticed in a concentration-dependent manner. In α-amylase inhibition assay at a minimum concentration 10 µg/mL, the fruit extract exhibited inhibitory percentage of 11.244±2.904% and maximum concentration of *M. calabura* fruit extract (320 µg/mL) exhibited 94.401±0.011% inhibitory activity. The positive control acarbose exhibited 25.293 ±0.667% to 92.562± 0.023% respectively. Fruit extract IC_{50} value (61.43 µg/mL) showed higher concentration than standard acarbose. Whereas strong inhibition observed when compared with α-glucosidase inhibition (Table 2). The α-glucosidase inhibitory concentration (IC_{50}) of fruit extract was 140.33 µg/mL. The percentage of inhibition at minimum concentration (10 µg/mL) of the extract showed 18.644±0.363% and maximum concentration of 640 µg/mL exhibited 68.886±1.275% inhibitory activity. The positive control acarbose exhibited 25.036±4.374% to 98.789±0.419% respectively.

Table 2: IC_{50} Values (µg/mL) for α-amylase and α-glucosidase inhibitory assay

Name of enzyme assay	IC_{50} values (µg/mL)	
	Acarbose	*M. calabura* fruit extract
Alpha-amylase	17.38	61.43
Alpha- glucosidase	26.12	140.33

*Values are represented as mean±SD (n = 3); statistical significant level at (P<0.05).

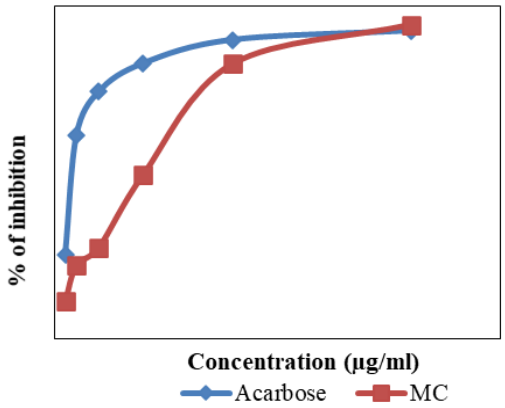

Fig 3: Inhibitory effect of aqueous methanolic extract of *M. calabura* fruit against α - amylase.

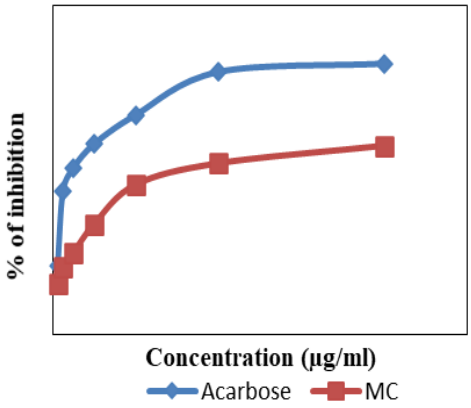

Fig 4: Inhibitory effect of aqueous methanolic extract of *M. calabura* fruit against α - glucosidase.

DISCUSSION:

Many people with type 2 diabetes mellitus need antidiabetic drugs to manage their condition, but medications may cause fewer side effects. So the currently available drugs are not much use in prevention or reduction of diseases. Herbal drugs have potential bioactive compounds with great medicinal properties to treat diabetes mellitus such as metabolic disorder to inhibit digestive enzymes involved in the digestion of carbohydrates. So, natural products are considered to be safer than synthetic drugs[20]. The present study comprehensively evaluated the major phytochemical constituents such as total phenols (51.84 mg of gallic acid) and flavonoids (23.37 mg of quercetin) of aqueous methanolic fruit extract. In previous study Preethi *et al.* investigated the phenol content of *M. calabura* fruit

extract and recorded highest content of phenol (1.486±0.028 mg/100g) followed by the different solvent extractions[21]. Anusuya *et al*., carried out with fruit extract with solvent mixture of (methanol/acetone/water) exhibits higher amount of total phenols [22]. Phenolic compounds of flavonoids, tannins, anthraquinones, lignins and other compounds of saponins and terpenoids are important bioactive chemical constituents commonly correlated with their antioxidant activities.

The phenolic compounds have redox properties which perform as hydrogen donors, reducing agent and singlet oxygen quenchers. *Muntingia calabura* fruit was evaluated by the free radical scavenging ability against DPPH and ABTS. This study exhibited the standard antioxidant ascorbic acid showed increased reducing power in a concentration dependent manner found a direct relation between antioxidants as an average proton donating ability. In the DPPH free radical scavenging activity, acceptance of electrons from antioxidant compounds which in turn changes the colour of the solution from violet to yellow[23]. However, the fruit extract scavenging of DPPH radical was found to be lower than standard ascorbic acid and the ABTS radical scavenging study confirms that fruit extract had antioxidant capability, reacts with potassium persulphate to produce a blue-green chromogen of ABTS radical cation[24]. In the occurrence of antioxidant reductant, the intensity of the coloured radical is decreased as similar to the ascorbic acid at the concentration of 20 µg/mL. This shows that *M. calabura* aqueous methanolic extract represents a good antioxidant ability to scavenge ABTS radical. In a previous study Gustavo *et al*., reported the scavenging possibilities due to the higher contents of total phenolics, flavonoids and anthocyanins[25]. Although there have been recent studies on *M. calabura* fruit had appreciable amounts of antioxidants like vitamin C and E[26] similar to *Begonia versicolor* leaves contain vitamin C reported by Ermi *et al* [27].

Effective antioxidant compound of gallic acid is also present in the fruit of *M. calabura*[28]. This beneficial effect of antioxidants in the plant can be attributed to the presence of secondary metabolites especially due to the hydroxyl group containing phenolic compounds such as flavonoid contents[29]. Thus, it could be concluded that *M. calabura* fruit can serve as a natural source of antioxidant and play an important role in adsorbing and neutralizing free radicals to prevent cell damages and oxidative stress related diseases like diabetes mellitus and cancer. Diabetes mellitus is a metabolic disorder and one of the major growing health problems. Most of the people have been taking Insulin and oral hypoglycemic agents as the only antidiabetic therapy. Thus, dietary herbal medicine has antioxidant potential to recover the cell damages of pancreatic beta cells and have hypoglycemia potential properties which stimulate the pancreatic insulin secretion and inhibit the carbohydrate hydrolytic enzymes. Alpha-amylase is the key enzyme that is responsible to catalyze the hydrolysis of α-1,4-glycosidic linkages of starch, glycogen and various oligosaccharides and disaccharides. The α-glucosidase enzyme hydrolyzes the disaccharides into simple sugars which are subsequently immersed through the small intestine thus causing postprandial hyperglycemia and may have useful effects on insulin resistance and glycemic index in diabetic people delay the absorption of carbohydrates from dietary sources and decrease postprandial glucose level[30-34]. By inhibiting the activity of α-glucosidase in the small intestine, this reduces glucose absorption by delaying carbohydrate digestion time may be useful in the management of diabetes[35]. In the literature there are no *in-vitro* studies dealing with the antidiabetic activity on *M. calabura* fruit. So the present *in-vitro* study proved that fruit aqueous methanolic extract had moderate inhibitory effects on α-amylase and α-glucosidase enzyme when compared to the standard acarbose. Due to the presence of inhibitors in the *M. calabura* fruit extract, the starch added to the α-amylase enzyme assay mixture is not degraded and gives a dark-blue colour complex in the starch iodine method which is responsible for containing phenol and flavonoid compounds and free radical scavenging ability on inhibitory potential of α-amylase and α-glucosidase. According to Thouri *et al*., flavonoids and their derivatives had ability to inhibit glucose transporter-1, blocking of glucose absorption and reduce the potency of digestive enzymes[36] and fruit extract also have hypoglycaemic action through stimulation of surviving ß-cells of islets of langerhans to release more insulin[37] and several flavonoids, glycosides present within the extract reduces the blood glucose reported by Sravan *et al*[38]. Also recorded fruit extract decreased the blood glucose level as compared to standard drug, Glibenclamide[39]. Terpenoids have been found to stimulate insulin secretion or functioning like insulin effect[40]. Saponin was found to be bioactive against diabetes and reduced fasted blood glucose and regulates the hyperglycemia associated with oxidative stress in type 2 Diabetes mellitus[41-43]. *Muntingia calabura* fruit had health promoting properties due to its antioxidant properties reported by Preethi *et al*[44]. So natural polyphenols can be used as a drug and as nutritional dietary supplements to cure or prevent various diseases and in extracting some of these compounds appear to be capable of improving the performance of pancreatic tissues by increasing the insulin secretion and inhibiting the carbohydrate hydrolyzing enzymes[45].

This study indicated that aqueous methanolic extract of *M. calabura* fruit contains phenols, possesses free radical scavenging ability and antidiabetic potential.

Therefore, the fruit extract might be more effective for type 2 diabetes mellitus and *in-vivo* studies need to be carried out to confirm these observations of *Muntingia calabura* fruit for pharmacological and therapeutic properties.

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CONFLICT OF INTEREST:

The authors declare no conflict of interest.

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RESEARCH ARTICLE

Bacterial Isolates and Antibiotic Susceptibility of Ear Infections in Al-Kindy Teaching Hospital, Baghdad, Iraq

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ABSTRACT:

Background: Ear infections can manifest in many forms depending on site of infection whether external, middle or internal ear and the culprit pathogen whether viral, bacterial or fungal. Acute middle ear infections are usually accompanied by aural discharge. **Objective:** 1. To get an overview on the bacterial pathogens involved in ear infections. 2. To assess the antibiotic resistance of bacterial pathogens. **Methods:** A cross sectional study conducted in Al-Kindy Teaching Hospital / Baghdad /Iraq. Swabs taken from 225 patients suffering from aural discharge were tested for culture and sensitivity for the duration of two years 2018-2019. Aural discharge is cultured by inoculating it into blood, MacConkey agar, chocolate agars and Sabouraud agar (for fungi). Then the antibiotic susceptibility and resistance is assessed by (Kirby-Bauer Method). **Results:** Then, by analyzing the percentage of pathogens involved in ear infections we have found that the highest percentage is for Pseudomonas aeruginosa (51%), followed by Staph, aureus (20%), Proteus vulgaris (11%). **Discussion:** Cefotaxime, which was known to be an efficient antibiotic against pseudomonas previously, has lost its effectiveness. Similarly, gentamycin is no longer effective against E.coli. **Conclusion**: Choosing the proper antibiotic in any bacterial infection is of tremendous importance. However, reassessment of antibiotic resistance profiles is vital and should be regarded as a routine task on regular intervals.

KEYWORDS: Antibiotic resistance, proteus, pseudomonas, otitis media.

INTRODUCTION:

Ear infection is an inflammation of the ear and ear discharge is one of the most common manifestations of ear infection[1, 2]. It has various forms, most commonly occurring in children presenting in the form of otitis media.

Due to the shorter length of the Eustachian tube, the higher frequency of ear infections in children is more horizontal in children than in adults [3, 4].About 65-330 million individuals worldwide suffer from ear infections, and 60% of them suffer from hearing impairment.

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Otitis media is a middle ear infection, typically associated with an upper respiratory tract infection. It may occur at any age But 60-80 percent of cases are children[5,6].

Asymptomatic or symptomatic otitis media infections can be present. Symptoms such as moderate to severe pain, ear rash discharge (pus), irritation and sometimes fever are characterized by acute symptomatic infections. Sources of ear infections include bacteria, viruses and fungi, with the most common cause being bacteria. Otitis media can be classified into 2 groups according to clinical presentation: Acute otitis media (AOM), Chronic suppurative otitis media (CSOM)[7, 8].

Several bacterial species have been linked to the infection, such as *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Proteus mirabilis* and *Staphylococcus*

aureus[9,10]. Highly resistant strains of both gram-positive and gram-negative bacteria, such as extended spectrum beta lactamase (ESBL), can inhibit the most effective antibiotics. [11, 12].

Bacteriology of aural discharge:
Bacteria can be aerobic (e.g. *Pseudomonas aeruginosa, Escherichia coli, S. aureus, Streptococcus pyogenes, Proteus mirabilis, Klebsiella species*) or anaerobic (e.g. Bacteroides, Peptostreptococcus, Proprionibacterium)[13, 14]. In chronic suppurative otitis media (CSOM) cases. Bacteria are rarely present in the skin of the external canal, but are likely to proliferate if trauma, inflammation, laceration or high humidity occurs [15, 16].

MATERIALS AND METHODS:

This is a cross sectional study conducted in Al-Kindy Teaching Hospital / Baghdad /Iraq. Swabs taken for 225 patients suffering from aural discharge were tested for culture and sensitivity for the duration of two years 2018-2019. Aural discharge is cultured by inoculating it into blood, MacConkey, chocolate and Sabouraud agars (for fungi).

Inclusion criteria:
Any patient with otorrhea for two weeks or more.

Exclusion criteria:
1. Patients with otitis externa
2. Patients with tympanostomy tubes
3. Immunocompromised patients
4. patients with other medical conditions that may affect interpretation of the effect of investigational drugs
5. Patients on any medications that may affect the interpretation (e.g., inhaled steroids).

After obtaining the formal approval from the Scientific and Ethical Committee in Al-Kindy College of Medicine, University of Baghdad, a verbal and written consent from each patient was taken. Randomization, Blinding were assured Aural swabs from patients with aural discharge were collected from ear in ENT department and MacConkey agar plates. Bacteria were identified by using Gram staining. Antimicrobial sensitivity testing was done on Mueller Hinton agar using Modified Kirby Bauer disc diffusion technique. 0.5 McFarland turbidity test inoculums were used as a standards were for comparison. The suitable antimicrobial discs used for Gram negative bacteria and gram positive. After keeping the appropriate antibiotics, the plates were incubated at 35C for the time of 16-18 hours. The zone of inhibition was measured and interpreted according the Clinical and Labarotary Standards Institute (CLSI).

The antibiotic susceptibility and resistance is assessed by (Kirby-Bauer Method) by using commercially obtained disks for the following antibiotics: CRO
CAZ =ceftazidime, CFM=cefotaxime, ATM=aztreonam, CFP=cefipime, FOX=cefoxitine, AZM=azithromycin, TMP=trimethoprime, CIP=ciprofloxacin, VAN=vancomycin, AK=amikacin, GN=gentamycin

The results were measured focusing on several demographic parameters which are age, sex. The type of pathogen whether bacterial or fungal in relation to its antibiotics results of culture and sensitivity.

The results were illustrated in frequencies and percentages using EXCEL software. Statistical analysis was conducted with Chi-Square test by implementing Graph-Pad 8 software and P value < 0.05 was used as level of significance.

RESULTS:

225 patients were recruited in this study comprising 108 males and 117 females. Their age and sex distribution are illustrated in Figure 1. The highest frequency is in the 11-20 years age group.

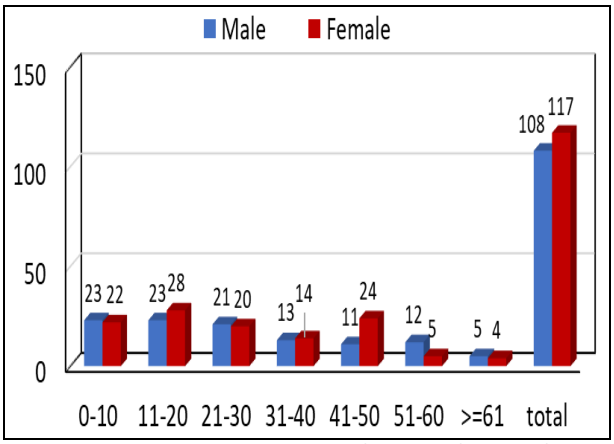

Figure 1: Frequency distribution of cases of ear infections according to sex and age groups. The highest frequency is in 11-20 yr age group. There is no statistical significance, p= 0.23

Then, by analyzing the percentage of pathogens involved in ear infections we have found that the highest percentage is for *Pseudomonas aeruginosa* (51%), followed by *Staph. aureus* (20%), *Proteus vulgaris* (11%), fungal infections (9%), *Klebsiella pneumonia* (4%), *Enterobacter aerogenes* (2%), *E.coli* (2%) and *Streptococcus pneumoniae* (1%).

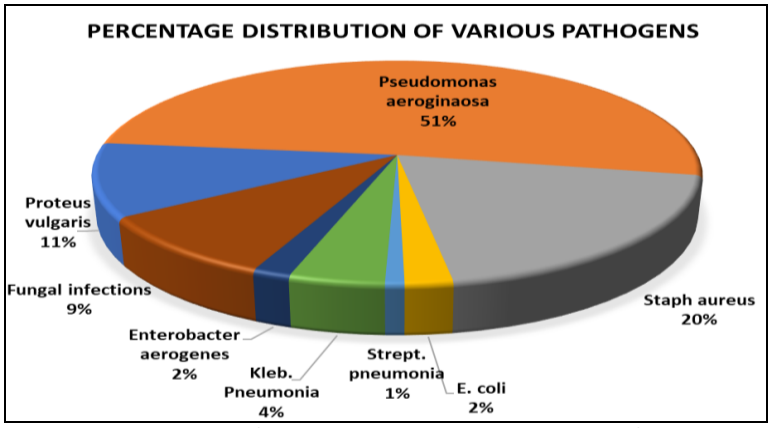

Figure 2: Pie chart representing the percentage of various pathogens involved in ear infections. The highest percentage is for Pseudomonas aeruginosa representing 51% of cases.

Table 1: Various pathogens involved in samples of aural swabs and their pattern of antibiotic resistance in terms of frequency and percentage of resistance.

	Pseudomonas aeruginosa		*Proteus vulgaris*		*Staph. aureus*		*E. coli*		*Streptococcus pneumonia*		*Klebsiella pneumonia*		*Enterobacter aerogenes*	
	No. of cases	**%**	**No. of cases**	**%**	**No. of cases**	**%**	**No. of cases**	**%**	**No. of cases**	**%**	**No. of cases**	**%**	**No. of cases**	**%**
CRO	3	27.27	1	16.67	3	50.0	3	100.0	0	0.00			1	0.00
CAZ	19	28.79	3	23.08	4	80.0	4	100.0	1	100.00	3	100.0	1	0.00
CFM	42	89.36	1	25.00	3	75.0	2	100.0			0	0.00	1	100.
ATM	13	19.7	2	25.00	1	25.0	2	66.67			1	33.33	1	0.00
CFP	11	31.43	0	0.00	1	25.0	1	100.0			0	0.00	2	0.00
FOX	14	82.35	2	22.22	0	0.00					0	0.00		
AZM	2	28.57	2	18.18	1	8.33	0	0.00			1	50.0		
TMP	1	50.0	1	33.33	2	25.00	1	0.00			0	0.00		
CIP	18	36.0	4	50.00	4	26.67	2	100.0			2	66.67		
VAN			0	0.00	1	3.23								
AK	16	21.33	1	5.88	1	6.67	0	0.00			1	14.29	2	0.00
GN	36	38.71	10	41.67	2	6.25	2	50.0	1	100.00	2	25.00	0	0.00

Looking deep into the causative pathogens and their resistance patterns we would get table 1, which illustrates details of their resistance patterns to various antibiotics in terms of frequency and percentage.

- *Pseudomonas aeruginosa* pattern of resistance shows the least resistance to aztreonam, azithromycin and amikacin.
- Regarding Proteus vulgaris resistance pattern it shows the least resistance to cefepime, vancomycin and amikacin.
- *Staphylococcus aureus* shows the least resistance patterns toward cefoxitin, vancomycin, gentamycin and amikacin.
- *E.coli* shows the least resistance pattern toward azithromycin, amikacin , trimethoprime.
- *Streptococcus pneumonia* shows high resistance toward gentamycin and ceftazidime.
- *Klebsiella pneumonia* shows the least resistance toward cefotaxime, cefoxitine, cefipime and trimethoprime.
- *Enterobacter aerogenes* show no resistance to all the available antibiotics in the test.

DISCUSSION:

The emerging problem of antimicrobial resistance is rapidly growing that day by day we have less arsenal of antibiotics to combat the new “superbugs” responsible for resistant infections.

Thus, we have conducted a study to explore the recent patterns of pathogens responsible for ear infections and their expressed antibiotic resistance.

In terms of age and sex distribution, the commonest age is in 11-20 years old which is compatible with international studies[17, 18]. However, there is no statistical significance as measured by Chi-Square test.

Pseudomonas aeruginosa represents the biggest share; 51%. This is compatible with a study reporting the pseudomonas as the main culprit pathogen accounting for 37% of cases, followed by Staph aureus 14%[19, 20].

Regarding Pseudomonas aeruginosa, Proteus vulgaris and Staph aureus, we had got good number of samples to get clear picture about their antibiotics resistance patterns.

Thus, we can say that in case of aural infection with *Pseudomonas aeruginosa*, the antibiotics of choice are: aztreonam, amikacin, ceftriaxone. Similar results, in another study, were obtained regarding the high efficacy of amikacin (resistance 8.3%) while regarding ceftriaxone and aztreonam which they didn't show to be effective as the resistance was 96.6% and 100%, respectively[19, 21]. This could be explained by the higher number of samples in the latter study.

While in case of aural infection with *Proteus vulgaris*, we should first think of using cefipime, vancomycin and amikacin.

For *Staph. aureus* aural infections, the first-choice antibiotics are cefoxitin, vancomycin, gentamycin and amikacin.

E.coli treatment could be successful when we use azithromycin or amikacin or trimethoprime.

Similar results were obtained regarded amikacin (resistance 5.7%), while trimethoprime was reported to show high resistance 71.4%[(13)]. This difference in results could be explained by the higher number of samples in the latter study.

The last three bacterial species, *Streptococcus pneumonia*, *Klebsiella pneumonia* and *Enterobacter aerogenes* results are not very relevant because of low number of samples that we couldn't come out with clear view of the resistance patterns.

Cefotaxime is a highly effective 3rd generation cephalosporin that was reported to be effective against *Pseudomonas aeruginosa* in early studies[22, 23]. It was introduced to Iraq several decades ago. However, in the present study it didn't show to be very effective against Pseudomonas nor Proteus. This can be attributed to extensive unjustified use of antibiotics.

The same thing can be said about gentamycin which has shown to be not effective against *E.coli* although it is Gram –ve and Gentamycin is 1st choice Gram –ve antibiotic[24].

CONCLUSION:

Choosing the proper antibiotic in any bacterial infection is of tremendous importance. However, reassessment of antibiotic resistance profiles over regular intervals is vital and should be regarded as a routine task. Since pseudomonas aeruginosa is the most common cause of ear infection, it should be considered in every single case and treated accordingly with the proper antibiotics like aztreonam, amikacin and ceftriaxone.

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RESEARCH ARTICLE

Antioxidant and Antimicrobial Activity of *Canavalia gladiata*

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ABSTRACT:

The present study deals with the antioxidant and antimicrobial activities of *Canavalia gladiata*. Antioxidant activity by nitric oxide and DPPH methods reveals that methanol extract of *Canavalia gladiata* shows good results when compared to the aqueous extract. It indicates that methanol extract of *Canavalia gladiata* shows maximum percentage of inhibition when compared to the standard drug (Ascorbic acid).Antioxidants help to neutralize free radicals, which are unstable molecules that are linked to the development of a number of degenerative diseases such as cancer, cardiovascular disesase, cognitive impairment immune dysfunction, cataract and macular degeneration. Antibacterial activity by Agar well diffusion method reveals that methanol extract of *Canavalia gladiata* shows better results when compared to the aqueous extract. It indicates that methanol extract of *Canavalia gladiata* shows maximum zone of inhibition when compared to the the standard drug (chloramphenicol).

KEYWORDS: *Canavalia gladiata* nitric oxide method, DPPH method, agar well diffusion method,Antimicrobial and Antioxidant activities.

INTRODUCTION:

canavalia gladiata is a popular plant commonly called as sword bean and belongs to the family Fabaceae and is used for various ailments[1,2]. It is commonly cultivated in almost Asian countries and European countries such as in India, Myanmar or adjacent China[3-5]. Sword Bean is a twining, nearly erect annual herb, some cultivars may also be semi-erect and the root system is deep, growing up to 6 ft. Stems are sparsely pubescent or glabrous and Leaf blades are elliptic or ovate-elliptic, base cuneate, apex acute or acuminate; stipules deciduous. Leaves[6-8] are shiny, trifoliate. Flowers are pink and white in colour .Flowers are bisexual, papilionaceous, all joined; ovary superior, style slender, curved, stigma is small. Pods are 30 cm long and 5cm wide. Seeds are elliptical. Seeds are red or red-brown, rarely black, pink or white.

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Fruits[9-11] are linear-oblong pod, slightly compressed, sometimes curved. The main chemical constituents are carbohydrates, alkaloids Phenolic compounds, flavonoids and amino acids such as cystin, tyrosin, and tryptophan. Seeds contain three crystalline globulins canavalin, concanavalin A and B etc**.**Six compounds were isolated and their structures were identified Gallic acid, Methyl gallate, 1,6-di-o- galloyl-β-D-glucopyranoside, B- sitosterolm, Lupeol and δ-tocopherol[1-15]. All parts of the plant have been used as crude drug for the treatment of vomiting, abdominal dropsy, kidney-related lumbago, asthma, obesity, stomach-ache, dysentery, coughs, headache, intercostal neuralgia, epilepsy, schizophrenia, inflammatory diseases and swellings[12-16].

MATERIALS AND METHODS:

The leaves of *Canavalia gladiata* were collected from the local area of Nellore, SPSR Nellore district (India) . The plant material (PARC/2020/4331-Voucher Specimen) was identified and authenticated by the botanist Prof.P.Jayaraman M.Sc, Ph.D. (PARC), Director Institute of Herbal Botany, Tambaram Chennai.

A) Methanol extract of *Canavalia gladiate*:
About 650 grams of the dry powder extracted with 2 litres of 95% ethanol by continous hot percolation using soxhlet apparatus. After completion of extraction it was filtered and concentrated to dry mass by vaccum distillation. A pale green colour residue was obtained. The extract was then stored in a dessicator.

B) Aqueous extract of *Canavalia gladiate*:
About 650 grams of the dry powder extracted with 2 litres of distilled water by continuous hot percolation method using soxhlet apparatus. After completion extraction, the aqueous extract was filtered and concentrated to dry mass by vaccum distillation .A green colour residue was obtained.

Antioxidant Activity:

Nitric oxide method[17-28]

The reaction mixture (3ml) containing sodium nitroprusside (10mM, 2ml), phosphate buffer saline (0.5ml) and extract or standard solution (0.5ml) will be incubated at 25 degrees Centigrade for 150 min. After incubation, 0.5 ml of the reaction mixture containing nitrate will be pipetted and mixed with 1 ml of sulphanilic acid reagent (0.33%i in 20% glacial acetic acid) and will be allowed to stand for 5 min for completing diazotization.Then 1 ml of naphthylethylene diamine dihydrochloride (1%) will be added, mixed and will be allowed to stand for 30 min.The absorbance of these solutions will be measured at 540 nm. against the blank solution The percentage inhibition will be calculated by comparing the absorbance values of control and test by using the formula.

$$(\%)\ \text{scavenged} = \frac{(\text{A control} - \text{A test})}{\text{A control}} \times 100$$

A control is the absorbance of the control reaction mixture. A test is the absorbance of sample of the extracts at different concentrations.

DPPH free radical scavenging activity[17-28]

The free radical scavenging activity will be measured in vitro by 1, 1-diphenyl-2-picryl-hydrazyl assay. About 0.3 mM solution of DPPH in 100% ethanol will be prepared and 1ml of this solution will be added to 3ml of the extract dissolved in ethanol at different concentrations (5-80mcg/ml).The mixture has to be shaken and allowed to stand at room temperature for 30 min .Absorbance will be measured at 517 nm using a spectrophotometer.The capability to scavenge the DPPH radicals will be calculated using the formula.

$$(\%)\ \text{scavenged} = \frac{(\text{A control} - \text{A test})}{\text{A control}} \times 100$$

A control is the absorbance of the control reaction mixture. A test is the absorbance of sample of the extracts at different concentrations

Determination of Antimicrobial Activity:

Fresh aqueous and methanolic extracts of *Canavalia gladiata* were used for the determination of antimicrobial activity.

Microorganism used:

Salmonella typhi

Method:

Agar well diffusion method[18-28]

From the above mentioned organisms, inoculums was prepared by inoculating the organisms in 10 ml of nutrient broth and incubated at 37 degrees centigrade for 18 hrs. Nutrient agar medium was poured in to each sterilized petridish and organism was inoculated. Wells were made in to the medium by using sterile cork borer and each sample of the extracts (100ul) was filled in to the wells of agar plates directly by using a micro liter syringe .Then the plates were incubated at 37 degrees centigrade for 24 hr.After incubation, the zone of inhibition was observed and measured in mm.

RESULTS:

Table 1: DPPH method

S. No	Type of extract	Concentration (ug/ml)	Absorbance	% Inhibition	IC50 (ug/ml)
1	Methanol extract	100	1.0953	34.37	
		200	0.8776	47.42	
		300	0.7788	53.34	291
		400	0.5628	66.28	
		500	0.5151	69.14	
2	Aqueous extract	100	1.3932	16.52	
		200	1.2596	24.53	
		300	1.1291	32.35	487
		400	0.9630	42.30	
		500	0.7634	54.26	
3	Ascorbic acid	100	0.9640	42.24	
		200	0.7624	54.32	

		300	0.6079	63.58	263
		400	0.3609	78.38	
		500	0.2978	82.16	

Table 2: Nitric oxide method

S.No	Type of extract	Concentration (ug/ml)	Absorbance	% Inhibition	IC50 (ug/ml)
1	Methanol extract	100	0.7473	32.87	
		200	0.6150	44.76	
		300	0.5420	51.32	290
		400	0.3948	64.54	
		500	0.3635	67.35	
2	Aqueous extract	100	0.8859	20.42	
		200	0.8179	26.53	
		300	0.7152	35.76	510
		400	0.6208	44.24	
		500	0.4881	56.16	
3	Ascorbic acid	100	0.6202	44.29	
		200	0.5171	53.55	
		300	0.4300	61.38	211
		400	0.2645	76.24	
		500	0.2191	80.32	

Table 3 :Antimicrobial activity of *Canavalia gladiata* by Agar well diffusion method against *S.typhi*

S.No	Name of the Extract	Concentration (µg/ml)	Zone of inhibition (mm)
1	Methanol extract	100	16
2	Aqueous extract	100	0
3	Std (Chloramphenical)	100	26

DISCUSSION:

Antioxidant activity by nitric oxide method and DPPH method states that methanol extract of *Canavalia gladiata* shows good antioxidant activity when compared to aqueous extract. In both cases as concentration increases, % of scavenging activity also increases for methanol extracts of *Canavalia gladiate* When compared to aqueous extracts. Antimicrobial activity by agar well diffusion method by using microorganism *salmonella typhi* states that methanol extract of *Canavalia gladiata* shows the better antagonist effect against the microorganism when compared to the standard drug chloramphenicol.

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RESEARCH ARTICLE

Identification of Bioactive Phytocomponents of Hydroalcoholic Extract of *Enhalus acoroides* by Gas Chromatography- Mass Spectrometry Analysis

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ABSTRACT:
Sea grass are good source of potent drug which has medicinal properties and able to cure human disease. One such marine plant is the *Enhalus acoroides* which belong to hydrocharitaeceae family. This marine species are widely distributed in the tropics of Indian and western Pacific Ocean and the species were collected from Ramanathapuram district for further process. This study reveals about the bioactive components present in *Enhalus acoroides* and identified its biological activity by Gas chromatography Mass spectrometry analysis using hydroalcoholic extract. The compounds present in *Enhalus acoroides* are Benzoic acid, 2- methyl 7- oxa bicyclol heptanes, 1, 3 Nonadiene, silane, ethoxytriethyl. Biological activities of the compounds present in the sample include antioxidant, hypocholesterolemic, antihypertensive, anti-inflammatory, anti-microbial, antiviral and anti-hepatotoxic effect.

KEYWORDS: Cancer, *Enhalus acoroides*, Gas Chromatography- Mass Spectrometry, Phytocompounds, Biological activity, 2-Butynediamide, Antioxidant.

INTRODUCTION:
All over the globe, non-communicable diseases including cancers, cardiac problem, diabetes and chronic respiratory illness plays a major threat to today's mankind health and development[1]. In 1985, World Health Organization estimated that approximately 65% of the world's population predominately relied on medicinal herbs for their primary health care illness[2]. The traditional medicines like Herbs and species makes human beings healthier[3]. Herbs are used from the ancient periods for the treatment of human ailments[4]. In India, around 2500 herbs have been identified for the valuable medicinal values[5]. Due to the folkloric properties of herbs, many developing countries use the plants for therapeutic remedies[6]. Knowledge in phytochemicals of plants is desirable not only for the therapeutic properties, but also helps in finding the economic phytoconstituents for the synthesis of chemical substances for the folkloric problems[7]. Phytochemicals plays a major role in the alternatives for the control of antibiotic resistant human pathogens[8]. Herbs possess several essential phytochemicals including flavonoids, phenolics, carotenoids and so on which possess antioxidant propeties[9]. Basically sea grass has high antioxidant activity and able to cure various type of cancers. Especially *Cymodoceae serrulata* has high cytotoxic effect on silver nano particles and capable of curing cervical cancer. It shows high inhibition rate[10]. Apart from potent drug application, sea grass may also be used as a fertilizer; nourishment since they are good in lipid, protein and fibre. Caroteniods and chlorophyll acts as an antioxidants and vitamins because they present in higher amount and hence it has a wide application on Pharmacological industry[11]. Seagrass is one of the essential medicinal plants. Sea grass is the marine plants which serve as a feedent for marine organisms and endangered species[12]. Over the Past years it has been proved that sea grass used for remedial purposes which may include stomach problems, skin diseases, fever, muscle pain, wounds[13]. *Enhalus acoroides* comes under hydrocharitaceae family which is a monotypic marine genus[14]. Leaves of *Enhalus acoroides* are larger and found in the sub tidal region. The extract of *Enhalus acoroides* show high antioxidant activity[15]. GC-MS analysis has various applications in finding the mode of action of medicinal herbs[16]. This study reveals about the bio-compounds present in the

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hydro alcoholic extract of *Enhalus acoroides* by Gas Chromatography Mass Spectrometry analysis and determined its pharmacological activities.

MATERIALS AND METHODS:

Collection and Authentication of *Enhalus acoroides:*

Sample has been collected from Devipattinam, Ramanadhapurm District on June, 2019 and authenticated from Dr. P.Jeyaraman, PhD., Director, Plant Anatomy Research Centre, Presidency College.

Preparation of Alcoholic Extract:

The sample was washed with tap water, shadow dried and grinded in herbal grinder. 20 grams of powder sample were transferred separately in to conical flasks. The conical flask containing 1000ml of hydro-alcohol (70%) were added and vigorously shaking for one hour using rotatory shaker and kept for 24 hours. Through whatman No.1 filter paper, the extracts were filtered after 24 hours. Store the filtrate at 4°C and the extract was used for GC MS analysis.

GC MS-Analysis:

GC-MS analysis was carried out on Shimadzu 2010 plus comprising a AOC-20i auto sampler and gas chromatograph interfaced to a mass spectrometer instrument employing the following conditions: column RTX 5Ms (Column diameter is 0.32mm, column length is 30m, column thickness 0.50µm), operating in electron impact mode at 70eV; Helium gas (99.999%) was used as carrier gas at a constant flow of 1.73 ml /min and an injection volume of 0.5 µI was employed (split ratio of 10:1) injector temperature 270 ºC; ion-source temperature 200 ºC. The oven temperature was programmed from 40 ºC (isothermal for 2 min), with an increase of 8 ºC/min, to 150ºC, then 8ºC/min to 250ºC, ending with a 20min isothermal at 280ºC. Mass spectra were taken at 70eV; a scan interval of 0.5 seconds and fragments from 40 to 450 Da. Total GC running time is 51.25min. The relative percentage amount of each component was calculated by comparing its average peak area to the total areas. Software adopted to handle mass spectra and chromatograms was a Turbo Mass Ver 5.2.00[17].

Identification of components:

By using the database of National Institute Standard and Technology (NIST) interpretation on GCMS was conducted since it has more than 62,000 patterns. The spectrum of the known components was compared with the spectrum of the unknown component stored in the NIST library. The name of the compound, molecular weight and structure of the phytocomponents were ascertained.

RESULTS AND DISCUSSION:

Identification of Bioactive Compounds in *Enhalus acoroides* by GC MS analysis:

Twenty compounds were identified in hydro alcoholic extract of *Enhalus acoroides* by GC-MS analysis.. The chromatogram obtained from the hydro alcoholic extract of *Enhalus acoroides* shown in Figure 1. The active phytocomponents along with their retention time (RT), molecular wirght, molecular formula and concentration (%) are presented in Table 1. Figure 2 shows the structure of identified prevailing compounds which includes Hexadecanoic acid (1.73%), methyl ester, 9-Octadecenoic acid (Z) (5.34%), 9,12,15-Octadecatrienoic acid, methyl ester, e, 2-Hexadecen-1-ol, 3,7,11,15-tetramethyl-, [R-[R*,R*-(E)]]l, 8,9,9,10,10,11-hexafluoro-4,4-Dimethyl-3,5-dioxatetracyclo and Stigmast-5-en-3-ol, (3.beta.,24S).

Table 1: Identification of Phytochemical compounds in the Hydro alcohohic extract of *Enhalus acoroides* using GC-MS

Peak	R. Time	Area %	Height %	Molecular Formula	Molecular Weight	Bioactive Compounds
1	9.990	0.18	0.56	$C_9H_{11}N_3O_2$	193	Benzoic Acid, 2-(3,3-Dimethyl-1-Triazenyl)
2	12.611	0.12	0.47	$C_7H_{12}O$	112	2-Methyl-7-Oxa-Bicyclo[2.2.1]Heptane
3	13.106	0.37	0.97	$C_8H_{14}O$	126	4-Hepten-3-one, 5-methyl-, (E)
4	13.225	0.13	0.27	$C_4H_4N_2O_2$	112	2-Butynediamide
5	13.367	0.20	0.43	C_9H_{16}	124	1,3-Nonadiene, (E)
6	13.548	1.73	4.41	$C_{17}H_{34}O_2$	270	Hexadecanoic acid, methyl ester
7	13.977	5.34	8.06	$C_{18}H_{34}O_2$	282	9-Octadecenoic acid (Z)
8	15.200	0.03	0.19	$C_{12}H_{22}O$	182	3-Dodecen-1-AL
9	15.336	1.12	3.14	$C_{19}H_{34}O_2$	294	9,12-Octadecadienoic acid, methyl ester, (E,E)
10	15.423	2.56	5.29	$C_{19}H_{32}O_2$	292	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)
11	15.540	1.33	2.86	$C_{20}H_{40}O$	296	2-Hexadecen-1-ol, 3,7,11,15 tetrame thyl-, [R-[R*,R*-(E)]]
12	15.600	1.39	2.04	$C_{10}H_{20}O_2$	172	Methyl 4-methyloctanoate
13	15.700	0.85	1.85	$C_{12}H_{12}F_6O_2$	302	8,9,9,10,10,11-HEXAFLUORO-4,4-dimethyl-3,5-dioxatetra cyclo
14	15.858	5.27	6.37	$C_{22}H_{42}O_2$	338	Laurinsaeure, 9-Decen-1-Ylester
15	15.908	2.52	6.64	$C_{15}H_{26}$	206	7-Pentadecen-5-yne, (E)
16	15.992	3.69	6.36	$C_8H_{20}OSI$	160	Silane, ethoxytriethyl
17	16.267	20.79	11.34	$C_{29}H_{39}NO_4$	465	2-(6-[1-ethyl-4-[4-(1h-pyrrole-2-carbonyl)-2,3,3a,4,5,7a-hexahydro-1h-inden-5-yl]-buta-1,3-dienyl]-5-methyl-tetra
18	16.415	19.12	12.85	$C_{29}H_{50}O$	414	Stigmast-5-en-3-ol
19	16.608	13.05	12.69	$C_{29}H_{50}O$	414	Stigmast-5-en-3-ol
20	16.660	20.20	13.22	$C_{29}H_{50}O$	414	Stigmast-5-en-3-ol

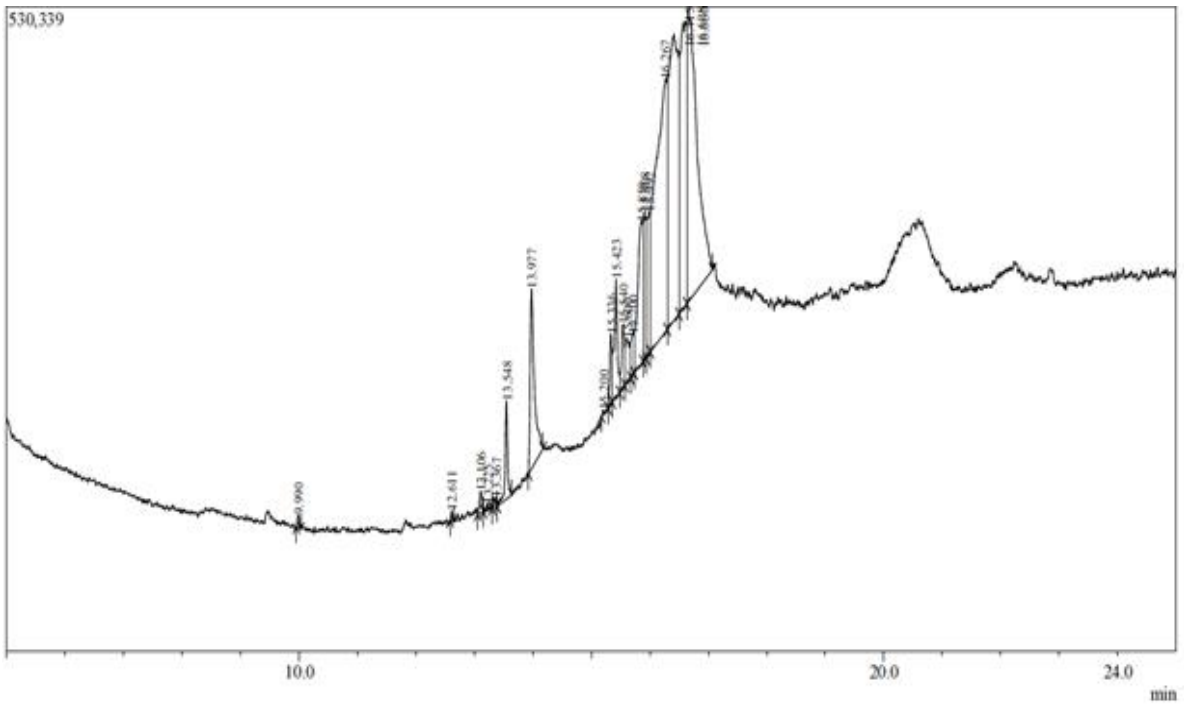

Figure 1: Chromatogram of hydroalcoholic extract of *Enhalus acoroides*

Table 2 shows the pharmacological activities of identified phytocomponents present in the hydroalcoholic extract of *Enhalus acoroides*. Alcholic compounds as well as fatty acids were present which have the ability to reduce stress, and also used as a disinfectant[18]. Cancer is a dreadful disease can be cured by nonadiene which has high anti- tumour activity[19]. One of the palmitic acid derivatives is the hexadecanoic acid which posses' hypocholestremic effect, hemolytic and also acts as alpha reductase inhibitor[20]. The compounds present in the hydro alcoholic extract of *Enhalus acoroides* shows many pharmacological activities which includes antiviral, antibacterial, anti-inflammatory, antihypertensive and also anticancer activity. Stigmast-5-en-3-ol compound has anti-diabetic activity which has insulin like effect and also lowers the cholesterol level[21]. The presence of many phytocomponents justifies the use of the seagrass for several ailments by traditional practitioners.

Table 2: Pharmacological activities of bio-compounds identified in the Hydro alcohohic extract of *Enhalus acoroides* using GC-MS

Peak	R. Time	Name of the compounds	Pharmacological Activity[22]
1	13.548	Hexadecanoic acid, methyl ester	Antioxidant, hypocholesterolemic, Anti androgenic, hemolytic, Alpha reductase inhibitor.
2	13.977	9-Octadecenoic acid (Z)	Antihypertensive, Increase HDL and decrease LDL Cholesterol
	15.336	9,12-Octadecadienoic acid, methyl ester	Antiinflammatory,hypocholesterolemic cancer preventive, hepatoprotective, nematicide, insectifuge, antihistaminic antieczemic, antiacne, 5-Alpha reductase inhibitor, antiandrogenic, antiarthritic, anticoronary, insectifuge
3	15.423	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)	Hypocholesterolemic, Nematicide Antiarthritic, Hepatoprotective, Anti androgenic, Nematicide, 5-Alpha reductase inhibitor, Antihistaminic, Anticoronary, Insectifuge, Antieczemic Anticancer
4	15.540	2-Hexadecen-1-ol, 3,7,11,15-tetramethyl-, [R-[R*,R*-(E)]]	Antimicrobial, Anti-inflammatory
5	15.700	8,9,9,10,10,11-hexafluoro-4,4-dimethyl-3,5-dioxatetracyclo	Antimicrobial
6	16.415	Stigmast-5-en-3-ol, (3.beta.,24S)	Anti-hepatotoxic, Antiviral, Antioxidant, Cancer¬ preventive, Hypocholesterolemic.

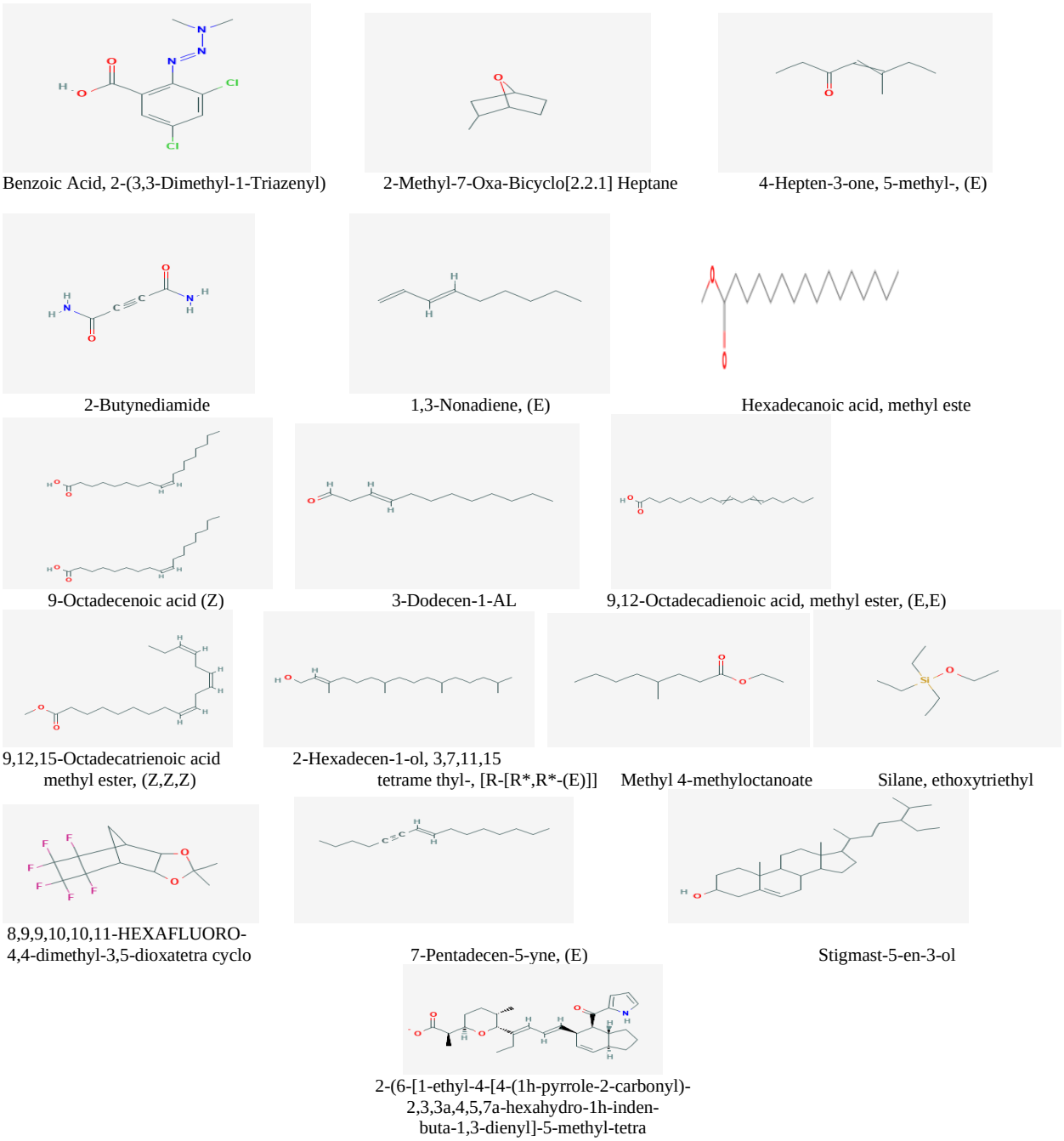

Figure 2: The Structures of Identified Phytochemical Compounds of Hydro alcoholic extract of *Enhalus acoroides*

In asthma patients 2-Hexadecen-1-ol produce therapeutic effects and also acts as a source of anti-inflammatory and antitumor agents[23]. Sermakkani *et al.*, narrates that hexadecane shows high antioxidant and anticancer activities[24]. The compound 9, 12, 15-Octadecatrienoic acid possess hepatoprotective, cancer preventive, nematicide, antieczemic, antihistamine, anticoronary, antiarthritic and antiviral properties[25, 26]. The methanol and hexane extract of *Enhalus acoroides* has antimicrobial activity. The hydroalcoholic extract shows high antioxidant activity which has ability to cure degenerative diseases[27]. The compounds like hexadecenoic acid and 12, 15-Octadecatrienoic acid has reduced growth of human and fish pathogens. Thus, the compound hexadecnoic acid has important application on pharmaceutical industry[28].

CONCLUSION:

The current investigation of Gas Chromatography- Mass Spectrometry analysis shows that there are 20 various phytocompounds were identified in the hydro alcoholic extract of *Enhalus acoroides*. This study reveals that

Enhalus acoroides has pharmacological activities like antioxidant, anti-inflammatory, anti-androgenic, anti-microbial, anticancer and antihepatotoxic. Due to it adverse pharmacological properties, the hydro alcoholic extract of *Enhalus acoroides* has the ability to cure many therapeutic diseases. Hence *Enhalus acoroides* might be used for developing novel drugs, and further study needs to elucidate the novel bioactive compounds and their mechanism of action.

CONFLICT OF INTEREST:

The authors declare no conflict of interest.

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RESEARCH ARTICLE

Investigating the potential of Quercetin enthused nano lipoidal system for the management of dermatitis

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ABSTRACT:

Objective: The present research work was undertaken to develop quercetin enthused nanolipoidal systems and its characterization. The objective was to investigate potential of prepared system in the management of DNCB induced dermatitis. **Method:** Nanolipoidal system was prepared in different combinations with quercetin, L-α phosphatidylcholine (SPC) and ethanol and characterized for particle size, polydispersity index (PDI), zeta potential, drug entrapment efficiency, percentage drug release, skin retention and skin permeation. Selected batches were further incorporated into Carbopol 934 base gel. The vesicles were in size range 324.19-359 nm while polydispersity index (PDI) ranges from 0.241-0.554 and for zeta potential, it was from -26.33 to -39.3 nm. Entrapment efficiency was from 23.77-94.68 %. Confocal laser scanning microscopy showed penetration depth of rhodamine enthused ethosome across rat skin up to 45.23 µm which was significantly higher than the rhodamine solution (10 µm). In dinitrochlorobenzene (DNCB) induced mice dermatitis model histopathology study showed a marked decrease in amount of inflammatory cell nucleus in mice treated with quercetin loaded ethosomal gel followed by 76.13% decrease in-ear swelling and ear mass respectively in morphology study. The conventional marketed formulation showed a nominal decrease in epidermal thickness. Further Primary irritation index was less than 0.4 indicating negligible irritation in all the groups. **Results:** The optimized formulation F6 with SPC and ethanol in the ratio of 20:80 displayed the highest drug content and entrapment efficiency of 94.68±1.14%. PDI was 0.241±0.11 and skin retention 7.7%. Batch F6 with vesicle size and zeta potential of 324.9±19 nm and -26.33 mV, respectively, was incorporated in Carbopol 934 base gel and the prepared gel was evaluated for morphology, spreadability, *in vitro*, *ex vivo* release study, and kinetics study and *in vivo* studies. **Conclusion:** The present study revealed that the developed ethosomal gel can be used for enhanced delivery of Quercetin *via* skin. The *in vitro* studies indicated that the gel serves as an efficient carrier for Quercetin. It showed its effectiveness in the management of dermatitis. Further, Quercetin loaded nanoethosomal gel formulation can be viewed as a promising drug delivery system for the management of dermatitis.

KEYWORDS: Transdermal drug delivery, Nanovesicular, Quercetin, Ethosomes, Cell viability.

INTRODUCTION:

Dermatitis is a common inflammatory disorder of the skin. It is also known as eczema which is a word derived from the Greek origin meaning 'boil over' or 'break out' (*ekzein*, *ek*-out, *zein*-boil). The words 'dermatitis' and 'eczema' are synonymously used as dermatitis is also derived from Greek origins and refers to skin inflammation (*derma*-skin, *itis*-inflammation) [1].

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Dermatitis is best characterized by atypical fluid secretions in the epidermal layer due to spongiosis. These secretions cause intercellular swelling, formation of intra-epidermal vesicles and juicy papules. Another symptom of this dermal disorder which intensifies the skin's degradation is constant itching or pruritus. Pruritus makes the skin thick or leather-like which reflects that it leads to 'lichenification'. Dermatitis has become a major stress causing factor among different diversity of patients and their families. It results from

multiple factors like the kind of genetic predisposition a person has, environmental aspects, improper functioning of the immune system and skin (as a barrier). As an immune disorder dermatitis can also make the patient hypersensitive and susceptible to allergic disorders like rhinitis, asthma, etc [2].

Since it majorly affects paediatrics, there is a 50% chance that the young ones suffering may develop asthma and 75% suffer from the risk of getting high fever. Therapeutic treatment of eczema has always been a major challenge for the pharmaceutical field. Commonly used conventional therapies include topical or systemic corticosteroids, topical calcineurin inhibitors, phototherapy, antibiotics, antihistamines and immunosuppressive agents. Although these therapies dominate the anti-dermatitis medications sectors, but they can lead to multiple local / systemic adversities. This is due to the fact that dermatitis patients require long-term therapeutic assistance. Topical treatment strategies are very effective but difficult to develop due to the barrier function of stratum corneum which balances the skin's integrity. It functions as an effective barrier to skin penetration of drugs and it is necessary to employ enhancement strategies. There are various innovative researches exploiting strategies for penetration enhancement. There are various penetration enhancement techniques like iontophoresis, sonophoresis, micro-needles, and electroporation. These holds promise for the use of these drugs in a better and successful manner that is patient-friendly. The novel drug delivery systems (NDDS) can also be significantly used to combat the drawbacks and side effects of conventional drug delivery systems. Conventional medication requires multiple-dose therapy that holds innumerable issues. It becomes complicated for the above-mentioned conventional systems to deliver the perfect number of active agents to the right target because each medication needs to be delivered in an optimal manner to the patient. However, novel systems like the transdermal drug delivery systems offer the potential advantage of directly delivering the drug at a controlled rate for a prolonged period [3-4].

The disorders like eczema, where deeper skin layers are involved; there is a need for vesicular carrier system for delivery of drugs though the skin layers that are an effective barrier to drug penetration. The objective of this system is to explore skin as a site for drug administration with better patient compliance. There are numerous advantages associated with vesicular systems that are like elimination of the first-pass metabolism, steady plasma levels maintenance, reduction of dosing frequency and subsequent decrease of dose-related side effects.

Vesicular systems like ethosomes show enhanced therapeutic efficacy due to improved dissolution profile, drug retention and stability. Ethosomal systems are novel permeation enhancing lipid carriers embodying ethanol containing lipid vesicles with inter-digitated fluid bilayers. In contrast to the liposomes and deformable liposomes, ethosomes have been shown to exhibit high encapsulation efficiency, skin deposition ability, and depth of skin penetration for a wide range of molecules including lipophilic drugs and are effective at delivering molecules to and though the skin [5-6]. Ethosomes can also encapsulate herbal phytoconstituents which have been proven to be effective against several types of dermatological disorders like dermatitis, psoriasis, acne, etc.[7-9]. Extracts from various herbs have been continuously developed and studied to provide relief from different types of inflammatory conditions. This is due to the presence of special active phytoconstituents. Herbal extracts or their specific active phytoconstituents lag behind due to their bioavailability. This can be combated by employing distinguishable vesicular carriers (like ethosomes itself). Thus, the well-established properties of phytoconstituents like toxicity, strengthening effects on immune system, availability in bulk, etc. can be utilized with the help of novel drug delivery systems [10- 11]. One of the many beneficial and constantly researched herbal phytoconstituent is quercetin.

Quercetin is a major health-benefitting, dietary flavonoid which is extensively found in food items like apples, onions, green tea, berries, red wine, buckwheat tea, etc. It is a potent pleiotropic molecule that can modulate multiple pathways. It also exhibits distinguishable biochemical and biological effects when employed as an active constituent against viral diseases, inflammatory disorder, cancer and cardiac issues [12]. Antioxidant effect of quercetin plays a very crucial role in its activity with respect to the signal transduction pathway, glutathione, environmentally induced reactive oxygen species (ROS) and enzymatic activity [13]. Quercetin has become an actively performing anticancer drug as it has entered the clinical trial phase. In contract to the beneficial effects of quercetin, it also falls behind due to low solubility (sparingly soluble in water), limited absorption (though oral routes) and minimal bioavailability. The drug delivery system which enhances the absorption of drug in the systemic circulation by penetration though topical application, can be used to bypass the drawbacks attached with quercetin [14].

The rationale behind the current research work was to achieve improved properties of quercetin by incorporating them in nanoethosomal gel form for the possible management of dermatitis.

MATERIALS AND METHODS:

Materials:

Quercetin was obtained from- SRL, poly ethylene glycol 400, and L-α phosphatidylcholine, were obtained from Sigma Aldrich, rest of the chemicals used were of analytical grade. Wistar rats (150–250 gm) were procured from the Central Drug Research Institute, Lucknow, India. The protocols for experiment were reviewed and approved by the Institutional Animal Ethics Committee (IAEC) as followed the guidelines of the Committee for the Purpose of safety and Supervision of Experiments on Animals (CPCSEA), Government of India[15]. **Registration number** [1492/PO/Re/S/11/CPCSEA 28/3/2017 and CSIR-IITR Lucknow, 54/GO/RBi /S/99/CPCSEA].

Methods:

Preformulation studies:

The Preformulation studies for the quercetin were done. The solubility was determined by the qualitative method. The partition coefficient was determined by the conventional shake flask method. The Drug excipient interaction was performed using FTIR (Perkin Elmer Spectrum, version 10.03.06)[16].

Preparation of Quercetin loaded Ethosomes:

Ethosomes were prepared by modified cold method. Weighed the quantity of L-α phosphatidylcholine and quercetin were taken in a beaker and added ethanol to it. The beaker was covered with parafilm to prevent evaporation of ethanol. The solution was stirred on a magnetic stirrer and the water was added drop by drop with the help of syringe at room temperature[17]. Stirring was continued for the next 15 min. Sonication was done for next 5 min to reduce the size of prepared vesicles. The dispersions were stored in refrigerator. All formulations used are shown in Table1.

Table 1. Formulation table for ethosomes preparation

Formulation Code	% L-α Phosphatidylcholine	Ethanol: Water
F1	1	10:90
F2	1	20:80
F3	1	30:70
F4	1	40:60
F5	2	10:90
F6	2	20:80
F7	2	30:70
F8	2	40:60
F9	3	10:90
10	3	20:80
F11	3	30:70
F12	3	40:60

Procedure for characterization of quercetin loaded ethosomes:

All the prepared batches were observed under phase-contrast microscopy with a magnification power of 100 X (Columbus). Photograph were taken using (Nokia 6.1 Plus). The optimized batch was observed by transmission electron microscopy (Malvern) with voltage of 200 kV for surface appearance and shape[18].

PDI, Size and Zeta Potential:

The ethosomes were dispersed in distilled water and 10 µl of diluted dispersion was placed on the carbon-coated grid then zeta potential vesicle size and polydispersity index were measured by zetasizer (Nano plus- Version 5.22/3.00 from BBAU, Lucknow)

Entrapment Efficiency:

The entrapment efficiency of quercetin loaded nanoethosomal vesicles was determined by the ultra-centrifugation method in which formulation was kept in a centrifugation tube and placed in the REMI Cooling centrifugation machine. Centrifugation was carried out at 10000 RPM at 4°C for 30 min. The clear supernatant was siphoned of clearly to separate the unentrapped drug, sediment was treated with 1 ml of 0.1 % Triton X 100 to lysis the vesicles and then diluted with PBS (6.8). Entrapment efficiency was calculated using the following formula[19]

$$\% \textbf{ Entrapment Efficiency} = \frac{\text{Amount of drug present in vesicles}}{\text{Total Drug incorporated}} \times 100$$

$$\% \textbf{ Loading Efficiency} = \frac{\text{Amount of drug present in vesicles}}{\text{Initial drug loaded}} \times 100$$

In vitro Studies:

In vitro study was performed using Franz diffusion cell (Ambient borosilicate fabricated) with glass, surface area available for diffusion as 2.54 cm^2. The dialysis membrane (50-LA, Himedia Lab. Pvt. Ltd) was placed in PBS (6.8) for 4 hours to attain saturation before starting the study. It was placed between the donor and receptor compartment and ethosomal dispersion (5ml) to be analyzed was placed into the donor cell compartment[20]. The receptor chamber was filled with PBS (6.8) and was maintained at 37±0.5°C with continuous stirring. The donor compartment was covered with parafilm to prevent evaporation. 1 ml aliquot from receptor phase solution were withdrawn at different time intervals (5, 1, 2, 3, 4, 5, 6, 7, 8, 24 h) and exact volume of medium was readded back into the medium[21] and quantification was done using UV Spectrophotometer (Shimadzo-1800) at 252 nm.

Ex vivo permeation and determination of the amount of remained drug in the skin:

In this study the dialysis membrane was replaced with rat skin. The skin was mounted between donor and receptor compartment with the stratum corneum side facing upwards into the donor compartment.[22] Skin surface was washed first with PBS pH 6.8 and then with

ethanol. The procedure was repeated twice to ensure that no traces of formulation were left onto skin surface. The permeation area of the skin was excised, weighed, and then cut into small pieces to extract the drug content present in skin with ethanol. The resulting solution was centrifuged (1500 RPM), and the quercetin concentration was measured and expressed as percent of initially added drug.

Permeation Data Analysis:

Study of release rate profile and data obtained from the *in vitro* drug release study are fitted in different kinetic equation: zero order as the cumulative percent drug release Vs time, first order release as the percentage release (RTR) Vs time, higuchi release kinetics as the cumulative percentage drug release Vs square root (SQRT) time, korsmeyer peppas release kinetics as the log mt/mi Vs log time.[23-24]

Calculation data of skin permeation parameters:

Cumulative amount of the drug passed per unit area was plotted as a function of time. The flux was calculated from the slope of the linear portion.[25] The permeability coefficient (Kp) of Quercetin though out rat skin was calculated using relation derived from Fick's first law of diffusion, which is expressed by the following equation:

$$Kp = \frac{J}{C}$$

Where J is the flux and C is the drug concentration in donor compartment.

Confocal Laser Scanning Microscopy (CLSM):

The capability of probe loaded ethosomes to penetrate though the skin was observed by confocal laser scanning microscopy. The probe Rhodamine B (0.03%) was used in place of drug. Diffusion study of probe loaded ethosomal dispersion was performed in a similar manner as discussed in previous section[26]. The skin was removed from the cell and thoroughly washed with distilled water at the end of the study$_{14}$. The treated area was cut out and sectioned using microtone. The skin specimen was observed under a confocal microscope. (Reni Shaw in via Raman Microscope Holmarc Honeycomb Tabletops (TT300-120) and penetration depth of Rhodamine B was measured[27].

Incorporation into a hydrophilic gel:

The pH was adjusted by adding triethanolamine dropwise. On the basis of entrapment efficiency, zeta potential, polydispersity index, and vesicle size F-6, F-11, F-1, F-8 were selected and incorporated into carbopol base gel. The gel base was prepared by dispersing the weighed quantity of Carbopol 934 P (1% w/w). The carbopol was soaked in distilled water overnight. Quercetin loaded ethosomal dispersion (5ml) was added in it with continuous stirring. [28]

***In vitro* Diffusion Study and Data analysis:**

In vitro diffusion study was performed for a hydroethanolic solution (HES), ethosomal gel formulation (G F-6, GF-11, G F-1, and GF-8), conventional gel formulation and standard gel formulation.[29] Using the same procedure as discussed in the previous section. Release data were fitted in different Kinetic equations.

For the release rate profile study, the data, collected from the *in vitro* drug release study were fitted in the different kinetic equations as done in the previous section.[30]

Characterization of quercetin loaded ethosomal gel pH, Viscosity:

The pH was determined by digital pH meter (RI-152-R).[31]The viscosity was measured by using Brookfield viscometer (Model No DV-III ULTRA) using spindle no 06 at 100 RPM.[32]

Texture, Spreadability:

Texture Analysis of optimized nanoethosomal gel was determined by the TAXT2 Texture Analyzer. [33]Spreadability was expressed as the time required for two slides to slip off from the gel for at fixed distance by applying constant weight attached (unit gm.cm/sec). Spreadability of the gel formulations is inversely proportional to the viscosity. The lesser the time taken for the slides to slip off from the gel, the better the spreadability of the gel. A greater spreadability indicated a smaller shear is needed to spread the gel.[34]

Drug Content:

Drug content of prepared gels were determined by dissolving an accurately weighed quantity of the gel (100mg) in 5 ml of ethanol. 1 ml was transferred to a volumetric (50ml) and appropriate dilutions were made with phosphate buffer pH 6.8.[35] The resulting solution was then filtered using a 0.45µm membrane filter and measured spectrophotometrically at 252 nm (Shimadzu-1800, Japan).

Stability Studies:

Drug loaded ethosomal gel was stored at 4°C and at 25°C for one month. The physical stability was examined by visual observation and pH determination. Gel formulation was assessed by change in pH.[36]

Statistical Analysis:

Statistical analysis was done by using one-way ANOVA (Microsoft Excel). The difference was considered significant when $P \leq 0.05$.[37]

Animal study:

Wistar rats of either sex (150-200 gm) with no previous drug treatment were used for in vivo studies. The animals were fed with standard diet and water Librium. Acclimation to laboratory hygienic condition was done for seven days before the start of experiment[38]. The experimentation on animal was performed as per Institutional Animal and Ethics Committee of Amity Institute of Pharmacy, AUUP, Lucknow.

Registration number [1492/PO/Re/S/11/CPCSEA 28/3/2017 and CSIR-IITR Lucknow, 54/GO/RBi /S/99/CPCSEA].

Dermatitis Induction:
Dinitrochlorobenzene (DNCB) induced animal model was used for the induction of Dermatitis in rat. 50 µl of 1% DNCB in acetone: olive oil (4:1) was applied on the inner and outer surface of left ear lobes of each animal from Group 2 -6 once daily for 5 days[27]. Eight hours before the final application weighed quantity of test (G-3) and marketed preparation (Clobetasol-Zydus Pharmaceuticals) was applied on the inner and outer sides of left ear lobes to each animal of group 3 to 6 as per protocol. Animal were sacrificed and ear of both sides were cut off using sharp surgical scissor. Then skin tissue from the right and left ear of rat were excised and sent for histological examination[39].

Histopathology:
The tissue was fixed in 10% neutral formalin solution for 24 h, and dehydrated with a sequence of ethanol-xylene series of solutions. Materials were filtered and embedded with paraffin (40–60°C)[40]. 10µ thickness was taken by microtone sections. The sections were processed in alcohol-xylene series and stained with haematoxylin-eosin dye. Histological changes were observed under a microscope. Photograph was taken from each slide[41].

Skin Irritation Study:
The hair on the dorsal side of the animal were removed by clipping a day before the experiment. The animals were divided as per the protocol and treatment was given as per the protocol on the shaven back area of skin. The treated area was covered with gauge and wrapped with a bandage. The bandage and test material were removed after 24 h and after 1 h, the treated area was examined and scored for skin irritation[42].
1. Moderate erythema (dark pink)
2. Moderate to severe erythema (light red)
3. Severe erythema (extreme redness).

Cell Viability Study:
Cell survival study using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT): The cell survival study was conducted using a MTT based assay. The cells were washed with 1mM phosphate buffer saline pH 7.2 and the 100 µl/well cells with OD 600 nm=1.0 were seeded on the 96 well plate[43]. A 5 µl MTT solution was added to each well and incubated for 1 h at 37 ^{0} C. After the incubation, the cells were centrifuged at 1000 rpm for 5 min and 150 µl of the solubilization buffer containing SDS and DMSO were added to the cell pellet[44]. After 15 min of incubation, the cells were centrifuged at 1000 X g for 5 min. The absorbance of the supernatant at 570 nm was recorded with the help of a spectrophotometer[45].

Reduced Glutathione:
The measurement of GSH was done by following the protocol of Rahman *et al*., 2006 with some modifications. In this 40 µl of tissue, homogenate was added to 0.4 M of Ellman's reagent and 60 mM phosphate buffer p H=7.5.[46]The reaction mixture was kept in an incubator for 1 h at 37^{0}C and absorbance was measured at 412 nm in a UV-Vis spectrophotometer[47]

Estimation of lipid peroxidation:
The estimation of malondialdehyde (MDA) content was done using the lipid peroxidation estimation kit. A 40 µl of tissue homogenate was added to the color developing solution containing thiobarbituric acid and acid reagent. This mixture was incubated in a water bath at 60^{0} C for two h to form a pink color solution of MDA-TBA adduct. The absorbance was measured at 512 nm in a UV-VIS spectrophotometer[48].

Total antioxidant activity using 2, 2-diphenyl-2-picrylhydrazyl (DPPH) assay:
The DPPH assay is a free radical scavenging method used to assess the total antioxidants present by following the protocol of Brand Williams *et al.,* (1995) with slight modifications. A 40 µl of the tissue homogenate was added to the 0.002 ml of 0.004% methanolic DPPH solution[49]. The reaction was incubated for 15 min at 37^{0}C and the absorbance was measured at 520 nm in the UV-Vis spectrophotometer.

Antioxidant Enzymes:
A. Catalase:
Catalase activity was measured according to the protocol of Beers *et.al.,* 1952 with some modifications. Briefly, 20 µl of tissue homogenate was added to 0.1% hydrogen peroxide (H_2O_2) in 2.9 ml of 1m M phosphate buffer pH 7.2. The time-dependent decomposition of H_2O_2 was monitored at 240 nm for 2 min in the UV-Vis spectrophotometer[50].

B. Superoxide Dismutase:
The estimation of superoxide dismutase (SOD) was done by following the protocol of Beauchamp and Fridovich, 1971. A 20 µl of tissue homogenate was

added to the reaction mixture containing Phenazine methosulphate, 0.052 M Nitroblue tetrazolium salt, Nicotinamide adenine dinucleotide, and sodium phosphate buffer pH=8.3 and incubated for 5 min[51].

Table 2. Entrapment efficiency, vesicle size, PDI and zeta potential of Quercetin loaded nanoethosomal batches

S. No.	Formulation Code	Entrapment Efficiency (%)	Vesicle Size (Nanometers)	Polydispersity Index (PDI)	Zeta Potential(mV)
1	**F1**	63.99±1.73	334±15	0.532±0.032	-37.2
2	**F2**	56.61±1.14	343±13	0.453±0.029	-38.3
3	**F3**	42.48±0.70	361±17	0.0342±0.054	-39.3
4	**F4**	23.77±2.00	359±18	0.453±0.022	-35.2
5	**F5**	45.29±1.11	359±12	0.327±0.037	-30.9
6	**F6**	94.68±1.14	324.9±19	0.241±0.11	-26.33
7	**F7**	52±0.83	342±14	0.354±0.019	-36.3
8	**F8**	57.39±2.11	341±11	0.421±0.032	-33.2
9	**F9**	54.2±1.83	337±14	0.387±0.029	-31.4
10	**F10**	57.85±0.64	339±16	0.421±0.027	-39.7
11	**F11**	86.36±1.21	329±17	0.114±0.031	-35.8
12	**F12**	50.55±0.76	332±16	0.554±0.024	-31.3

After incubation, the reaction was stopped by glacial acetic acid and the supernatant was added to the n-butanol, mixed and centrifuged at 2000 rpm for 10 min. The absorbance was read at 560 nm in the spectrophotometer[52].

RESULTS AND DISCUSSION:

Visualization of vesicles by phase contrast microscopy and Transmission Electron Microscopy:

Phase contrast micrograph and TEM image of selected batch (F-6) showed the spherical surface and unilamellar structure of Ethosome[53].

Zeta Potential, Vesicle Size, and Polydispersity Index:

The vesicle sizes of ethosomes were in between 324-359 nm and Poly dispersity index (PDI) ranged from 0.24-0.45 and zeta potential varied from -26.33 to -39.7 mV. (Table-2)

Evaluation of Ethosomal Gel:

pH

pH of quercetin loaded nanoethosomal gel was found to be **7.02- 7.12.**

Viscosity:

Viscosity of selected gel was found between **5300-6600cps** while viscosity of base gel was **11000 cps** and standard gel was **5500cps**. (Table-4)

Gel was measured on PP 25 which showed 26.67 Pascal yield point (red point) and yield point 0.571 Pascal Viscosity of selected gel was found to be 10,000 CPS at share rate of 10 while at the shear rate of 15.8 shows 6729 cps viscosity. Result shows the highest viscoelastic property of gel and gel has good stability property. (Table- 3, 4)

Table 3. Stability study at different pH

Formulation Code	Initial pH Day 0	4°C One month	25°C One month
GF-1	7.06±0.03	7.03±0.03	7.05±0.03
GF-2	7.1±0.01	7.08±0.01	7.05±0.01
GF-3	6.8±0.06	6.7±0.06	6.6±0.06
GF-4	6.9±0.07	6.8±0.07	6.6±0.07

Characterization of Gel

***In vitro* Study**

On the basis of entrapment efficiency, zeta potential, poly dispersity index and vesicle size F-6, F-11, F-1 and F-8 were selected and incorporated into carbopol base gel. (Figure-1)

Table 4. Characterization of Gel

Formulation	Colour	Appearance	Spreadability	Drug Content	Ph	Viscosity (cps)
GF-1	Yellowish	Homogeneous	34.07±0.72	96.65±1.4	7.06±0.03	5300
GF-6	Yellowish	Homogeneous	33.03±0.56	81.65±1.9	7.1±0.01	6600
GF-8	Yellowish	Homogeneous	35.1±0.71	79.47±1.4	6.8±0.07	6200

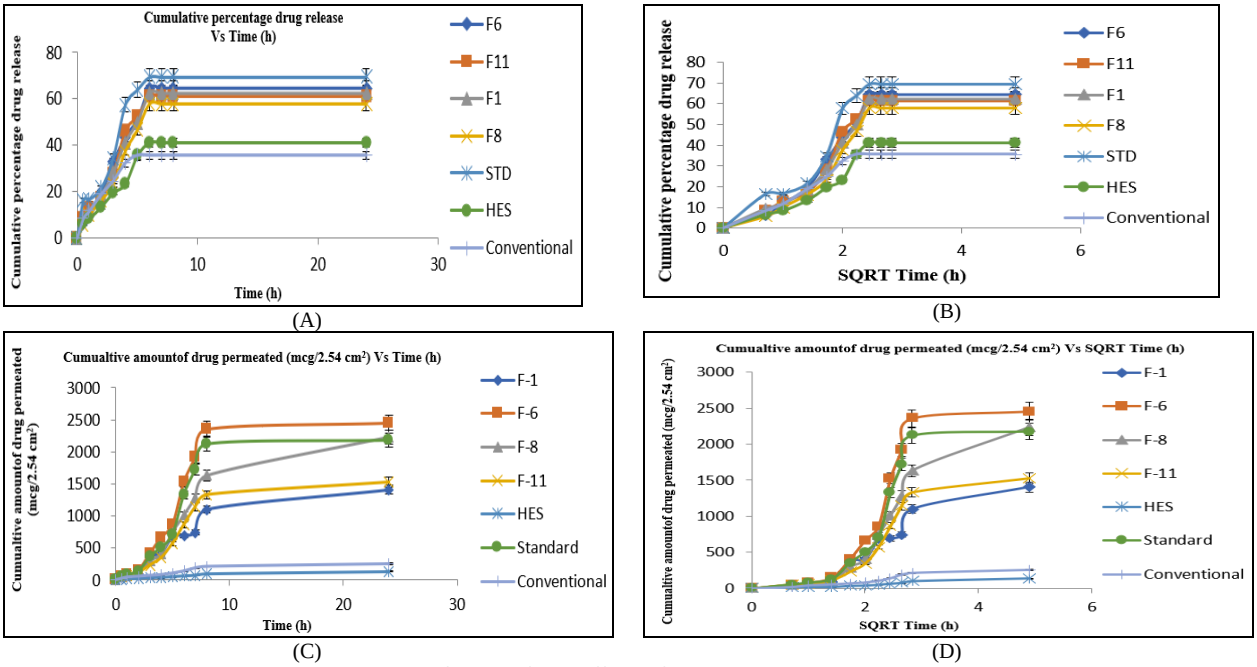


Figure 1. (A, B, C, D) Cumulative drug release after 24 h from different formulations though animal skin

Ethosomal gel formulation GF-1 to GF-4, conventional gel and HES were also evaluated for *in vitro* drug release study. Results showed that cumulative percentage drug release for GF-3 loaded ethosomal gel was significantly higher than quercetin loaded conventional gel at the end of 0-24 h. ($p>0.05$) These results suggested that ethosomal gel can enhance the skin permeation of the drug (Figure-1). The data obtained from *in vitro* release study of ethosomal gel and conventional gel was fitted in various kinetic models to assess the release rate profile. Results indicated that Higuchi Kinetic model was best fitted as R^2 value for this was highest among all. This implies slow and steady release by the process of diffusion as proposed by Higuchi. (Table-5,6)

Table 5. Permeated drug, flux and permeability coefficient of different formulations

Formulation Code	Permeated amm at 24 h ($\mu g/cm)^2$	Flux ($\mu g/cm2$ /h)	Permeability coefficient (Kp) × 10−3 (cm/h)
F-1	1406.45±451.32	58.60±0.63	5.86±0.032
F-6	2454.10±293.78	102.25±3.22	10.22±0.63
F-8	2233.90±768.21	93.07±0.51	9.307±0.051
F-11	1526.19±152.18	63.59±0.57	6.359±0.043
Standard	2178.51±165.23	90.77±0.65	9.077±0.37
HES	134.75±23.66	5.61±0.37	0.561±0.011
Conventional	252.86±32.76	10.53±0.21	1.053±0.09

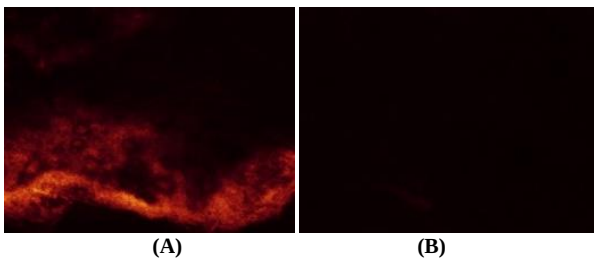
Table6. % Drug Residual in different formulation

S. No.	Formulation Code	Amount Used (mg)	Amount Remained (mg)	% Drug Permeated
1.	F-1	2	0.162	8.1
2.	F-6	2	0.154	7.7
3.	F-8	2	0.205	10.25
4.	F-11	2	0.179	8.95

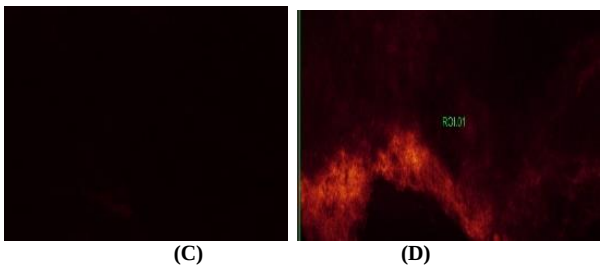
Table 7. Kinetics assessment of release profile of Quercetin loaded ethosomal Gel by using animal skin.

Formulation Code	Zero order Model R^2	First order model R^2	Higuchi order model R^2	Kosermeyer and Peppas model R^2
F 6	0.8147	0.0375	0.9414	0.1857
F 11	0.7936	0.0376	0.9315	0.1522
F 1	0.8076	0.0372	0.9029	0.1594
F 8	0.7865	0.0377	0.9099	0.1614

The transdermal flux ranged from 102.25±3.22 ($\mu g/cm^2$ /h) of F6 to 5.61±0.37 ($\mu g/cm^2$ /h) of (HES) and permeability coefficient ranged from 10.22±0.63 (cm/h) of F6 to 0.561±0.011 (cm/h) (HES) respectively. The result indicated that the flux of optimized batch F6 was 18.22 time higher then hydroethanolic solution. Results obtained from skin retention study showed that 7.7 % drug retained in skin. (Table-7)



(A) **(B)**



(C) (D)

Figure 2. Confocal laser scanning graph (A- HES at the depth of 45.23 micron), (B- F-6 at the depth of 45.23 micron), (C- HES at the depth of 10 micron), (D- F-6 at the depth of 10 micron)

CLSM was used to trace the penetration depth of fluorescence marker Rhodamine B loaded across rat skin; it was observed that it reaches up to the depth of 45.23-micron CLSM into the rat skin which was significantly higher than the Rhodamine B solution at the depth of the rat skin HES solution of Rhodamine B showed negligible fluorescence. This study proves the enhanced permeation profile of Rhodamine B loaded ethosomes into a deeper layer of the skin whereas Rhodamine B hydroethanolic solution remained confined to superficial layers.

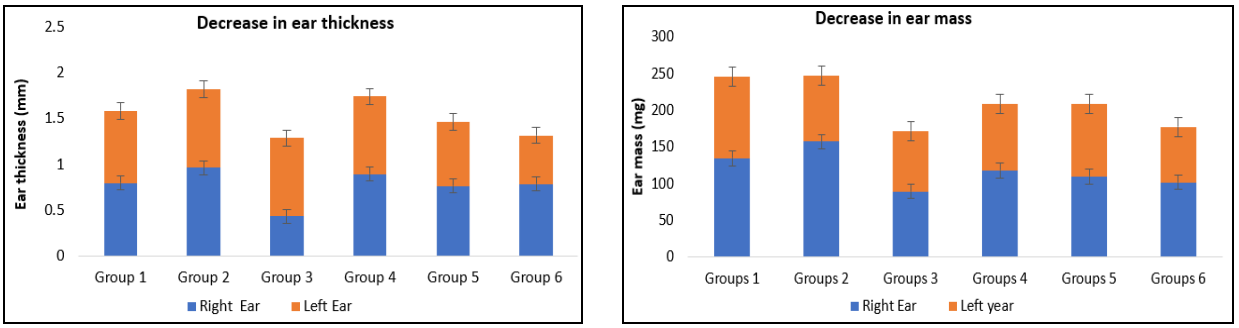
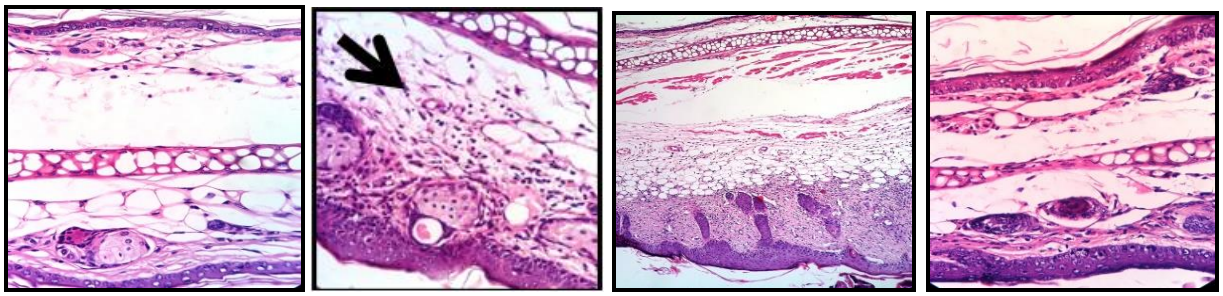


Figure 3. Bar graph Representation for an increase in ear thickness and mass

Ear swelling changes (including mass and thickness) took place in right ear lobes of DNCB induced dermatitis animal. Mass and thickness differences between left and right ear were used to evaluate the induced ear swelling with the help of digital micrometer. Ear swelling changes (including mass and thickness) were observed in right ear lobes of DNCB induced dermatitis animal. The decrease in ear thickness and mass after the last treatment has been represented as a bar graph in Figure 3. In dinitrochlorobenzene (DNCB) induced rat dermatitis model histopathology study showed a marked decrease in the amount of inflammatory cell nucleus in rat treated with quercetin loaded ethosomal 76.13% decrease in ear swelling 71.3 % ear mass respectively in morphology study. The conventional marketed formulation showed a nominal decrease in epidermal thickness, ($p>0.05$) (Figure-4)



A B C D

Figure 4. Histological examination of dermatitis (A-Control, B-Disease Control, C-Treated with Quercetin loaded nanoethosomal gel, D-Treated with marketed formulation

A: Control animals showing normal epidermis, dermis and auricular cartilage (H. and E. X 100)

B: DNCB induced animals showing both side skin of ear pinna showing marked infiltration of mononuclear cells i.e. lymphocytes in dermis and epidermal hyperplasia. H. and E.X 100

C. Animal treated with Quercetin loaded nanoethosomal gel showing one side of ear pinna whereas other side is almost normal. There was reduction infiltration of inflammatory cells i.e. lymphocytes and epidermal hypertrophy of the affected side. (H. and E.X 100)

D. Animals treated with marketed formulation showing marked epidermal hyperplasia (straight line) with moderate infiltration of inflammatory cells in dermis, edema (bold arrow) and fibrosis STANDARD H. and E.X 100

Cell Viability Assay:

The viability was conducted by the tetrazolium based colorimetric assay for the study. The mitochondrial enzyme present in the viable cell produce formazan, a

blue product by the reduction of tetrazolium salt 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). The results obtained in the dermatitis induced rat showed significant decreased of 13.77% cell viability when compared with the 0 day control rat. The marketed formulation which was used as a standard and the new formulation used as a test showed insignificant decline in the cell viability when compared to the control. Upon comparison with the dermatitis induced rat results obtained demarcate increased cell viability. The data indicate that the test formulation (F-6) used in the study has the efficacy of healing the dermatitis in the defined time without itself undergoing cellular damage. While there is no significant difference between base gel and conventional gel.

The *in vivo* anti-oxidant enzyme (Catalase) was found to be (32.30%) less in dermatitis induced rat while the treated and the standard showed remarkable increase in catalase activity as compared to control. From the above result we can conclude that the dermatitis healing capacity of the test formulation used in the study is due to antioxidants and the enzyme involved is the Catalase. While there is no significant difference between base gel and conventional gel.

2, 2 diphenyl-1-picrylhydrazyl (DPPH) is a colorimetric estimation of the total anti-oxidant activity. The results obtained indicate that the dermatitis induced rat showed a significant low (4.25 %) antioxidant capacity. Interestingly the treated and standard showed rise in DPPH value when compared is the control rat. The rise in DPPH levels upon application of the test formulation prove to be a potent healer with immerse antioxidant properties. While there is no significant difference between base gel and conventional gel.

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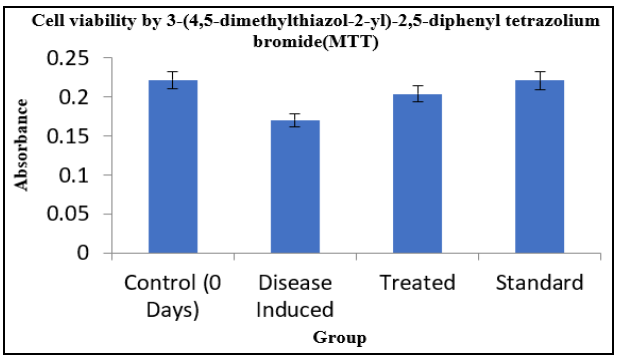

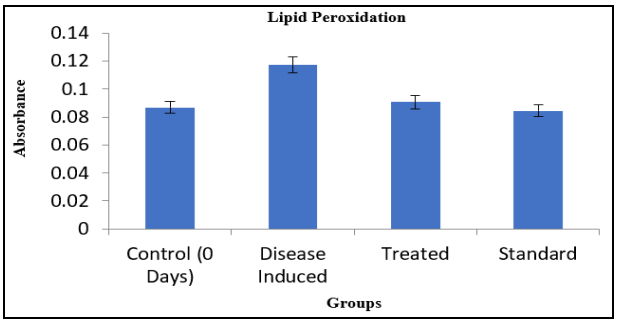

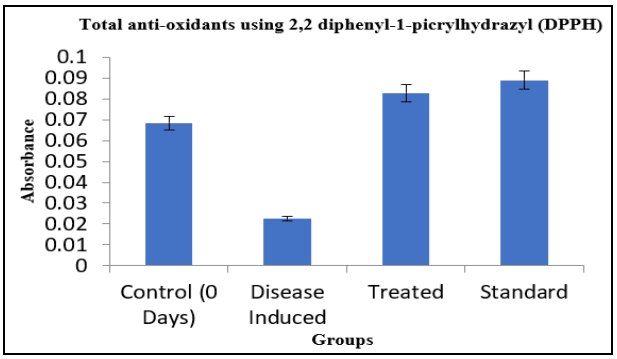

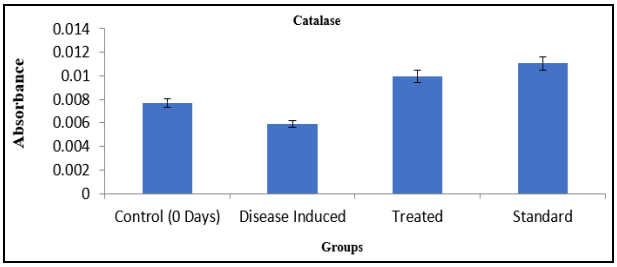

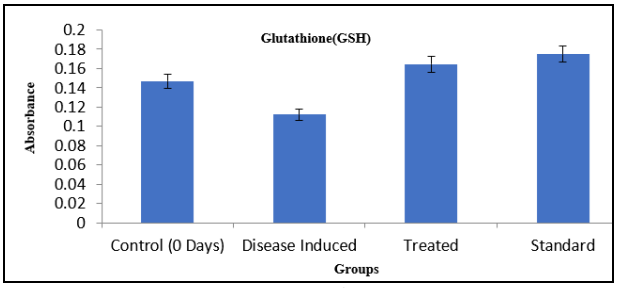

Figure 5. Bar graph representation of Cell viability assay

LIST OF ABBREVIATIONS

API – Active pharmaceutical ingredient
BCS –Biopharmaceutical classification system
FTIR – Fourier transform infrared spectroscopy
QC – Quercetin
CLSM - Confocal Laser Scanning Microscopy
TEM - Transmission Electron Microscopy
PDI - Polydispersity Index
MTT -3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
SOD - Superoxide Dismutase
DPPH - 2, 2- diphenyl-1- picrylhydrazyl
GSH - Gluthiaone
DMSO- Dimethyl Sulfoxide
PBS - Phosphate Buffer Solution

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RESEARCH ARTICLE

The duration of finishing and polishing of acrylic removable orthodontic appliances in the Dental Technology Study Program at Universitas Airlangga, Surabaya, Indonesia

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ABSTRACT:

Objective: This study was intended to quantify the duration of finishing and polishing of removable orthodontic appliances by Dental Technology Study Program students. **Material and Methods:** This descriptive observational study employed total sampling. Fifteen of 6th semester students participated in the study, each of them producing three removable orthodontic appliances with the duration of finishing and polishing being recorded. Data was analyzed descriptively. **Results:** The duration of the finishing and polishing of orthodontic plates by Dental Technology Study Program students varied widely from 9 to 420 minutes with a mean of 71.20 minutes, both between individual students and between the first, second and third orthodontic plates. **Conclusion:** It is concluded that the duration of finishing and polishing in the fabrication of an orthodontic plate by the students was between 9 and 420 minutes. The duration varied depending on the design of the appliances, the skill of the students in processing or when performing the finishing and polishing.

KEYWORDS: Orthodontic appliances, Finishing, Polishing, Acrylic dust, Dental technician.

INTRODUCTION:

Removable orthodontic appliances consist of active components, retentive components and acrylic plates[1]. The baseplate performs several functions, such as perpetuating the strength of the active component to anchorage, blocking unwanted tooth shift and protecting the palatal spring. It can be modified to produce anterior and posterior bite planes[2]. Since ages, polymethyl methacrylate (PMMA) has been used in dentistry[3]. Acrylic orthodontic plates are generally made from cold cured acrylic material. Compared with the heat cured variety, cold cured acrylic is more economical and less time-consuming. Cold cured acrylic consists of monomer liquid that consists of methyl methacrylate (MMA) and polymer powder that consist of PMMA[4]. MMA monomer is flammable, colourless and transparent with a sharp and pervasive odour. Various case studies have reported that MMA causes lung, skin and eye irritation[5].

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Dental technicians can potentially be exposed to harmful substances, one possible risk being caused through the inhaling of dust that may contain toxic substances[6]. Dust is routinely produced during the process of making removable orthodontic appliances. After the acrylic has set, finishing and polishing with the abrasive material is carried out. The resulting dust can endanger health, pneumoconiosis being one example of this risk[7].

Dental technicians need to exercise caution in anticipating the potential negative impact of their contact with dust by minimizing its duration. Research into the degree of exposure to dust that a dental technician might experience when manufacturing a removable orthodontic appliance, especially when finishing and polishing have yet to be undertaken. The study reported here was conducted to quantify the required duration of finishing and polishing involved in the manufacture of a removable orthodontic appliance.

MATERIAL AND METHODS:

Ethical Aspects:

This study was approved by Universitas Airlangga Faculty of Dental Medicine Health Research Ethical

Clearance Commission (Certificate No: 043/HRECCFODM/V/2018). The subjects were explained about the procedure and asked the informed consent regarding the commencement of this study.

Study Design and Sample:

This descriptive observational study was conducted between March and May 2018 at the Dental Technology Study Program, Universitas Airlangga, Surabaya. The inclusion criteria were orthodontic acrylic plates in any designs made by sixth-semester students of the Dental Technology Study Program. Total sampling was employed, and a group of 15 students participated in the study, each of whom produced three orthodontic acrylic plates. The exclusion criteria were the acrylic plates that broke from processing stage or not an orthodontic plate.

Data collection:

Acrylic processing was conducted after the application of springs and clasps to the dental cast. After that, the model was soaked in water for approximately ten minutes before the separation material (Meliosep, Germany) was applied to the surface of the dental cast. This was allowed to dry before the acrylic processing, involving the use of cold cured acrylic material (Ortho Resin, UK) was carried out with layering or sprinkle technique. At this point, the model was put into a polyclav (Remanium Dentaurum, Germany) filled with water heated to a temperature of 130F / 50℃ at a pressure of two standard atmospheres and left for 30 minutes (6). The acrylic plate was subsequently removed from the dental model and made ready for finishing. The finishing time was calculated through a stopwatch (Casio, Japan). Finishing was performed using a handpiece and micromotor (Saeshin Strong 204, South Korea) incorporating a bur for use on an acrylic material. Finishing was undertaken to reduce the excessively large acrylic area, with the acrylic plate being smoothed with sandpaper.

Data Analysis:

The polishing process involved the use of a slurry pumice cone and a polishing machine (Silfradent, Italy), followed by brushing with kryte to produce a shiny plate. The finishing and polishing times were recorded. In cases where the finishing and polishing process was not carried out continuously, the interruption was not considered. Data were collected and tabulated by Microsoft Excel software and analyzed descriptively to calculate the mean value.

RESULT:

The duration of the finishing and polishing of removable orthodontic plates is shown in Table 1. From table 1 it appears that the time required for finishing and polishing 1st, 2nd and 3rd plate have a big variety and also among all students. The average finishing and polishing time for 3 orthodontic plates was 71.2 minutes. The fastest time was obtained by the 14th student with 9 minutes, while the longest was the 10th student, 420 minutes. of the 1st, 2nd and 3rd plate, the fastest was achieved by the 13th student with a time of 18.35 minutes and the longest was by the 10th students with a time of 217.67 minutes.

Table I. Duration of finishing and polishing of removable orthodontic plates (minutes)

Student	1st plate	2nd plate	3rd plate	Mean (x)
1	21	27	25	24.33
2	65	115	45	75
3	38	90	20	49.33
4	55	34	73	54
5	41	180	54	91.67
6	33	25	20	26
7	35	30	25	30
8	45	35	45	41.67
9	117	123	90	110
10	420	120	113	217.67
11	131	103	43	93.33
12	70	50	145	88.33
13	15	25	15	18.33
14	9	19	48	25.33
15	121	125	123	123

DISCUSSION:

Work is an integral part of everyday life[8] . Producing product in scheduled time is important to satisfy customer needs[9] and time management is needed to enhance the productivity[10]. Before insertion into the mouth of the patient, the orthodontic plate must be well-polished. Rough surfaces will render it uncomfortable, while an increase in plaque accumulation and biofilm formation will reduce the sheen of the acrylic plate[11]. Orthodontic appliances are made from self-cured acrylic with higher porosity and greater surface roughness[12]. In order to obtain a smooth and shiny acrylic plate, both finishing and polishing are performed. While acrylic polishing can be carried out chemically, the disadvantages of this method include a reduction in mechanical resistance compared to mechanical polishing, adverse effects on resin resistance and structure and a greater degree of deformation[13]. Therefore, the performance of the procedure on self-cured acrylic plates is contraindicated[14]. Research also demonstrated that mechanical polishing produces a smoother surface than its chemical equivalent[15,16].

Safety is an issue of basic significance in all fields [17] and environmental awareness is a matter of concern[18]. The mechanical finishing and polishing of acrylic plate produce dust that can endanger health. Based on the results of this study, the duration of a dental technician's contact with dust when finishing and polishing an orthodontic acrylic plate varies between 9 and 420 minutes. In fact, the calculation of a meantime cannot be considered absolute due to differences in the design of

appliances and operators, although the latter had received the same training in the manufacture of a removable orthodontic appliance.

The results indicated that the duration of the finishing and polishing process performed by dental technicians varied. In the case of the first student, the duration of finishing and polishing was 21, 27 and 25 minutes. The time required is relatively short and stable with an average duration of 24 minutes. This relatively short period can be achieved by the skillfulness of the operator performing the finishing and polishing processes. Besides, acrylic processing was conducted effectively with the result that the amount of excess acrylic which increases the finishing time was minimized.

The second student recorded 65, 115 and 45 minutes. These results fluctuated, probably because each job has a different degree of difficulty. In the second acrylic plate, a longer duration of 115 minutes was required. In this case, an error occurred in the installation of the maxillary Coffin spring which lacked symmetry around the median line and whose repair prolonged the finishing time.

With the third student, the duration of finishing and polishing of the second acrylic plate was longer due to the addition of a maxillary Coffin spring. A design incorporating Coffin springs prolongs the finishing and polishing process because it is completed twice, once before and once after the Coffin spring has been installed. In the case of the fifth student, the second acrylic plate underwent extremely lengthy (180 minutes) finishing and polishing. This is because, after polishing, the acrylic plate did not fit into the working model (rocking) and, consequently, had to be remade.

Concerning the ninth student, the average duration of 110 minutes was probably due to the lack of involvement of skilled dental technicians in the finishing and polishing process. For the tenth student, the first plate required 420 minutes. This maximum duration was caused by several factors, including the acrylic plate being of such thickness that it required a protracted finishing time or it being so thin after finishing that the acrylic process had to be repeated. The mean duration of finishing and polishing in the case of the tenth student was the highest of all the samples (217 and 67 minutes). It would appear that this student was less skilled in completing the finishing and polishing process causing it to require a relatively long time.

The thirteenth student recorded the fastest times with an average of 18 and 33 minutes. Due to the skill of the operator, the acrylic processing was completed effectively, efficiently and carefully with the result that the finishing and polishing process could be undertaken more easily. The shortest time was achieved in the case of the fourteenth student, the first acrylic plate requiring only nine minutes. This relatively brief duration was achieved because the procedure was performed on the lower jaw which is comparatively smaller than the upper jaw.

The results of this study indicate that several factors influence the duration of the finishing and polishing of acrylic plates, such as appliance design and operator skills. The addition of the maxillary Coffin springs prolongs the finishing and polishing process which is divided into two phases: after the acrylic plate has been rendered smooth and shiny, the Coffin springs are added while finishing and polishing is performed on the embedded part.

Dental laboratory air has a high level of pollution due to grinding dust, especially extraordinarily fine, invisible particles capable of penetrating the lungs and causing severe damage [19]. The potential risk is one of inhaling dust during finishing and polishing. This physical hazards can affect the overall health of workers[20]. Preventive measures can be taken by providing a workspace of an optimal standard. Dental laboratories must be spacious, clean, have efficient air ventilation and a separate work table for finishing and polishing [21,22].

During finishing and polishing, dental technicians should wear respirable dust masks preventing the inhalation of acrylic dust. The majority of dental technicians persist in using surgical masks even though they prove less useful for respirable dust whose particles are sufficiently small to penetrate them. Also, the use of gloves and glasses is also essential in order to achieve optimal protection [23,24].

It was concluded that the duration of finishing and polishing in the manufacture of an orthodontic acrylic plate by Dental Technology Study Program students ranged between 9 and 420 minutes. The duration varied according to appliance design and the skill of the students when processing or completing the finishing and polishing. Precautions, skill, the speed of working and personal protective equipment are required to minimize the danger of dust causing respiratory diseases.

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RESEARCH ARTICLE

An assessment of cytotoxic potentiality of *Aloe barbadensis* on the root meristem cells of *Trigonella foenum-graecum* L.

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ABSTRACT:
The leaves of *Aloe barbadensis* are used in traditional and modern systems of medicine. The aim of this study was to evaluate the cytotoxic potentialities of aqueous solution of *Aloe barbadensis* leaves on *Trigonella foenum-graecum* root tip meristem cells using a cytogenetic approach. Treatments with various concentrations of *Aloe barbadensis* leaf extract to *Trigonella foenum-graecum* root meristem cells showed mito-inhibition and induced several chromosomal aberrations as chromsomal breakage, fragmentation, scattering, stickiness etc.

KEYWORDS: *Aloe barbadensis*, *Trigonella foenum-graecum*, Chromosomal aberrations, Mito-inhibition.

INTRODUCTION:

The virtues of ***Aloe barbadensis*** (Syn. *vera* **Family: Liliaceae)** have been recorded for thousands of years by many ancient civilizations, including Egypt, Persia, Greece, India and Africa. The name was derived from the Arabic alloen meaning 'bitter' because of the bitter liquid found in the leaves. In 1500 B.C. Egyptians recorded use of the herbal plant in treating burns, infections and parasites. The aloe plant has long (upto 20 inches long and 5 inches wide), triangular, fleshy leaves that have spikes along the edges. The fresh parenchymal gel from the center of the leaf is clear. The sticky latex liquid is derived from the yellowish green pericyclic tubules that line the leaf. This is the part that yields laxative anthraquinones. *Aloe vera* leaf contains major Glycosides – Anthracene derivatives: Hydroxyanthraquinone derivatives (25-40) viz., aloin (= barbaloin, a mixture of aloin A and B, the disstereoisomeric 10-C glucosides of aloe-emodin anthraone) and 7-hydroxyaloin isomers. Minor contain includes aloe emodin, chrysophanol, chromone derivatives viz., aloeresin B (= aloesin, upto 30%) with p-coumaryl derivatives aloeresins A and C and the aglycone aloesone. The active principle of aloe is a mixture of glycosides called aloin.

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Numerous aloe species around the world are used for conditions ranging from dermatitis to cancer. *Aloe* gel's greatest use is as a skin salve and vulnerary for minor burns, canker sores and epithelial injuries. *Aloe* latex is a potent laxative and it should not be used during pregnancy. However, allergic reactions to aloe have been reported. Long term use of anthraquinone laxatives may results in laxative dependence, dehydration, potassium depletion and weakness. The cytotoxicity of *Aloe vera* gel and their results confirmed that *Aloe vera* gel contained low molecular wt. toxic compounds[1]. Cytotoxicity and genotoxicity of *Aloe* was also analysed[2]. In *vitro* study of free radical scavenging activity of flavonoids of *Aloe vera* was done[3]. In *vivo* and *in vitro* investigation of cytotoxic and antitumor activities of polyphenolic leaf extract of *Hibiscus sabdariffa* against breast cancer cell lines[4] and antimicrobial and anticancer activities of Banana leaves (*Musa acuminata*) and Olive leaves (*Olea europaea* L.) as by-products was carried out[5].

MATERIAL AND METHODS:

To obtain Stock solution, 1ml gel of *Aloe vera* is dissolved in 1000ml of distilled water. The required ascending concentrations of 100 ppm, 250 ppm, 500 ppm, 750 ppm and 1000 ppm were prepared by serial dilution method from Stock solution. The *Trigonella foenum-graecum* seeds were grown in sterile moist sand at 25°C to 30°C for obtaining the roots. First of all, roots were cut without any treatment to find control value of

mitotic index for *Trigonella foenum-graecum* root meristem.

The roots were then treated with different concentrations of *Aloe vera* leaf gel aqueous solution for two hours, four hours and six hours and after that roots were cut carefully and then fixed in Carnoy's solution (3 ethanol: 1 Acetic acid) for 24 hrs. and transferred to 70% alcohol for preservation. The root tips had been hydrolyzed in 1N HCl for 2-3 minutes and squashed in 2% acetocarmine for cytological studies. The slides were temporarily sealed and examined. The mitotic index had been calculated[6]. Chromosomal aberrations and their percentage in each treatment were recorded. Chromosomal aberrations were photographed microscopically. To examine recovery, treated roots were left in distilled water for 24 hrs. and mitotic index was recorded.

RESULTS:

All the used concentrations of aqueous leaf extract of *A. barbadensis* inhibited the mitotic activity of *T. foenum-graecum* (2n=16) root meristem. The percentage value of mitotic index decreased gradually with an increase in concentration and duration of the treatment (Table 1). The observed mitotic index for *T. foenum-graecum* root tip cells in controlled condition was 19.43%. When the root tips were treated with 100 ppm aqueous extract, the mitotic index declined to 18.05% (2 hours), 17.52% (4 hours) and 16.08% (6 hours). The percentage of mitotic index further decreased to 16.89% (2 hours), 16.00% (4 hours) and 14.90% (6 hours) by the treatment of 250 ppm concentration of aqueous leaf extract of medicinal plant. The treatment of 500 ppm maintained the declining trend in percentage value of mitotic index and recorded mitotic index was 15.77% (2 hours), 14.97% (4 hours) and 13.87% (6 hours). Inhibition of mitotic index continued in 750 ppm treatment which was 14.52% (2 hours), 13.94% (4 hours) and 12.83% (6 hours). The treatment of 1000 ppm of aqueous solution of *Aloe* leaf extract showed maximum inhibition in mitotic index with percentage value of 13.37% (2 hours), 12.91% (4 hours) and 11.11% (6 hours).

An increase in the mean percent value of recovered mitotic index was observed after giving a recovery period of 24 hours duration to the treated *T. foenum-graecum* root tips in all the used concentrations. Minimum recovery mitotic index was recorded in 2 hours treatment of 500 ppm concentration and maximum recovery in mitotic index was noticed in 2 hours treatment of 750 aqueous concentration of *A. barbadensis* leaf extract (Table 1).

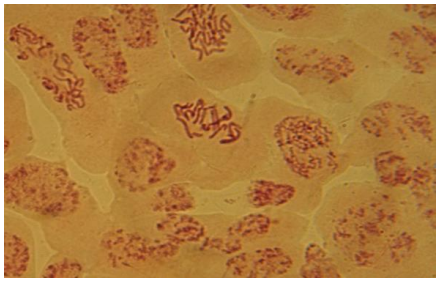
Chromosomal aberrations induced in treated root tip cells of *T. foenum-graecum* have been shown in Table 1 and Photoplate-1(A-E). At interphase and prophase, no nuclear or chromosomal aberration was recorded.

The anomalies observed at metaphase were breakage of chromosome, disturbed metaphase, chromosomal extrusion, fragmentation, polyploidy, scattering and stickiness of chromosomes, out of which breakage and scattering of chromosomes were recorded in all the used concentrations during the course of study. The mean percentage values of chromosomal breaks observed were 1.11% (100 ppm), 2.38% (250 ppm), 2.35% (500 ppm), 2.50% (750 ppm) and 1.26% (1000 ppm). Disturbed metaphase (Photoplate-1A) was noticed during 100 ppm and 250 treatments only with mean percent value of 1.11% and 1.19%, respectively. Chromosomal extrusion was found during treatments by all aqueous concentration of *Aloe* leaf extract except 250 ppm with mean percent value of 2.22% (100 ppm), which declined to 1.17% in 500 ppm, but increased to 1.25% (750 ppm) and 1.26% (1000 ppm). Fragmentation of chromosome (Photoplate-1B) was caused only by treatments with higher concentrations and percent value of fragmentation recorded was 1.25% (750 ppm) which increased upto 2.53% in 1000 ppm treated mitotic cells. Polyploidy (Photoplate-1C) was induced only during treatments by lower concentrations with percent value of 1.11% (100 ppm) and 1.19% (250 ppm). was found to be most common with mean percent value of 2.22% (100 ppm) which declined to 1.19% (250 ppm) and 1.17% (500 ppm) but raised to 2.50% (750 ppm) and 2.53% (1000 ppm). Stickiness of chromosomes was not observed during 100 ppm and 500 ppm treatments. The mean percent values of stickiness recorded were 2.38% (250 ppm), 1.25% (750 ppm) and 1.26% (1000 ppm).

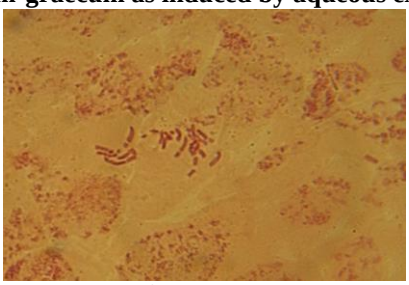
Chromosomal extrusion (Photoplate-1E) was only aberration observed at anaphase Scattering of chromosomes (Photoplate-1D)in root tip cells treated with all the used aqueous concentrations of *Aloe* leaf extract except 100 ppm. The mean percent values found to be were 5.12% (250 ppm), 2.22% (500 ppm), 2.32% (750 ppm) and 2.12% (1000 ppm). During treatments no abnormality was found at telophase.

PHOTOPLATE-1:

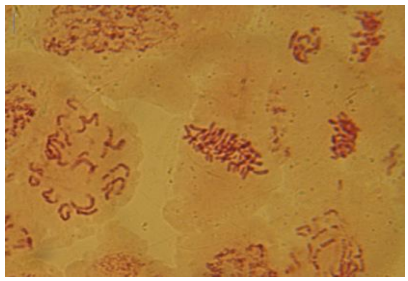
Chromosomal aberrations in T. foenum-graecum as induced by aqueous extract of Aloe barbadensis leaf.



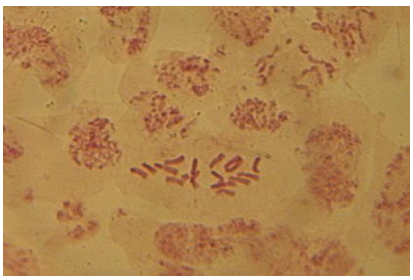
Disturbed metaphase (Photoplate-1A)



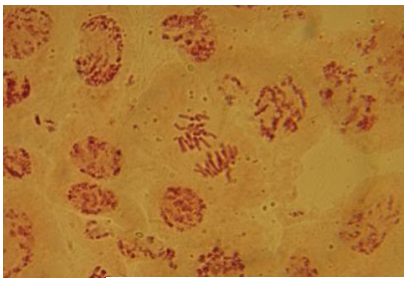
Fragmentation of chromosome (Photoplate-1B)



Polyploidy (Photoplate-1C)



Scattering of chromosomes (Photoplate-1D)



Chromosomal extrusion (Photoplate-1E)

Table 1: Mitotic index, frequency of aberrations and their percentage as induced by aqueous solution of *Aloe barbadensis* leaf extract on *Trigonella foenum-graecum* (2n =16)

Concentration	**100 ppm**				**250 ppm**			
Duration (in hrs)	**2**	**4**	**6**	**% of abr. (Conc wise)**	**2**	**4**	**6**	**% of abr. (Conc wise)**
Mitotic index(in %)	18.05+2.13	17.52+1.86	16.08+1.2		16.89+3.27	16.00+1.76	14.90+1.36	
Recovered Mitotic Index (in %)	18.73	18.61	17.40		17.55	17.14	15.88	
Types of aberrations	-	-	Br 'M'	1.11	Br 'M'	-	Br 'M'	2.38
	-	-	Dst 'M'	1.11	-	-	Dst 'M'	1.19
	-	Ex 'M'	-	2.22	-	-	-	-
	-	-	-	-	-	-	-	-
	-	Poly 'M'	-	1.11	-	Poly 'M'	-	1.19
	Sc 'M'	-	-	2.22	Sc 'M'	-	-	1.19
	-	-	-	-	-	Stk 'M'	-	2.38
	-	-	-	-	-	Ex 'A'	Ex 'A'	5.12

Table 1 continued

Concentration	**500 ppm**				**750 ppm**				**1000 ppm**				**Control**
Duration (in hrs)	**2**	**4**	**6**	**% of abr. (Conc wise)**	**2**	**4**	**6**	**% of abr. (Conc wise)**	**2**	**4**	**6**	**% of abr. (Conc wise)**	
Mitotic index (in %)	15.77 +1.36	14.97 +2.09	13.87 +2.09		14.52 +1.51	13.94 +2.20	12.83 +1.89		13.37 +2.87	12.91 +1.74	11.11 +1.72		19.43
Recovered Mitotic Index (in %)	16.23	16.11	15.29		16.20	15.28	14.58		14.50	13.91	12.06		
Types of aberrations	Br 'M'	-	Br 'M'	2.35	Br 'M'	Br 'M'	-	2.50	-	Br 'M'	-	1.26	No **aberration**
	-	-	-	-	-	-	-	-	-	-	-	-	
	-	-	Ex 'M'	1.17	Ex 'M'	-	-	1.25	Ex 'M'	-	-	1.26	
	-	-	-	-	-	-	Fr 'M'	1.25	-	-	Fr 'M'	2.53	

	-	-	-	-	-	-	-	-	-	-	-	-	
	Sc 'M'	-	-	1.17	Sc 'M'	-	Sc 'M'	2.50	Sc 'M'	-	Sc 'M'	2.53	
	-	-	-	-	Stk 'M'	-	-	1.25	-	Stk 'M'	-	1.26	
	-	-	Ex 'A'	2.22	-	-	Ex 'A'	2.32	Ex 'A'	-	-	2.12	

Abbreviations – Br 'M'- Breakage of chromosomes at metaphase, Dst 'M' – Disturbed metaphase, Ex 'M' - Chromosomal extrusion at metaphase, Fr 'M' – Fragmentation of chromosomes at metaphase, Poly 'M' – Polyploidy at metaphase, Sc 'M' - Scattered metaphase, Stk 'M' - Stickiness of chromosomes at metaphase, Ex 'A' - Chromosomal extrusion at anaphase.

DISCUSSION:

Two parameters have been employed in the present investigation to evaluate cytotoxic potentiality of medicinal plant *Aloe barbadensis* leaf extract. The first parameter dealt with the effect of aqueous extracts of undertaken medicinal plant *Aloe barbadensis* on mitotic index of *Trigonella* root cells. Whereas the second parameter comprises of aberrated behaviour of chromosomes and their respective structural changes during mitosis, when treated with aqueous leaf extract of selected medicinal plant.

All the concentrations (100 ppm, 250 ppm, 500 ppm, 750 ppm and 1000 ppm) of *Aloe barbadensis* leaf extract were found to be mitodepressive in all the treatments given to *Trigonella* root meristems.

Mitotic activity is a fundamental mechanism of the growth and development of eukaryotic organisms and is affected by some internal or external factors. The process of mitosis can be influenced by external factors such as plant extracts, alkaloids and other synthetic chemicals. It has been suggested that reduction in mitotic index might be due to reduction in the synthesis of either protein or DNA or both[7]. The process of mitosis might be inhibited due to the blockage of DNA synthesis or due to inhibition of spindle formation[8].

The chromosomes in their number and morphology are constant and specific in each and every species. Any structural or numerical alteration in chromosomal constitution of an organism or cell may cause an imbalance in genetic architecture of the cell. Generally, these changes are not expressed in the normal morphology and physiology of the organism as most of the chromosomal changes are not potential mutations. Some of the structural changes in chromosomes may affect germ cells and pass on to next generation through sexual reproduction.

Aqueous leaf extract of *Aloe barbadensis* was found to be potentially capable of inducing chromosomal breakage, fragmentation, stickiness and scattering, polymerization of chromosomes at metaphase and disturbed metaphase and chromosomal extrusion at anaphase in *Trigonella* root meristem cells. Chromosomal breakage has been observed in *V. faba* root tip chromosomes treated with some medicinal plant extracts[9] and also in *Trigonella* root meristem cells when treated with PEG6000[10]. Stickiness and fragments of chromosomes were found in root meristem cells of *Vicia faba* treated with *Plantago* husk[11] and in *Allium cepa* root tip cells treated by Orthosiphon[12]. Chromosomal breaks are induced in all treatments in a dose dependent manner except 1000 treatment, but fragments are observed maximally in1000 ppm treatment. Scattering of chromosomes was observed in cultured cells of *Hemerocallis* hybrid[13]. Sticky chromosomes were found while analyzing dose response of medicinal plant *Viola odorata* on meiotic and mitotic chromosomes of *Trigonella*[14]. Sticky chromosomes, fragments and anaphasic bridges were found while assessing mitodepressive and clastogenic effects of aqueous extracts of the lichens *Myelochroa lindmanii* and *Canoparmelia texana* (Lecanorales,Parmeliaceae) on meristematic cells in plant bioassays[15]. Stickiness usually leads to the formation of anaphase and telophase bridges and this end up inhibiting metaphase and cytokinesis respectively and thus hampering cell division. Stickiness might be due to the ability of the extracts to cause DNA depolymerization and partial dissolution of nucleoproteins, breakage and exchanges of the basic folded units of chromatids and the stripling of the protein covering of DNA in chromosomes[16].

Polyploidy plays an important role in plant evolution and constitutes an important mechanism of diversification and creation of genetic variability. Polyploidy induction was carried out in Tunisian *Trigonella foenum-greaum* L. populations.[17]

In *vitro* cytotoxic studies on methanolic leaf extract of *Mussaenda erythrophylla* Schumach and Thonn was done and extract was found to be cytotoxic against all the cell lines under investigation. However, the extract was found to be more potent and effective against A-549 (Human Lung cancer cell lines) with IC50 value 30.68 µg/ml[18]. The study revealed that ethanolic extract of whole plant of *Andrographis echioides* L. exhibited apoptotic activity that in turn induced cytotoxicity in HT-29 colon cancer cell lines[19] and *S. hispida* is effective against free radical mediated diseases[20]. Evaluation of antioxidant and cytotoxic potential of different extracts from the leaves of *Aegle marmelos* L.[21], antioxidant activity of aqueous extract of *Barleria*

mysorensis and *Furcraea foetida* leaves[22] and of methanolic extract of *Boerhaavia diffusa* L had been conducted[23.]

The chromosomal aberrations have been accepted as indicator of genetic damage[24] or mutagenecity detected through the ability to induce aberration has been suggested as a reliable and sensitive test for biologically active substances [25,26,27,28]. The investigator has an opinion that the present study will definitely open new approaches for research.

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RESEARCH ARTICLE

The hidden danger in Syria: Silent Celiac Disease

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ABSTRACT:
Celiac disease (CD) is a disorder caused by an aberrant autoimmune response to gluten ingestion in genetically susceptible individuals. Its prevalence nears 1%, and it is not considered typical in the Middle East because of the lack of reports in the Arabic countries. Therefore, this study aimed to define the prevalence of celiac disease in the Syrian community. Healthy young adults and volunteers ranging between 5–65-year-old were tested from December 2018 till May 2019. Samples from the participant's sera were tested for anti-tissue transglutaminase antibody (tTG) IgA/IgG by ELISA using the human recombinant transglutaminase antigen. One hundred participants with a mean ± SD of 22 ± 14 years-old were recruited in the study. Only one participant was suspected of having celiac disease. The mean ± SD of anti-tTG IgA/IgG values were 9.81 ± 9.61 for IgA and 6.64 ± 6.60 for IgG. The study shows no significant difference in IgA or IgG titers between males and females at P = 0.7, P = 0.8, respectively. The estimation of seroprevalence of CD antibodies in a healthy Syrian population is close to 1%. We suggest that the underdiagnosed CD might pose a high risk in Syria, contrary to previous thoughts.

KEYWORDS: Celiac disease, Tissue transglutaminase, diagnosis, Gluten.

1. INTRODUCTION:
Celiac disease (CD) is a chronic inflammatory disease affecting the upper small intestine principally and is triggered by intolerance to the gluten in genetically predisposed persons. Gluten is a protein found in wheat consumed mainly in Western countries, nearing an average intake of 10 to 20grams/person/day[1-4]. Prolamin and glutelin proteins are the main components of gluten. Both contain glutamine and proline residues abundantly, which promote deamination during digestion through tissue transglutaminase (tTG) enzyme[5].

This action leads to inflammation of the mucosa and villous atrophy and poor assimilation consequently. The well-known manifestations of CD are often signs and symptoms linked to malabsorption. Including diarrhea, steatorrhea, and loss of weight or growth failure. It has been recognized that TNF-α and IL-6 are upregulated in CD which have a proinflammatory role aggravating inflammation in intestine[6-12] and other tissues[13-24]. The reveal of tissue transglutaminase (TTG) as the antigen triggering autoantibodies production in coeliac disease has given serological tests a more critical role in diagnosing this condition, such as considering histopathological confirmation of CD no longer mandatory in the 2012 ESPGHAN criteria for coeliac disease in children[25]. However, this approach was reinforced in the 2020 ESPGHAN criteria[26-29]. However, testing for IgA anti-TTG is well accepted as the standard test for coeliac disease worldwide. In addition, these tests reflect good sensitivity and specificity.

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The clinical picture of CD varies from overt malabsorption to a silent form, and it is believed that there may be more than five undiagnosed patients for each diagnosed patient[30]. This has led to the concept of a "celiac iceberg," wherein the majority of the patients have silent or latent CD. Screening studies in children have revealed that the silent form is more frequent. It was found that 8 patients with CD; 3 of them presented with atypical CD and 5 with silent CD[31]. Patients with untreated CD are at risk of diverse complications, including osteoporosis, infertility, lymphoma, and other autoimmune disorders, such as diabetes mellitus and thyroiditis[32,33]. Treatment with a gluten-free diet precludes most of these complications. This raises the importance of early diagnosis and treatment, especially in subjects with silent CD. It is impossible to identify asymptomatic patients without screening. This study indicates that despite improved recognition rate of diagnosis, there are still asymptomatic patients who remain undiagnosed. Awareness should be increased, especially for individuals who are at high risk for disease development.

2. MATERIALS AND METHODS:

2.1 Patients:

This study was a cross-sectional seroprevalence study of CD among a total of 100 people for celiac disease. The study included males and females with age ranged 5- 65 years old and attended private hospitals and clinics in Syria during the period between December 2018 - May 2019. A questionnaire was used to get information about the patients' health.

2.2 Sample collection:

Five milliliters of venous blood were collected from all the subjects under good aseptic precautions using disposable latex gloves and syringes. The collected blood samples were left to clot naturally in the tubes at room temperature. They were then separated by centrifugation at 1500 x g for 10 minutes to be used for the serological tests. All samples were labeled by a serial number and the person's name, then the samples were immediately frozen at - 20ºC. For each subject a questionnaire was obtained that included information about the patient's name, contact address, age, gender, education status, knowledge about CD, whether previously diagnosed as CD patient, or if the presence of any 1st degree relatives diagnosed with CD, as well as information about the presence of any history of chronic diarrhea, anemia, abdominal complains, a hepatic disease, diabetes, tumors and thyroid anomalies.

2.3 Ethical Aspects:

Ethical approval was obtained from the Research Ethics Committee of the University of Damascus. Each patient agreeing to participate in the study signed a consent form after fully explaining to them the study's objectives and the expected benefits from participating in it. A parental approval was obtained for minor participants. Healthy subjects with serologically confirmed celiac disease were informed that they have silent celiac disease and were advised to reveal this information to their physicians. Moreover, an overview of the disease was given to every volunteer diagnosed as having a silent celiac disease to make him/her familiar with the different symptoms of the disease.

2.4 Anti-tissue Transglutaminase IgA and IgG antibodies:

This test was carried out to detect the Anti-Tissue Transglutaminase antibodies type IgA and IgG by "ELISA." The test was done while adhering to the instructions of Manufacture's Company (Diametra / Italy). This Elisa Kit provides Quantitative measurement of IgA/IgG class antibodies against tissue transglutaminase in the human serum or plasma. Anti-Tissue Transglutaminase IgA/IgG test depends on binding of serum or plasma antibodies coating the human recombinant tissue transglutaminase into the microplates. The antibodies in calibrators, controls or pre-diluted patient samples bind to the inner surface of the wells. Thirty minutes of incubation was allowed, the microplate is splashed by wash buffer to remove non-reactive serum constituents. An anti-Human-IgA/IgG horseradish peroxidase conjugate solution recognizes IgA/IgG class antibodies bound to the immobilized antigens. After that, a chromogenic substrate solution that contains TMB is dispensed into the wells. After 15 minutes of incubation a halting solution is added to stop the color development. The color of the solution changes into yellow, and the amount of color is directly proportional to the concentration of IgA/IgG antibodies present in the original sample.

2.5 Statistical analysis:

Data are presented with mean ± standard deviation. Spearman rank was used. Statistical tests were performed utilizing Graph Pad Prism version 5.0 (Graph Pad Software, Inc., San Diego, CA). A P-value < 0.05 was considered a significant result.

3. RESULTS:

A total of 100 subjects who came to private hospitals and clinics compromised of both sexes; males (n=51) and females (n=49), and containing both children younger than 18 years old (n=47) and older or equal to 18 years old (n=53). Collection of samples was carried out from September 2018 to May 2019. The age range of participants was 5-65 years with. The distribution of subjects into six age groups according to gender showed that the majority was between 5-15 years (43%). The groups had females more than males except for the 16-25 years age group (Figure 1).

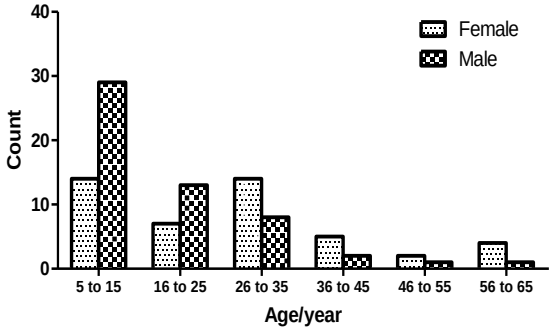

Figure 1: The distribution of Syrian nationals according to age

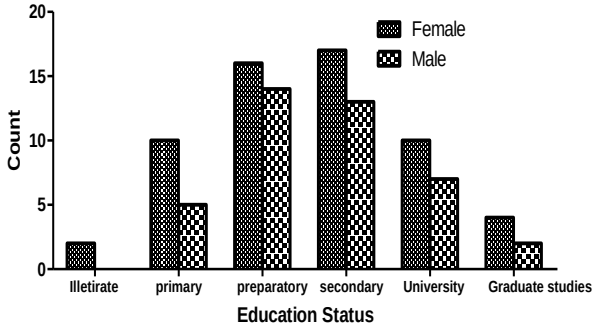

Figure 2: The distribution of Syrian nationals according to educational status

Table 1: Distribution of age and education according to gender among sampled subjects

Demographic characteristic	Frequency%			X^{2*}	P-value
Age	**Total**	**Male**	**Female**	9.8	0.0017
5 to 15	43(43%)	29	14		
16 to 25	20(20%)	13	7		
26 to 35	22(22%)	8	14		
36 to 45	7(7%)	2	5		
46 to 55	3(3%)	1	2		
56 to 65	5(5%)	1	4		
Illiterate or read and write	2(100%)	0(0%)	2(0%)	0.14	0.7
Primary	15(15%)	5(5%)	10(10%)		
Preparatory	30(30%)	14(14%)	16(16%)		
Secondary	30(30%)	13(13%)	17(17%)		
University	17(17%)	7(7%)	10(10%)		
Graduate studies	6(6%)	2(2%)	4(4%)		

* X^2 value is for linear –by-linear association

According to education status, the distribution of subjects illustrates that the proportion of females who finished university education was slightly higher than those who did not finish their studies after high school. However, it was not significant compared to males (Figure 2).

The distribution of CD according to age was significantly different between females and males X^2= 9.8, P=0.0017. Whereas for the education status X^2= 0.14, P=0.7 (Table 1).

Serological Results:

One subject (1 %) was positive for tTG Ig A/tTG Ig G. In contrast, the remainder 99 (99 %) were clear cut negatives for tTG IgA/tTG Ig G. The mean ± SD for anti-tissue trans-glutaminase Ig A is 9.81±9.61 Au/Ml while the mean ± SD for anti-tissue transglutaminase Ig G is 6.64±6.60 AU/ml. Our results showed a weak but significant correlation between age and IgA or IgG titers, (Rho=0.2 P=0.03), (Rho= 0.3 P=0.001), respectively. According to the gender aspect, there was a weak, but significant correlation between IgA and IgG titers, Rho= 0.3, P=0.049 for men and Rho=0.36, P=0.007 for women. No difference in IgA or IgG titers between males and females P=0.7, P=0.8 respectively.

4. DISCUSSION:

Over the previous years, the prevalence of CD has intensely amplified in many countries, encouraging an increased burden on humanity and healthcare organizations. Once regarded as a condition manifesting mainly in children, CD has appeared as a common diagnosis in adults. The prevalence of CD has been extensively studied through Europe, North America, and Oceania. In contrast, studies on the frequency of CD lack from Africa, Asia, Latin America, and the Middle East. Future epidemiological studies are warranted in these nations to evaluate the worldwide epidemiology of the disease broadly. The overview of noninvasive and accurate serological tests for CD near the end of the 20th century made the diagnosis of CD more well-organized and cost-effective. Diagnosis strategies for CD have also shifted over time[34-36], leading to increased diagnoses. For example, the European Society for Paediatric Gastroenterology Hepatology and Nutrition guidelines now permit serological diagnoses of CD without biopsy validation in children who meet certain clinical, genetic, and serologic criteria. As observed in Southeast Scotland, United Kingdom, 56% of children in 2016 were diagnosed without biopsy[37]. Increased recognition of CD by physicians has led to assessing patients with "nonclassical" presentations (i.e., extraintestinal symptoms). Screening strategies have also been adopted

to recognize CD in asymptomatic individuals belonging to groups hypothetically at-risk such as those with type 1 diabetes, autism, or first-degree relatives with CD[38]. This may be reproduced in the significant increase of celiac-specific serologies requested in healthcare settings. A recent meta-analysis estimated the prevalence of CD to be 0.6% in Asia, 0.5% in Africa, and 0.4% in South America[39]. Global epidemiology studies have documented the quickly growing frequency of inflammatory bowel disease in several regions. An analogous rise in the occurrence of CD is probably arising as countries in these areas are embracing more industrialized societies.

Some studies in the Middle East regarding the diagnosis of CD cases didn't include a large population number. To our knowledge, there was only one published study on CD prevalence in Syria. This study was a cross-sectional seroprevalence of CD among Syrian individuals who were undergoing medical examination. The target population consisted of adolescents and children only (males and females) who were asymptomatic at sampling time. The choice of this group of subjects in our study was adequate as detecting silent CD in Syrian population was the aim of the study; since effortful persuasion to join the study wasn't needed as these healthy subjects came to the medical centers for medical laboratory examinations in the first place. The slight dominance of male participants is explained by female's refusal to participate in the study more than males. According to this study1:100 among Syrian individuals, CD prevalence is higher compared to the prevalence reported in the general population of Western Europe. The majority in Denmark was 1 : 3 30, Finland 1 : 1 30, Germany 1 : 500, Italy 1 : 1 84 and Netherland 1 : 1 98[40]. This high prevalence among Syrian nationals emphasizes the accuracy of the term CD iceberg, which points to the many CD cases left undetected and undiagnosed in the population. Celiac disease prevalence in Syria is found to be 1%. This estimation challenges the previous belief about celiac disease being an uncommon diagnosis in Syria. This requires the enhancement of the awareness about celiac disease among the community as well as among doctors. This finding entails a trial and a chance to shed attention and awareness about celiac disease to Syria. There are two main explanations for the low prevalence reported in some regions; the ignorance about the disease is the main one. The other important reason is the low consumption of gluten (which does not trigger an autoimmune response if not consumed by high titers). A third possible explanation is the improvement in the quantity and quality of the processing of cereal. In conclusion as long as novel clues regarding the causes of the CD come out, the views on the epidemiology of CD will keep changing, calling for new plans to aid in the management and prevention of CD globally.

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AUTHORS' CONTRIBUTIONS:
A.A. wrote the first draft of manuscript. S.S., I.A., edited and commented on the manuscript. All authors read and approved the final manuscript.

COMPETING INTERESTS:
The authors declare that they have no competing interests.

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RESEARCH ARTICLE

Indirect Electrochemical Determination of Chlorpropamide Through Its Interaction with Valsartan Using Square Wave Voltammetry

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ABSTRACT:
A square wave voltammetric technique coupled with three electrode detection system consist of hanging mercury drop electrode (HMDE) as working electrode, 1mm platinum wire as an auxiliary electrode (Pt-wire) and silver/silver chloride saturated potassium chloride (Ag/AgCl.sat.KCl) as reference electrode was used to determine the chlorpropamide indirectly through its interaction with valsartan, chlorpropamide gives no reduction peaks in the studied range. The effect of pH and the stability of the measurement were examined calibrations curve of chlorpropamide was constructed and the relation between current and concentration of chlorpropamide was linear with R^2 value = 0.9944. The limit of detection for chlorpropamide was 4.89 x 10-9 M through its interaction with valsartan.

KEYWORDS: Chlorpropamide, Valsartan, Interaction, Square Wave Voltammetry.

INTRODUCTION:
Chlorpropamide 1-[(p-chlorophenyl)sulfonyl]-3-propylurea (Fig. 1). It is a drug in the sulfonylurea class used to treat diabetes mellitus type two[1]. Its mechanism of action involves the stimulation of insulin release from the β-cells of the pancreas in response to a glucose load[2].

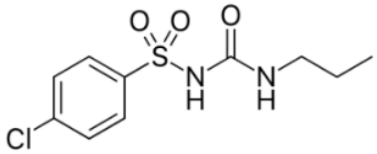

Fig. 1: Chemical structure of Chlorpropamide

The chlorpropamide was determination by several methods including high performance liquid chromatography (HPLC)[3,4,5]. A spectrophotometric method is used by British Pharmacopeia[6], Spectrophotometric[7], titrimetric[8], thin layer chromatography[9,10], gas chromatography[11,12].

Charge-transfer complexes are formed by the interaction between electron donors and electron acceptors[13].

Valsartan is chemically N-(1-Oxopentyl)-N-[[2'-(1H-tetrazol5-yl) [1, 1'-biphenyl]-4-yl] methyl]-L-valine (Fig. 2), it is an angiotensin receptor blocker, has been widely used for the treatment of hypertension[14].

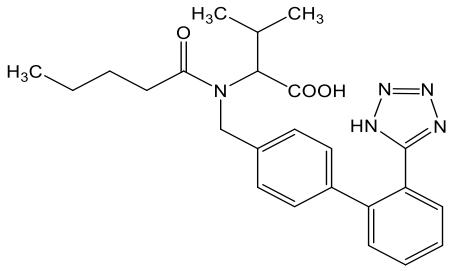

Fig. 2: Chemical structure of Valsartan

Several methods used to determination of valsartan such as UV–Spectrophotometric[15], Liquid Chromatography-Mass Spectrometry[16], High Performance Liquid Chromatography with Fluorescence Detection[17], High Performance Thin Layer Chromatographic[18].

Electrochemical methods used to study behaviour of many materials[19,20], mercury electrode have been studied electrochemical behaviour for other materials[21,22] and

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the interactions studied by electrochemical methods on dropping mercury electrode[23].

Indirect determination of drugs is preferable because most of them free from interference so in this work we tried to determine chlorpropamide indirectly by its interaction with valsartan, this interaction can be synergistic through the increase or enhance the activity of drug, so the interaction can leads to overdose effect if patient take more than one drug and one of them increase the effect of the others. In the other hand if one drug decrease the activity of the other drug it may cause to have any therapeutic use because of under dosage[24,25,26].

In our previous work, valsartan gives stable well-defined reduction peak at (-1.07) V versus Ag/AgCl.sat.KCl on HMDE working electrode in phosphate buffer (pH=7.0) supporting electrolyte[27].

MATERIAL AND METHODS:

Chemicals and reagents:

All chemicals used were analytical grade (Fluka, BDH) and used without purification. The pure chlorpropamide and valsartan were kindly supplied by Sammira drugs industry. Stock solution of each drugs were prepared by dissolving an appropriate amount of chlorpropamide and valsartan in absolute ethanol. The supporting electrolyte was phosphate buffer (mixed appropriate amount of dipotassium hydrogen phosphate (K2HPO4) and potassium dihydrogen phosphate (KH2PO4).

The procedure of measurement involves the place of buffer solution in polarographic cell and the oxygen was removed by passing nitrogen gas for 5min prior the measurements, then the polarogram was recoded for known concentration of valsartan (9.8 x 10^{-4} M) then the sequence addition of chlorpropamide were added and then the polarogram was recorded for each addition under the optimum condition and the calibration curve was constructed.

Instrumentation:

All the electrochemical measurements were performed using a 797 polarographic analyzer computrace supplied by Metrohm, Switzerland, coupled with a three-electrodes cell, HMDE as working electrode, 1mm Pt-wire as an auxiliary electrode and Ag/AgCl.sat.KCl as reference electrode.
A digital pH-meter model pH 211 supplied by HANNA company, Portugal, was used for pH-measuring.

RESULTS AND DISCUTION:

Valsartan gives well-defined stable reduction peak at (-1.07) V versus Ag/AgCl.sat.KCl reference electrode, the effect of pH was examined, calibration curve was constructed at the optimum conditions[27].

Chlorpropamide has no reduction peak at the studied potential range so a suggested method for its indirect determination through its interaction with valsartan will be convenient for analytical purpose.

Interactions of valsartan with chlorpropamide:

A polarogram of 9.9 x 10^{-5} M valsartan was recorded under its optimum conditions, the polarograms were recorded for a sequence-additions of chlorpropamide stock solution (10^{-3} M) in phosphate buffer (pH=7) as supporting electrolyte.
The reduction peak current of valsartan decreases with increasing additions of chlorpropamide (Fig. 3).

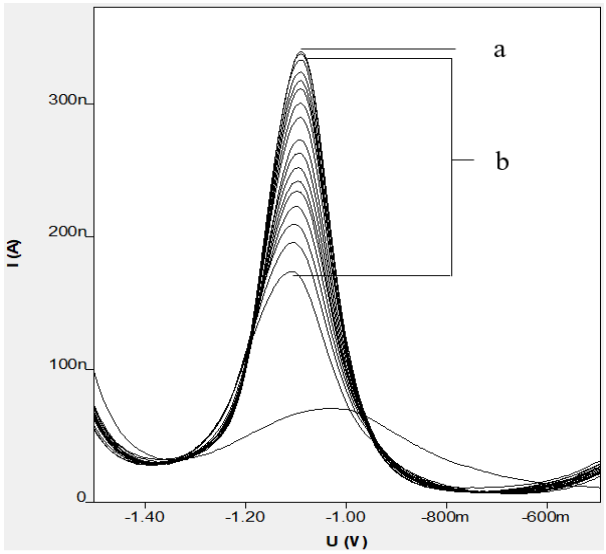

Fig. 3: The reduction peak of valsartan (9.9x10^{-4} M) (a) with the sequence additions of chlorpropamide (10^{-3} M) (b)

The relation between reduction peak current of valsartan and chlorpropamide added concentrations in the studied concentration range was linear with R^2 value = 0.9663 (Fig. 4).

The linearity of calibration curve suggest a method for the quantitation analysis of chlorpropamide.

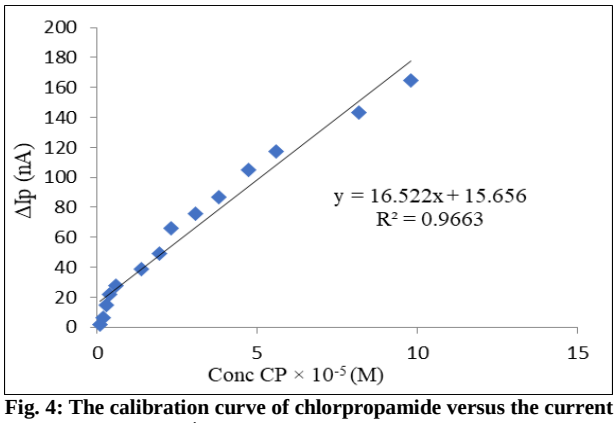

Fig. 4: The calibration curve of chlorpropamide versus the current of valsartan (9.9x10^{-4} M) through interaction with chlorpropamide (stock solution 10^{-3} M)

Stability of interaction

To study the stability of interaction peak a voltammogram of 9.8×10^{-4}M valsartan with 3.9×10^{-6}M chlorpropamide was recorded under the mentioned optimum conditions of valsartan in phosphate buffer pH=7 versus time, the results obtained are shown in table 1, it is clear that the interaction reduction peak current is stable within the time studied.

Table 1: Stability of interaction reduction peak current (9.8×10^{-4}M valsartan with 3.9×10^{-6}M chlorpropamide) using phosphate buffer pH=7

Time (min)	Ep of interaction (V)	Ip of interaction (nA)
0	-1.1	309
5	-1.1	307
10	-1.1	307
15	-1.1	308
20	-1.1	309
25	-1.1	309
30	-1.1	308
35	-1.1	306
40	-1.1	305
45	-1.1	305
50	-1.1	306
55	-1.1	306
60	-1.1	305

Binding constant:

Binding constant was calculated according to following equation[28]:

$$ln(Ip/(Ip°\text{-}Ip)) = ln(1/([CP])) - lnK \ldots\ldots\ldots\ldots\ldots\ldots\ldots (1)$$

where K is the binding constant.
Ip° : the reduction peak currents of the free valsartan.
Ip : the reduction peak currents of VAL-Chlorpropamide complex.

A plot of ln(Ip/(Ip°-Ip)) versus ln(1/[CP]) gives a linear relationship with R^2 value = 0.9635, the intercept represent the binding constant which equal 8.8851 M^{-1} (Fig. 5).

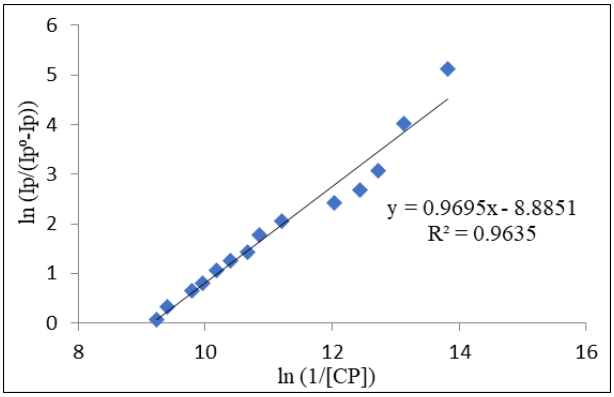

Fig.5: Plot of ln (Ip/(Ip° –Ip)) vs ln (1/[CP]) in high concentration

Calibration curve at low concentration:

To obtain lower detection limit of chlorpropamide through its interaction with valsartan, a sequence-additions of chlorpropamide (stock solution 10^{-5} M) were added to the polarographic cell containing 9.9 x 10^{-6} M valsartan and the polarograms were recorded under the optimum conditions (Fig. 6).

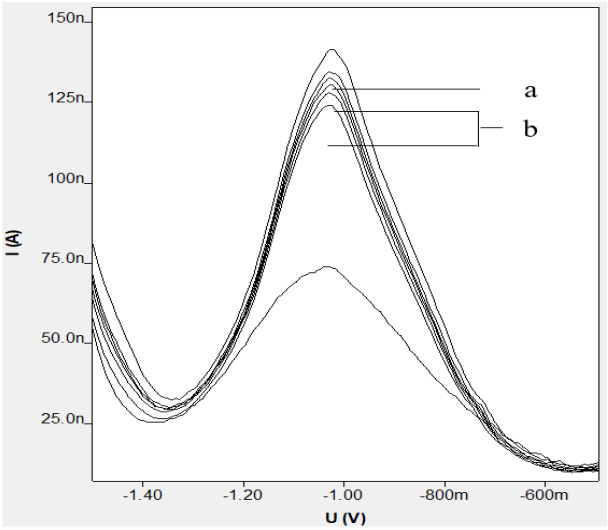

Fig. 6: The reduction peak of valsartan (9.9 x 10^{-6} M) (a) with the sequence additions of chlorpropamide (10^{-5} M) (b)

The results obtained are shown in figure 7 and figure 8.

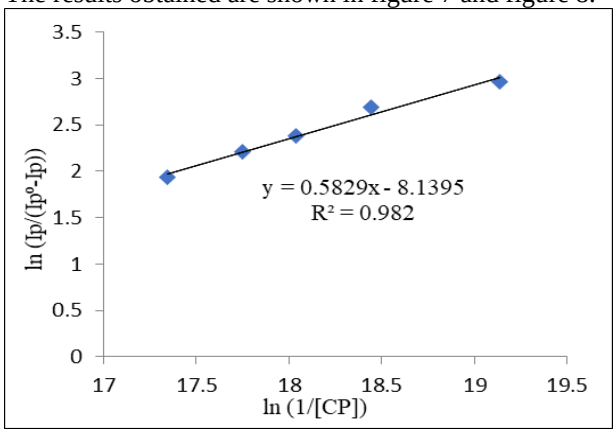

Fig.7: Plot of ln (Ip/(Ip° –Ip)) vs ln (1/[CP]) in low concentration

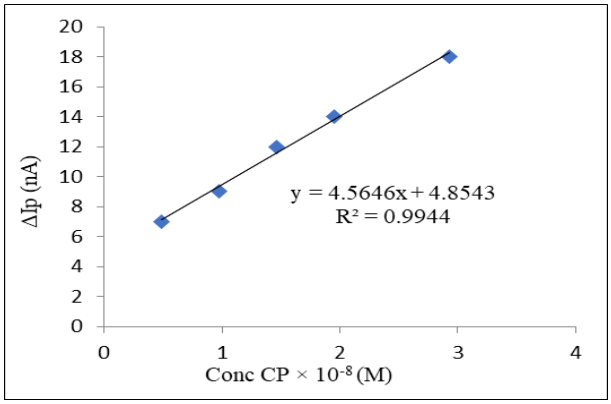

Fig. 8: The calibration curve of chlorpropamide versus the current of valsartan (9.9 x 10^{-6} M) through interaction with chlorpropamide (stock solution 10^{-5} M)

The detection limit of chlorpropamide was $4.89x10^{-9}$ M.

CONCLUSION:

The chlorpropamide can be indirectly determine by its interaction with valsartan. The suggest method was simple, sensitive and accurate, so through our suggested method we reached low concentrations of chlorpropamide ($4.89x10^{-9}$ M).

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RESEARCH ARTICLE

Screening plants extracts for Antifungal activity against *Rhizoctonia solani*

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ABSTRACT:

The adverse effect of pesticides used for controlling pests and the diseases caused by them is extensively rising. To overcome this harmful environmental impact, alternative methods are being studied and developed. Out of many possible methods, one effective method is to use plant extracts which embodies natural substances having antifungal properties. In an attempt towards enhancement of sustainable antifungal approach, four different concentrations of four plant extracts *Citrus limon*, *Azadirachta indica*, *Ocimum gratissimum* and *Acalypha indica*, were tested for their antifungal activity against *Rhizoctonia solani*, which is a serious threat for plants, and is responsible for considerable crop and yield losses. The aim of this study is to minimize yield losses and to regain the attention of formers towards exploiting natural resources for diseases control. From the results, it can be concluded that different concentrations of plant extracts have caused substantial inhibition in the mycelial growth of *R. solani*. The leaf extract of *Ocimum gratissimum* was highly effective in inhibiting mycelial growth, at the concentration of 500mg/ml of extract followed by *Citrus limon*, *Azadirachta indica*, and *Acalypha indica*. These plants might have potential for the development of natural fungicide for the management of diseases caused by fungal pathogens. Also, fungicides developed from these botanical extracts can prove to be highly effective for the management of plant diseases and would be simply obtainable, nonpolluting, biodegradable and economical.

KEYWORDS: Antifungal activity, disk diffusion method, medicinal plants, rhizoctonia solani, zones of inhibition.

1. INTRODUCTION:

Plants are infected by an extensive range of pathogens among which fungi are considered to instigate varieties of rot diseases. Many pathogens including *Rhizoctonia solani*, one of the most recognized species of genus *Rhizoctonia* is responsible for the destruction of crops worldwide, reducing the market values of crops and making them unsuitable for our consumption.

R. solani belongs to phylum *Basidiomycota* is a plant pathogenic fungus with wide host range including economically important agricultural crops such as rice, wheet, tomato, potato and pepper etc.[1-6]. Chemical fungicides are frequently used for fungal rots and are very common for the management of these diseases. But the use of these compounds has ensued the debut of new pathogens which are resilient to these fungicides and are also adverse for human health and natural environment. Consequently, plant beneficial microbes and plant extracts shown attractive features of inhibiting growth of plant pathogens. Among the bacterial groups, *Pseudomonas sp.* and *Bacillus sp*. are demonstrated as most potent fungal inhibitors[7,8,9]. In the other side, the extracts, which are obtained from the plants are thought to be extra adequate and less precarious as compared to

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the manufactured compounds and hence can be used as a substitute for antifungal compounds. Extracts obtained from numerous botanicals have gotten immense recognition and also gained scientific attention for their microbial activity such as antibacterial, antiviral, and antifungal activity.

Solvent extraction is the most preferred one for the extraction of secondary metabolites from plants and also successfully demonstrated for antimicrobial compounds. Methanolic extracts from *Ammannia baccifera Linn*.[10] and ethanolic extract from *Centella asiatica*[11] shows growth inhibition for human pathogen *Candida albicans*. Some of Family Fabaceae plant's methanolic extract were also shows good inhibition against *Aspergillus niger*[12]. Similarly, ethanolic extract of *Cleome rutidosperma*[13], *Cyclamen hederifolum*[14] and *Wattakaka volubilis*[15] were demonstrated as a potent source of antifungal compounds. Curcumin shows inhibitory as well as synergistic effect with known antifungal drugs against *Candida albicans*[16]. Antibacterial activity of *C.limon*[17], *Azadirachta indic*[18], *Ocimum gratissimum*[19 20] and *Acalypha indica*[21,22] were found to be a good source of antimicrobial agents against various pathogens. In the present study, considering the tremendous future of plants as sources for antimicrobial medicine pertaining to bactericidal and antifungal agents, a scientific investigation was initiated to screen the native flora for antifungal activity from *Citrus limon, Azadirachta indica, Ocimum gratissimum* and *Acalypha indica* against *R. solani* which is one of the major pathogenic fungi infecting wide range of plants.

2. MATERIALS AND METHODS:

2.1 Sample Collection:

Fresh leaves of Citrus *Citrus limon, Azadirachta indica, Ocimum gratissimum* and *Acalypha indica* were collected from Potheri, Chengalpattu district, Tamil nadu. The samples were collected in separate plastic bags on sunny days in order to avoid any moisture content on the samples.

2.2 Microbial culture and growth conditions:

The fungal culture of *R. solani* MTCC4633 was ordered from (Microbial Type Culture Collection and Gene Bank), CSIR Institute of Microbial Technology, Chandigarh. The fungus was grown on Potato Dextrose Agar (PDA) medium at room temperature and was maintained with periodic sub culturing.

2.3 Preparation of plant extracts:

The disease-free healthy leaves were selected and washed from all the sides with running tap water to take off the dust particles. After washing with tap water for three times, and once with sterile water, the leaves were kept for shade drying for two weeks. The dried leaves were powdered using an electric blender and stored in different zip lock covers. Ten grams of leaf powder was dissolved in 100ml of methanol, in 250ml conical flasks for each plant sample. The samples were kept in an upright position in the shaker incubator for 48 hours, maintained at room temperature at 120rpm. The solvent was changed after 24 hours for effective extraction. After running for 48 hours, the colorless solvent was filtered through Whatman No. 1 filter paper, to get the supernatant. The supernatant was collected in separate petri plates and was allowed to air dry for solvent to evaporate. Then the extract was stored in the eppendorf tubes in -20 degrees for further analysis of antifungal activity[18].

2.4 Assessment of Antifungal activity:

Antifungal activity of the extracts was screened by disk diffusion method. The disk diffusion test also known as agar diffusion test, or Kirby–Bauer test is a test of the antibiotic sensitivity of bacteria or fungus (Magaldi et al., 2004). DMSO was used as negative control and the solution of clotrimazole and lignocaine hydrochloride was used as a positive control. Different concentrations of plant extracts were dissolved in DMSO to different concentrations such as 50mg/ml, 100mg/ml, 200mg/ml and 500mg/ml for each plant sample. Discs were impregnated with 25µl of plant extracts from each concentration using micropipette. Then the discs were left to dry for drying at RT for 2 to 3hrs. In a PDA plate, mycelium of fungus was placed in the center of the plate. Then, the impregnated discs containing the diffused extracts were placed separately on the PDA plate. The plates were incubated for 48 hours at RT. After the incubation, the plates were examined for clear zone of inhibition in the vicinity of the disc and recorded[18].

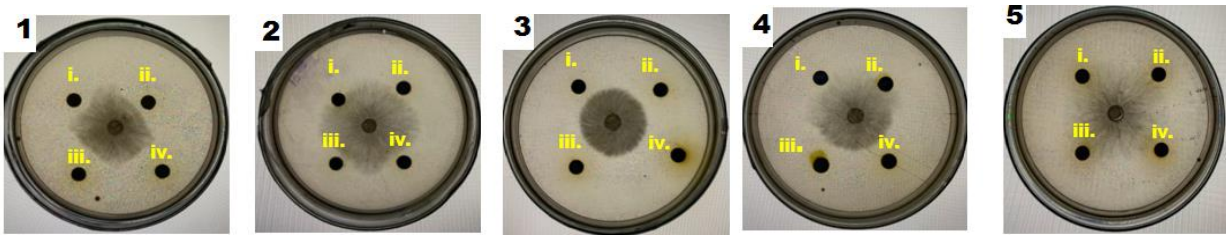
3. RESULTS AND DISCUSSION:

For antifungal screening of plant extracts from four plants belonging to different families as mentioned were evaluated against *R. solani*, one of the most pathogenic fungus responsible for major crop loss worldwide. The plant leaf samples for selected plants were collected, air dried and powdered. The powder were extracted with ethanol for bioactive compounds against *R. solani*. Among the 4 plants screened, *Ocimum gratissimum* and Citrus Limon shows better inhibition. Zone of inhibition by the plant extracts were observed after 48 hours and measured using ruler (Table 1). It was observed that different concentration of extracts showed substantial inhibition of fungus. After screening diffusates for their antifungal potential, *Ocimum gratissimum* appeared the most effective inhibition in radial mycelial growth of pathogen and followed by *Citrus limon* (Fig. 1). The other plant extracts were found to be less effective.

These report were supported by the studies of *O. gratissimum* to have antimicrobial, antifungal[23], and the hexane fraction of this inhibits dermatophytes[24]. Similarly, the other species of *Ocimum* also reported as a potent source of a antibacterial and antifungal compounds[25,26].

Table 1: Zone of inhibition of plant extracts against R. solani

Name of the plants	Zone of inhibition(mm)			
	50 mg/ml	***100 mg/ml***	***200 mg/ml***	***500 mg/ml***
Citrus limon	-	4.0	5.8	7.5
Azadirachta indica	-	-	3.0	5.0
Ocimum gratissimum	3.0	6.0	7.0	9.0
Acalypha indica	-	-	4.0	5.0



i—50mg/ml ; ii—1000mg/ml; iii—2000mg/ml; iv—5000mg/ml

Fig. 1. Disk diffusion method results after 48 hours of incubation. 1. *Citrus limon* 2. *Azadirachta indica* 3. *Ocmium gratissimum* 4. *Acalypha indica and* 5. Contro (DMSO) . Four different concentration like i. 50mg/ml, ii. 100mg/ml, iii. 200mg/ml and iv. 500mg/ml were used.

Other plants selected for the present study *C.limon* were also demonstrated to posses potent medicinal property. Ethanolic extract of *C.limon* were studied against *Candida glabrata*, *Aspergillus niger*, *Saccharomyces cerevisiae* and *Candida parapsilosis*[27,28,29,30] The bark and leaf extract of *Azadirachta indica* were demonstrated to antibacterial activity[31]. It is used as traditional medicine for treating several diseases like infections of the upper tract, cold, flu and pneumonia. In a previous study, It was also demonstrated that the aqueous extracts of neem cake shows inhbition against sporulation of *C. lunata*, *H. pennisetti*, and *C. gloeosporioides* f. sp. *Mangiferae*[32] and methonolic extract inhibit *Aspergillus flavus*, *Alternaria solani*, and *Cladosporium*[33] *A. indica* contain acalyphin and also flavonoids, and the extracts of this herb are frequently utilized asthma and pneumonia[34].

4. CONCLUSIONS:

The results of this study have shown that the methanol extracts of *Citrus limon*, *Azadirachta indica*, *Ocimum gratissimum*, and *Acalypha indica* have boundless possibility as antimicrobial agents in the management of risky and destructive pathogens. The study unconcealed that escalating the concentration of botanical diffusates lessens the mycelial growth of fungal pathogenic agents radially when performed in vitro. The plant extracts are more adequate and less risky as compared to the manufactured compounds and hence might be used as a substitute for manmade antifungal compounds. The results from this initial research to look at the potential use of these botanical diffusates for controlling the diseases induced by *R. solani* perceived to be encouraging with regards the mycelial growth of the plant. Additional detailed investigation of the bioactive constituents of such plants for similar mechanism of action will support the development of new complementary and reasonable antifungal chemicals to a great extent which will be economical and highly effective in reduction of crop losses worldwide.

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Knowledge, Perception, and Belief on Antibiotic Use in The Community of Panyabungan, Mandailing Natal District, Indonesia

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ABSTRACT:
Antibiotics are organic compounds produced by certain microorganisms and toxic to the other microorganisms. Irrational antibiotic use can cause serious health problems, especially bacterial resistance to antibiotics. This study aimed to determine the extent of knowledge, perception and belief on antibiotic use in Panyabungankota Subdistrict Mandailing Natal District. This study used cross-sectional design, total sample 198 people taken by purposive random sampling. Research data retrieval time during December 2017. In this study the questionnare used consisted of four sections they are demographic, knowledge, perceptions and belief. Analysis of the data used are univariate and bivariate analysis with chi-square method. From the result of the research, it was found that the public knowledge regarding antibiotic use was 37.9% belongs to good category, whereas society perception regarding antibiotic use was 69.7% belong to enough category and belief regarding antibiotic use was 74.7% belong to enough category. The result of statistical test showed significant relationship among public knowledge of antibiotic use and the characteristics of sex, age, last education, and work, p<0.1. The result of statistical test showed no significant relationship among perception and belief of antibiotic use and the characteristics of sex, age, last education, and work, p>0.1. and the result showed significant relationship between knowledge and beliefs, p<0.1, the result showed no significant relationship between knowledge and perception, p>0.1. and the result showed significant relationship between perception and beliefs, p<0,1. Based on the results of this study it can be concluded, that knowledge, perceptions and belief of antibiotic use still need to be improved.

KEYWORDS: Antibiotic, Belief, Knowledge, Perception, Resistance.

INTRODUCTION:

Antibiotics are substances produced by a microbe, especially a fungus, which can inhibit or kill other microbes. Antibiotics are essential drugs and can be used to eradicate various infectious diseasese[1]. Antibiotics are one of the most frequently prescribed, sold, and used drugs worldwide. Frequent use of antibiotics inappropriate results in increased germ resistance to antibiotics[2]. Antibiotic resistance has become a problem worldwide.

According to the Minister of Health of the Republic of Indonesia[3], Indonesia was ranked 8th out of 27 countries with a high burden of drug resistance to Multidrug Resistance (MDR) in the world based on World Health Organization (WHO) 2009 data.

Research conducted by Lim and Teh in Putrajaya, Malaysia, states that 83% of respondents do not know that antibiotics do not work to fight viral infections, and 82% of respondents do not know that antibiotics cannot treat coughs and flu[3]. Some respondents' statements included stopping the use of antibiotics when the symptoms improved and taking fewer antibiotics than the doctor prescribed would be healthier than taking all the antibiotics prescribed[4].

Research conducted by Widayati in Yogyakarta stated that around 71% do not have Moderately precise knowledge about the use of antibiotics, more than half

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believe antibiotics can prevent the disease from getting worse (74%). Low than half believe that antibiotics do not affect. Another study conducted in Lithuania stated that more than 61.1% of respondents had low knowledge of antibiotics[6]. Nearly half of respondents considered antibiotics to be effective against viral infections (26%) or bacterial and viral infections (21.7%). As many as 47.7% felt the common cold was an appropriate indication for antibiotic use[7].

Based on the results of a previous study conducted in Jordan taken from a random sample of 1,141 adults, 67.1% believed that antibiotics treat colds and coughs. 28.1% of antibiotics were abused as analgesics. A total of 11.9% of the women showed knowledge that antibiotics during pregnancy and breastfeeding are safe for consumption, and 55.6% used them as prophylaxis against infection. 49.0% used antibiotics without consulting a doctor, while 51.8% used antibiotics based on relative recommendations. Also, 22.9% of physicians prescribe antibiotics by telephone, and ≥ 50.0% routinely prescribe antibiotics to treat common cold symptoms[8].

Public misconceptions about using antibiotics can potentially lead to inappropriate treatment, where people believe antibiotics are "extraordinary drugs" or "powerful drugs" that can prevent and cure every symptom or disease. Knowledge and belief are factors that can influence individual antibiotic use behavior. By itself, knowledge is not Moderate to change behavior but plays a vital role in shaping opinions and attitudes. The consequences of using antibiotics with Low experience have the potential to lead to misunderstandings about their use. Given that the inappropriate use of antibiotics in the community continues to be a problem in every country, the provision of knowledge information related to the correct use of antibiotics is enforced. However, the condition of such information is still relatively rare, especially in Indonesia.

MATERIAL AND METHODS:

Type of Research:

This type of research is a descriptive study with a cross-sectional method supported by primary data, in the form of data obtained directly through filling out questionnaires answered by respondents.

Time, Place, Population and Sampel of Research:

The research was conducted in December 2017. The location of the research data collection was located in Panyabungan District, Mandailing Natal Regency, Indonesia. Through filling out questionnaires by respondents directly. Panyabungan Kota sub-district consists of 14 villages and seven sub-districts with a population of 15,117 people and total of sampel 198 people.

Evaluation of Knowledge, Perception, Belief:

Evaluation of Knowledge:

In the assessment of the knowledge, there are 14 (fourteen) question questions; each correct answer on the questionnaire is given a value of 2, wrong answers are given a value of 0, and not knowing is given a value of 0. The measurement scale for knowledge can be categorized:

a. good, if the value of the respondent is 22-28 (> 75%)
b. Moderate, if the respondent's value is 15-21 (50% - 75%)
c. Low, if the respondent's value is 0 - 15 (<50%)

Evaluation of Perception:

In the perception assessment consisting of 5 statements, each statement strongly disagrees given a value of 5, disagree is given a value of 4, is not sure that it is given a value of 3, agrees to be given a value of 2, and strongly agrees will be given a value of 1. The measurement scale for perception can be categorized:

a. good, if the respondent's value is 20-25 (> 75%)
b. enough, if the respondent's value is 12-19 (50% - 75%)
c. Low, if the respondent's value is 0 - 11 (<50%)

Evaluation of Belief:

In the trust assessment consisting of 4 statements, each statement strongly disagree given a value of 5, disagree is given a value of 4, is not sure that it is given a value of 3, agrees is given a value of 2, and strongly agrees is given a value of 1, the measurement scale for trust can be categorized:

a. good, if the value of the respondent is 16-20 (> 75%)
b. Moderate, if the respondent's value is 10 - 15 (50% - 75%)
c. Low, if the respondent's value is 0 - 9 (<50%)

Validity and Reliability:

The validity test is used to measure whether a questionnaire is valid or not; a questionnaire is declared valid if the questionnaire's questions can reveal something that will be measured by the questionnaire. A reliability test is a tool for measuring a questionnaire, which is an indicator of a variable. A questionnaire is reliable or reliable if a person's answer to a statement is consistent from time to time. To find out whether a variable is reliable or not, a statistical test is carried out by looking at the Wilcoxon rank test value, where the reliability requirement is p value> α (0.05)[9].

Analysis of Data:

This bivariate analysis is used to determine the relationship between the independent variable and the dependent variable. In this study, bivariate analysis was carried out by using the Chi-square test, where the test conditions have been met in the research data. If the p-value <0.1, it can be concluded that there is a

relationship between the variables studied. Meanwhile, if the p value> 0.1 means that the statistical calculation results are not significant or there is no significant relationship between the variables studied.

RESULT AND DISCUSSION:

Demographics of Respondents

This research was conducted in Panyabungan Kota District, Mandailing Natal Regency. With demographic data of respondents consisting of gender, age, education, and occupation. The following is an illustration of the frequency distribution of the characteristics of the respondents in (Table 1).

A total of 198 respondents were involved in this study. Characteristics of respondents based on gender were women as many as 121 respondents (61.1%) while men were 77 respondents (38.8%). Based on the age, 91 respondents (45.9%) were aged between 19-30. Based on the most recent education, 60 respondents with high school education (30.3). Complete data can be seen in Table 1.

Respondents' Knowledge Level About Antibiotics:

For testing the level of public knowledge of usage antibiotics made several knowledge questions consisting of knowledge general about antibiotics. This result is useful as information to find out the extent of the respondent's knowledge regarding the importance of knowledge respondents regarding antibiotics (Tabel 2.).

Table 1: Distribution of Respondent Characteristics

Variabels	Total	Percentage (%)
Gender		
Male	77	38,8
Female	121	61,1
Age		
19-30	91	46,0
31-42	61	30,8
43-45	36	18,82
55-70	10	5,1
Educations		
Primary School	38	19,2
Junior High School	43	21,7
Senior High School	59	29,8
Bachelor	58	29,3
Occupations		
Government Employees	60	30,3
Entrepreneur	23	11,6
Housewife	16	8,1
Farmer	94	47,4
Student	5	2,5

Table 2: Distribution of respondents' knowledge of answers to antibiotics

No.	Questions	Yes (%)	No (%)	Unknown (%)
1.	Antibiotics are drugs used to kill bacteria	149 (75,2)	30 (15,1)	19 (9,5)
2.	Antibiotics can be used to treat diseases caused by viruses.	96 (48,4)	74 (37,3)	28 (14,1)
3.	Antibiotics can be used to treat colds and coughs.	111 (56,0)	63 (31,3)	25 (12,6)
4.	Antibiotics should be used as well have a fever	69 (34,8)	92 (46,4)	37 (18,6)
5.	Antibiotics can kill bacteria normal living in the channel digestion	83 (41,9)	84 (42,4)	31 (15,6)
6.	Normal bacteria live inside the digestive tract is good for health	103 (52,0)	56 (28,2)	39 (19,6)
7.	Antibiotics are drugs that are used as a pain reliever and fever	67 (33,8)	101 (51,0)	30 (15,1)
8.	Penicilin is antibiotic	112 (56,5)	51(25,7)	35 (17,6)
9.	Antibiotics can cause an allergic reaction	95 (47,9)	67 (33,8)	31(15,6)
10.	Antibiotics have side effects.	87 (43,9)	78 (39,3)	33 (16,6)
11.	Excessive or inappropriate use of antibiotics can cause the antibiotic to become inactive or resistant	102 (51,5)	53 (26,7)	43 (21,7)
12.	It doesn't matter if the antibiotics are stopped when the complaints have disappeared.	83 (41,9)	72 (36,3)	55 (27,7)
13.	Low than prescribed antibiotics is better than the total amount prescribed	51 (25,7)	92 (46,4)	55 (27,7)
14.	New or expensive antibiotics are better	50 (25,2)	104 (52,5)	44 (22,2)

Table 3: Regression and Optical characteristics of ATN and LOS

Category	Number	Percentage (%)
Good	75	37,9
Moderate	69	34,8
Low	54	27,3
Total	198	100%

Table 2 and 3 shows that 198 respondents know about antibiotics. It is found that the respondents' knowledge is meager. It can be seen that 13 out of 14 questions, not more than 60% of respondents know about antibiotics' correct problems. As many as 149 (75.2%) of

respondents knew that antibiotics were drugs used to kill bacteria, but only 74 (37.3%) of them knew that antibiotics could not be used to kill viruses. This study is not much different from other studies in Yogyakarta, Putra Jaya, and Shah Alam. The majority of them know antibiotics are used to kill bacteria (76%, 78.3% and 80.3%) when as many as 71%, 83% and 60 % of respondents don't know that antibiotics cannot be used to kill viruses[10,11,12].

The public knowledge test data that has been carried out on 198 respondents shows that respondents have good knowledge of 75 respondents 37.9%, Moderate knowledge of 69 respondents 34.8%, and Low knowledgeable 54 respondents 27.3%. The results of these data illustrate the knowledge of the community in Panyabungan Kota District.

Respondents' Perception Level About Antibiotics:

Table 4: Distribution of respondents' perception of answers to antibiotics

No.	Questions	Totally agree (%)	Agree (%)	Not Sure (%)	Disagree (%)	Totally Disagree (%)
1.	Whenever I have a cold or a cold, I will immediately use antibiotics	29 (14,6)	63 (31,8)	41 (20,7)	50 (25,2)	15 (7,5)
2.	I used to stop antibiotics when I felt better.	19 (9,5)	97 (48,9)	28 (19,1)	38 (19,1)	6 (3,0)
3.	If you have flu symptoms, I will ask the doctor to prescribe antibiotics	20 (10,1)	63 (31,8)	48 (24,2)	65 (32,8)	7 (3,5)
4.	If anyone in my family is sick, I usually give antibiotics.	14 (7,0)	64 (32,3)	48 (24,2)	65 (32,8)	7 (3,5)
5.	I used to keep the antibiotic antibiotics in stock.	18 (9,0)	66 (33,3)	45 (22,7)	53 (26,7)	16 (8,0)

Table 5: Regression and Optical characteristics of ATN and LOS

Category	Number	Percentage (%)
Good	20	10,1
Moderate	138	69,7
Low	40	20,2
Total	198	100%

Table 4 dan 5 shows the statements related to public perceptions of antibiotics. Based on the study results, it can be seen that the public perception of antibiotics is still low. This is because only Low than 40% of respondents have the correct perception about antibiotics, such as not immediately using antibiotics if the flu, not immediately stopping antibiotics when recovered, not asking doctors to prescribe them. If you have flu symptoms and don't keep antibiotics in stock. This study's public perception shows the same condition as the public perception in Putra Jaya and Shah Alam. In these two studies also showed that Low than 50% of respondents showed the correct perception of using antibiotics when flu or colds. Antibiotics can be stopped if they feel cured and ask doctors to prescribe antibiotics if they experience flu symptoms. But these two studies show a good perception compared to our study regarding antibiotics can be shared with family members and the habit of keeping antibiotics as supplies for emergencies[13]. Based on the data analysis carried out, most respondents' attitudes were in a good category as many as 20 respondents, 10.1%. Meanwhile, in the moderate category, 138 respondents were 69.7%, and in the Low category, 40 respondents were 20.2%. These results illustrate the public perception of the use of antibiotics in the Panyabungan Kota sub-district.

Respondents' Belief Level About Antibiotics:

Table 6: Distribution of respondents' perception of answers to antibiotics

No.	Questions	Totally agree (%)	Agree (%)	Not Sure (%)	Disagree (%)	Totally Disagree (%)
1.	I believe that antibiotics can cure any disease.	18 (9,0)	63 (31,8)	59 (29,7)	44 (22,2)	14 (7,0)
2.	I believe antibiotics can prevent the disease from getting worse.	7 (3,5)	99 (50)	64 (32,3)	27 (13,6)	1 (0,5)
3.	I believe wounds on the skin heal faster by sprinkling powder antibiotics on them	12 (6,0)	84 (42,4)	64 (32,3)	35 (17,6)	3 (1,5)
4.	I believe that antibiotics have no side effects.	17 (8,5)	36 (18,1)	66 (33,3)	59 (29,7)	20 (10,1)

Table 7: Regression and Optical characteristics of ATN and LOS

Category	Number	Percentage (%)
Good	11	5,6
Moderate	148	74,7
Low	39	19,7
Total	198	100%

Table 6 and 7 shows the statements related to public beliefs about antibiotics. Based on the results of the study, it was found that as many as 40.8% of respondents had the wrong belief that antibiotics could cure any disease, 29.7% were unsure, and only 29.2% stated that antibiotics were not drugs that could cure any disease. More than 50% of respondents believed that antibiotics could be used as a medicine to prevent the disease from getting worse, 32.3% were unsure, and 14.1% disagreed. As many as 48.4% of respondents had confidence that powdered antibiotics could be used as wound medicine, 32.3% of respondents were unsure, and only 19.1% disagreed. If the skin wound is caused

by an infection, it must be treated with antibiotics, and then an appropriate antibiotic is used in the form of an ointment. Based on the research, it was found that 26.2% agreed that antibiotics had no side effects; only 39.8% of the respondents believed that antibiotics also had side effects. This is not much different from a study in Yogyakarta which showed that 40% believed that antibiotics could cure any disease, as well as the statement that antibiotics could be used as skin wounds, and 24% believed that antibiotics had no side effects. This shows that public confidence in these antibiotics can lead to antibiotic resistance[14].

Based on the data analysis carried out, most respondents' attitudes were in a good category as 11 respondents, 5.6%. Meanwhile, in the moderate category, 148 respondents were 74.7%, and in the low category, 39 respondents were 19.7%. These results illustrate the public's trust in the use of antibiotics in Panyabungan Kota sub-district.

The relationship between the characteristics of the respondents and the level of knowledge

This analysis indicates whether there is a relationship between each characteristic of respondents with the level of knowledge. More data can be seen in Table 8

Table 8: The relationship between the characteristics of the respondents and the level of knowledge

Variabels	The level of knowledge			P value
	Good	Moderate	Low	
Gender				
Male	21	31	25	0,049
Female	54	61	29	
Age				
19-30	38	36	17	0,038
31-42	25	20	16	
43-45	9	9	18	
55-70	3	4	3	
Educations				
Primary School	12	11	15	0,000
Junior High School	7	18	18	
Senior High School	14	24	21	
Bachelor	42	16	3	
Occupations				
Government Employees	8	39	6	0,000
Entrepreneur	19	31	44	
Housewife	42	18	0	
Farmer	1	4	0	
Student	5	7	0	

In Table 8, the comparison of gender characteristics between men and women, it can be seen that the comparison of attitudes about antibiotics between the two characteristics shows a difference, namely that women's knowledge has a better level of knowledge. The correlation between gender and knowledge obtained a significant P value of 0.049 (<0.1), which indicates a significant relationship between the sex of the respondent and the level of knowledge.

In the correlation between age and knowledge level, 18-30-year-olds have a good level of knowledge. The correlation between age and knowledge obtained a significant P value of 0.038 (<0.1), which indicates a relationship indicating a significant relationship between the age of the respondent and the level of knowledge.

The correlation between the last education and the level of knowledge shows that the last education bachelor has better knowledge, followed by Senior high school, Junior High School and the lowest is Primary schools. This indicates that a person's high level of education will affect the level of knowledge they get. According to Suhardi (2009), one of the factors that influence a person's knowledge is education. The higher a person's education, the easier the person will receive information to obtain more knowledge. The correlation between the last education and the level of knowledge got a significant value of P 0.000 (<0.1), which indicates a significant relationship between the respondent's last education and the level of knowledge.

On the correlation between work and knowledge level, which shows that civil servants have better knowledge. The correlation between work and knowledge obtained a significant value of P 0.000 (<0.1), which indicates a significant relationship between the respondent's job and the level of knowledge. In relation to the level of knowledge and work, work indirectly contributes to influencing a person's knowledge level. This is because work is closely related to social and cultural interaction factors, while social and cultural interactions are closely related to information exchange, and this will certainly affect a person's level of knowledge[15].

From the results obtained, it can be concluded that from the four characteristics of the respondents, the latest education, occupation, age and gender affect the level of knowledge. In accordance with the results of Singgih Putra Ambada research in Surakarta, which shows that the last education category, the respondent's occupation, age, and gender affect the level of respondents' knowledge about antibiotics[16]. Study conducted by yogesh stated that self-medication of antibiotics on the basis of incomplete knowledge should be avoided and proper education must be required to those who are treating themselves with self-medication practice in order to avoid antibiotics resistance[17]. And also antibiotics resistance can cause Emergence of Superbugs (bacteria highly resistant to antimicrobial agents) has severely threatened therapeutic[18]. Our result showed that 31,0% agree that antibiotics are ineffective in treating coughs and colds, study conducted by patil also showed that 33% did not know that antibiotics are ineffective in treating coughs and colds[19].

CONCLUSIONS:

The level of public knowledge on antibiotics use in the community in Panyabungan City District, Mandailing Natal District can be categorized as good 75 (37.9%), moderate category 69 (34.8%), and category Low 54 (27.3%) as a whole the level of knowledge is classified as good. The level of belief on antibiotics use in the community in Panyabungan District, Mandailing Natal District can be categorized as good 11 (5.6%), moderate category 148 (74.7%), and category low 39 (19.7%) overall, the level of belief is moderate. The level of community perceptions about antibiotics in the community in Panyabungan Kota District, Mandailing Natal Regency can be categorized as good 20 (10.1%), moderate category 138 (69.7%), and category low 40 (20.2%) the overall perception of society about is moderate.

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CONFLICT OF INTEREST:

The authors declare no conflict of interest.

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RESEARCH ARTICLE

An observational study amongst anti-diabetic patients related to the anti-diabetic drugs prescription at tertiary care centre of northern India

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ABSTRACT:

Being a chronic metabolic disorder, Diabetes mellitus (DM) affecting six percent of the world population. The WHO defines diabetes mellitus as "A metabolic disorder of multiple aetiology characterized by chronic hyperglycemia with disturbances of carbohydrate, fat and protein metabolism resulting from defects in the insulin secretion, insulin action, or both". Oral antidiabetic drugs (OADs) are the first line treatment for type 2 diabetes mellitus in management of the disease. Rational use of oral antidiabetic drugs (OADs) and insulin are used for the prevention of diabetes mellitus complications. Prescription of rational use of the drugs is a complex issue to achieve the goal that patients receive medications appropriate to their clinical needs, in doses that meet their own individual requirements for an adequate period of time, and at the lowest cost to them and their community. Rational use of the drugs in populations can be effectively studied with drug utilization reviews. The aim is to study the drug prescribing pattern of anti-diabetic drugs in newly diagnosed type 2 DM patients.

KEYWORDS: Observational study, anti-diabetic patients, anti-diabetic drugs, drug prescription, tertiary care centre, India.

INTRODUCTION:

Diabetes mellitus (DM) is a chronic metabolic disorder and six percent of the world population is affected by it[1-3]. The WHO defines diabetes mellitus as "A metabolic disorder of multiple aetiology characterized by chronic hyperglycemia with disturbances of carbohydrate, fat and protein metabolism resulting from defects in the insulin secretion, insulin action, or both"[2-8]. Many studies on Indian population shows that 4% of the adults suffered from diabetes mellitus in the year 2000 and it is expected to rise to 6% by the year 2025[9]. Oral antidiabetic drugs (OADs) are the first line treatment for type 2 diabetes mellitus in management of the disease[5]. Rational use of oral antidiabetic drugs (OADs) and insulin are used for the prevention of diabetes mellitus complications[6,10,11].

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Prescription of rational use of the drugs is a complex issue to achieve the goal that patients receive medications appropriate to their clinical needs, in doses that meet their own individual requirements for an adequate period of time, and at the lowest cost to them and their community[7,12-15]. Rational use of the drugs in populations can be effectively studied with drug utilization reviews. The aim is to study the drug prescribing pattern of anti-diabetic drugs in newly diagnosed type 2 DM patients.

MATERIAL AND METHOD:

It is a prospective observational study of prescription event and adverse drug reaction monitoring to drugs used for diabetes mellitus type-2 treatment. This study was carried out on the patients at Department of Medicine Rama Medical College, Kanpur. The duration of study was 6 months. The study started only after the approval of Institutional ethics committee.

The various study tools that will be used are the Suspected Adverse Drug Reaction Reporting Form

issued by Central Drugs Standard Control Organization (CDSCO) under Pharmacovigilance Programme of India (PvPI) which will record all the information, such as name, age, sex, weight, other relevant history including pre-existing medical conditions, details of suspected adverse drug reactions and details of suspected medications that the patients might be taking.

Data will be aggregated according to disease profile and other relevant information required for the study. Causality assessment was done using Naranjo's causality assessment scale[8].

Inclusion criteria:

- Newly diagnosed patients of diabetes mellitus type-2.
- Patients with age more than 18 years.
- Patients of either sex
- Patients having base line (pre treatment) biochemical parameters other than blood sugar (i.e. liver function test, kidney function test) within normal range.
- Patients having no associated comorbidities.

Exclusion criteria:

- Patients who were unwilling to participate and did not give consent in the study
- Patients who were unable to give interview.
- Patients with incomplete medical records.
- Patients with chronic liver disease such as cirrhosis, chronic hepatitis and acute viral hepatitis
- Terminally ill patients
- Patients with concurrent major psychiatric illness and/or concurrent major medical illnesses

Data Management and Analysis:

Statistical analysis:

Categorical variables were presented in number and percentage (%). Qualitative variables were compared using Chi-Square test /Fisher's exact test as appropriate. A p value of <0.05 was considered statistically significant. Statistical analysis was performed using GraphPadQuickCalcs software available online at **http://graphpad.com/quickcalcs/**. The data was entered in MS EXCEL spreadsheet and analysis was done using Statistical Package for Social Sciences (SPSS) version 21.0.

RESULT:

Demographic and health characteristics:

A total of 120 patients were diagnosed with type II diabetes. The total number of males in the study was 68 (56.7%) while females were 52 (43.3%) (Tab. 1).

Tab. 1: Gender wise distribution

Gender	N (%)
Male	68(56.7%)
Female	52(43.3%)
Total	120(100%)

Age wise distribution of patients showed 6(5%) were aged between 21 – 30 yr, 13(10.8%) between 31 – 40 yr, 35(29.2%) between 41-50 yr, 45(37.5%) between 51-60 yr, 18(15%) between 61-70 yr and 3(2.5%) in >70 yr [**Tab. 2].**

Table 2: Age wise distribution

Age (Yrs)	N (%)
21 - 30	6(5)
31 - 40	13(10.8)
41 – 50	35(29.2)
51 – 60	45(37.5)
61 – 70	18(15)
>70	3(2.5)
Total	120(100)

Duration of diabetes was between 16 and 20 yr in 12(10%) patients, 11-15 yr in 21(17.5%) patients, 6-10 yr in 36 (30%) patients and ≤5 yr in 51 (42.5%) patients **[Tab. 3].**

Tab. 3: Duration of Diabetes Distribution

Duration of diabetes (Yr)	N(%)
16 - 20	12(10%)
11 - 15	21(17.5%)
6 - 10	36(30%)
< = 5	51(42.5%)

Drug Prescription Pattern of oral antidiabetics:

Drug Prescription Pattern of oral antidiabetics it was observed out of 120 patients monotherapy was prescribed to 55 patients (Metformin was to 40 patients, Glimipride/Gliclazide to 15 patients that accounted for 33.3% and 12.5% of total respectively), combination therapy was prescribed to 65 patients (Metformin plus Glimipride/Gliclazide to 36 patients, Metformin plus Glimipride/Gliclazide plus Glitazone to 10 patients and Metformin plus Dipeptidyltransferase IV inhibitor to 19 patients that accounted for 30%, 8.3%, and 10.5% of total respectively) [**Tab. 4].**

Table 4: Drug Prescription Pattern of Oral Antidiabetic

Oral Antidiabetic Drug	
Monotherapy	**N(%)**
Metformin	40(33.3%)
Glimipride/Gliclazide	15(12.5%)
Combination Therapy	
Metformin plus Glimipride/Gliclazide	36(30%)
Metformin plus Glimipride/Gliclazide plus Glitazone	10(8.3%)
Metformin plus Dipeptidyltransferase IV inhibitor	19(10.5%)

DISCUSSION:

The total number of males in the study was 68 (56.7%) while females were 52 (43.3%). This is in agreement with previous reports as shown in study by Singh et al[9,16] who included 55% of males while 45% were females, similarly study by Saravanan et al[10] included 60% males rest 40% of females, another study included 51% males while 49% were females.

Age wise distribution of patients showed 6(5%) were aged between 21 – 30 yr, 13(10.8%) between 31 – 40 yr, 35(29.2%) between 41-50 yr, 45(37.5%) between 51-60

yr, 18(15%) between 61-70 yr and 3(2.5%) in >70 yr.It was observed from age wise distribution that maximum no. of patients with diabetes 37.5% were aged between 51 – 60 yr and then 29.2% between 41 – 50 yr. This was found in agreement with study done that showed almost similar observations, maximum no. of patients with diabetes 37% were between 51 – 60 yr and 26% were between 41 – 50 yr[17,18].

Duration of diabetes was between 16 and 20 yr in 12(10%) patients, 11-15 yr in 21(17.5%) patients, 6-10 yr in 36 (30%) patients and ≤5 yr in 51 (42.5%) patients. It was found that maximum no. of patients 42.5% had ≤5 yr duration of diabetes. Similar pattern was seen in study done by Singh et al[9] where 43.6% of patients had ≤5 yr duration of diabetes. In contrast study by Bhattacharjee et al[10,19.20] showed 29.5% of patients had ≤5 yr duration of diabetes where as maximum no. of patients 36.5% were in the timeframe of 5–10 yr duration of diabetes.

Drug Prescription Pattern:

In Drug Prescription Pattern of oral antidiabetics, out of 120 patients monotherapy was prescribed to 55 patients (Metformin was to 40 patients, Glimipride/Gliclazide to 15 patients that accounted for 33.3% and 12.5% of total respectively), combination therapy was prescribed to 65 patients (Metformin plus Glimipride/Gliclazide to 36 patients, Metformin plus Glimipride/Gliclazide plus Glitazone to 10 patients and Metformin plus Dipeptidyltransferase IV inhibitor to 19 patients that accounted for 30%, 8.3%, and 10.5% of total respectively).It was observed that Metformin was the maximum prescribed drug in monotherapy as well as maximum prescribed drug of all oral antidiabetics too.

CONCLUSION:

Out of total 120 patients male constituted more as compared to women. Age wise distribution showed maximum patients belong to age group between 41-50 yr, which appeared to be the most vulnerable group. Duration of diabetes was ≤5 yr seen in maximum patients.

Drug Prescription Pattern of oral antidiabetics showed that metformin was the most commonly prescribed drug followed by combination therapy of metformin plus glimipride/gliclazide.

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RESEARCH ARTICLE

Formulation design and Evaluation of Extended-Release Tablets of Oxybutynin for Effective Management of Overactive Bladder Syndrome

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ABSTRACT:

Overactive bladder syndrome (OAB) is a chronic condition with a composite of symptoms and has a significant negative impact on the physiological and psychological well-being of the patients. The present study is aimed at developing extended-release formulations of oxybutynin that is effective in reducing the side effects associated with the drug by maintaining a steady state concentration with minimal fluctuations in plasma drug concentration and thereby achieves improved patient compliance. The extended-release drug delivery of tablets can be achieved by preparing the matrix tablet of oxybutynin chloride with klucel HF in core tablet by wet granulation technique and functional coating with a combination of aquacoat ECD-30 and hypromellose E5 followed by film coating with opadry. For confirmation of compatibility, the pure drug and its physical mixtures were subjected to FTIR studies. All the formulations have shown acceptable limits in all precompression and post compression parameters. The *in vitro* release studies in 0.1N HCl and 6.8 phosphate buffer revealed that the optimized formulation F10 extend the release of the drug to 91% at 24 hours and the release profile was similar to the innovator's product as revealed by the similarity factor study. The release kinetics study revealed that the release of the drug followed diffusion mechanism. Stability studies of the selected formulation tablets were carried out at 25°C ±2°C/60% RH±5% and 40°C ±2°C /75%±5% RH for different time period and all the parameter was within the limits after storage period. Thus the extended release matrix tablets of oxybutynin chloride developed in this study have immense potential to develop into a marketed product following the testing in animals and human volunteers.

KEYWORDS: Oxybutynin, extended release, enteric coating, film coating, *in vitro* release.

INTRODUCTION:

Overactive bladder syndrome (OAB) is a chronic condition with a composite of symptoms that affect the quality of life of the patients. OAB is commonly associated with heterogeneity in symptoms, underreporting of symptoms and underestimation by patients, which disguises its prevalence among millions of people all over the world. About 546 million people would have been affected by OAB by 2018, with an estimated annual cost burden of 3.9 billion upon the world economy [1,2].

The International Continence Society defines overactive bladder as "urinary urgency, usually accompanied by frequency and nocturia, with or without urgency urinary incontinence (UI), in the absence of urinary tract infection (UTI) or other obvious pathology." The overall prevalence of OAB increases with rising age and is more common among women, though gender is not considered a risk factor among population [3]. OAB symptoms have a significant negative impact on the physiological and psychological well-being of the patients.

The sensation of urgency in OAB is considered to be related with disruption of normal muscle structure, impulsive excitability of the bladder and increased smooth muscle coupling triggered by pathological partial denervation of the bladder smooth muscle [4]. Behavioural modification by educating and training to

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improve pelvic floor muscle strength and urge control techniques is the first line of treatment for OAB [5]. The second line of treatment for OAB is treatment with antimuscarinic medications which inhibits the parasympathetic stimulation and reduces detrusor contraction by competing with acetylcholine for the muscarinic receptors [6].

Oxybutynin is a commonly used and most studied antimuscarinic drug used over three decades in the treatment of urgency urinary incontinence (UI) associated with OAB [7]. Oxybutynin elicits its therapeutic effect by blocking the carbochol-induced contractions by its intrinsic binding to muscarinic receptors [8]. Yet the treatment is often discontinued by patients owing to the high incidence of side effects, including dry mouth, constipation and blurred vision [9]. The antimuscarinic side effects listed are dose limiting and can be managed by reducing initial dose and subsequent dose titration of treatment [10]. Extended-release formulations of oxybutynin are effective in reducing the side effects associated with the drug by maintaining a steady state concentration with minimal fluctuations in plasma drug concentration and thereby achieve improved patient compliance [11]. OROS® oxybutynin uses osmotic pressure to deliver the drug for 24 h in a controlled fashion with well-tolerated and effective clinical outcomes in patients with OAB, in particular elderly patients using multiple medications [12]. The usage of extended-release oxybutynin was observed to improve tolerability and treatment adherence among patients with lesser side effects [13].

The present investigation aims at the development of extended release matrix tablets containing Oxybutynin chloride for the effective management of OAB and also to compare the release profile of optimized formulation with that of innovator's product.

MATERIALS AND METHODS:

Materials:

Oxybutynin chloride was a gift sample from Aurabindo Pharma, Hyderabad. Hydroxy Propyl cellulose (HPC) (Klucel HF USP) was purchased from Ashland industries, Europe. Aquacoat ECD-30 USP (Ethyl Cellulose 30 % aqueous dispersion) and Hypromellose E5 (hydroxyl propyl methyl cellulose) were obtained from FMC, U.S.A and DOW Chemical company, Michign respectively. Lubritab® (Hydrogenated Cottonseed oil) was obtained from JSR Pharma Ltd. Opadry yellow, opadry pink and opadry grey were obtained from Colorcon Chemicals. All other solvents and reagents used in the work were of analytical quality.

Methods:

Precompression studies:

Pre-compression characterization of API and formulation blend:

The drug sample was evaluated for its colour, odour, appearance, melting pointand solubility characteristics. The API was also subjected to particle size analysis and micromeritics evaluation. The compatibility between drug and the excipients was studied using FT-IR spectroscopy [14,15]. The pre-compression characteristics such as angle of repose, bulk density, tapped density, carr's index and Hausner's ratio was setermined for the formulation blends. [16,17]

Formulation of Oxybutynin ER tablets:

Preparation of core tablets:

The extended release tablet of oxybutynin was formulated by wet granulation technique. Briefly, oxybutynin chloride, klucel HF, pharmatose 200 M, aerosol and avicel PH101 were sifted through #40 while hydrogenated vegetable oil was sifted through #60 and were collected separately. The ingredients of the core tablet were dry mixed in a rapid mixer granulator for 10 min with impeller ran at slow speed and chopper off. The granulation was done in two steps with the binder solvent added first at high impeller speed and slow chopper speed followed by the addition of binder solution. Then kneading was carried out for 120 s with slow chopper and fast impeller speed. The drying of the wet mass was done in a rapid mixer dryer at 60°C until the loss on drying was not more than 2 % w/w. Dry granules were sifted through # 60, mixed and blended together with lubritab and magnesium stearate in a double cone blender. The core tablet blend thus obtained was compressed using 7.2 mm standard concave shaped plain dies. The composition of the core tablets, enteric layer coating and film coating is shown in table 1.

Enteric coating:

The enteric coating solution was prepared by dissolving Hypromellose E5 in purified with continuous stirring until a clear solution was formed. To the above solution, aquacoat ECD-30 was added with stirring to form a uniform dispersion. Triethyl cellulose and talc were then added to the above dispersion and was kept under continuous stirring, during the coating process. The coating parameters were set as shown in table 2. The coating process was continued till target weight build up was achieved. Once the desired weight was achieved, pan speed was reduced and spraying of enteric coating dispersion was stopped and the tablets were warmed at the temperature of 38°C – 40°C for one hour.

Film coating of Tablets:

The film coating solution was prepared by slowly dissolving opadry (aqueous moisture barrier system) in

water followed by homogenization for 20 minutes**.** The solution was kept under continuous stirring, during the coating process. The coating was continued till target weight build up was achieved. The coating parameters were followed as shown in table 2. On completion of coating process, the tablets were warmed at 40°C for one hour and then stored in containers until further use. The specifications of various parameters used in enteric coating and film coating are shown in Table 2.

Table 1: Formulation of Extended Release tablets of Oxybutynine Chloride.

INGREDIENTS	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11
Oxybutanin chloride	15	15	15	15	15	15	15	15	10	15	5
Lactose (Pharmatose)	34.5	54.5	54.5	62.5	72.5	82.5	72.5	72.5	77.5	72.5	82.5
HPC(Klucel)	70	50	50	40	40	30	40	40	40	40	40
MCC(Avicel PH-101)	60	60	60	60	60	60	60	60	60	60	60
Purified water	-	q.s	-	q.s	q.s	q.s	q.s	q.s	q.s	q.s	q.s
Isopropyl Alcohol	-	-	q.s	q.s	-	-	-	-	--	-	-
Aerosil 200pharma	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Hydrigenated vegetable oil (Lubritab Type1)	20	20	20	20	10	10	10	10	10	10	10
Light.Magnesium.Sterate	-	-	-	2	2	2	2	2	2	2	2
Core tablet weight	**200**	**200**	**200**	**200**	**200**	**200**	**200**	**200**	**200**	**200**	**200**
Extended release coating	10%	10%	5%	5%	5%	5%	5%	2.5%	5%	5%	5%
Aquacoat ECD -30	9.6	9.6	4.8	4.8	4.8	4.8	3.2	2	4	4	4
Triethylcitrate PG/NF	1.6	1.6	0.8	3.2	3.2	3.2	4.8	2	4	0.8	0.8
HPMC E5 cps	6.4	6.4	3.2	3.2	3.2	3.2	4.8	2	4	4	4
Talc	2.4	2.4	1.2	1.2	1.2	1.2	1.2	0.6	1.2	1.2	1.2
Purified water	q.s	q.s	q.s	q.s	q.s	q.s	q.s	q.s	q.s	q.s	q.s
ER coated Tablet weight	**220**	**220**	**210**	**210**	**210**	**210**	**210**	**205**	**210**	**210**	**210**
Film coating											
Opadry Grey	5.5	5.5	5.25	5.25	5.25	5.25	5.25	5.125	-	5.25	
Opadry Pink	-	-	-	-	-	-	-	-	5.25	-	-
Opadry Yellow	-	-	-	-	-	-	-	-	-	-	5.25
Purified water	q.s	q.s	q.s	q.s	q.s	q.s	q.s	q.s	q.s	q.s	q.s
Total tablet weight	**225.5**	**225.5**	**215.25**	**215.25**	**215.25**	**215.25**	**215.25**	**210.125**	**215.25**	**215.25**	**215.25**

Table 2: Coating Parameters

Parameter	Specifications	
	Enteric coating	Film Coating
Inlet Temperature	45-50°C	50-60°C
Exhaust Temperature	38-42°C	40-42°C
Atomization pressure	1.2 kg/cm^2	1.2 kg/cm^2
Coating pan speed	6-8 rpm	6-8 rpm
Spray rate	2-5 g/min	2-5 g/min
Needle gun diameter	0.8 mm	0.8 mm

Evaluation of Core, Enteric coated and Film Coated tablets: [18, 19]

The core tablets, enteric coated tablets and the filem coated tablets were tested for various post compression characteristcs such as thickness, hardness, friability, weight variation and drug content.

***In vitro* Dissolution Studies:**

The *in vitro* drug release studies were carried out using USP type III (reciprocating cylinder) dissolution apparatus operated at 25 dpm. The dissolution was performed in 250 ml of simulated gastric fluid (0.1N HCl) for 2 h followed by testing in phosphate buffer pH 6.8 for the remaining period with the temperature maintained at 37 ± 0.5°C. An aliquot (5ml) was withdrawn at specific time intervals up to 24 h and drug content was determined by UV-visible spectrometer at 220.0 nm. Sample quantification was based on previously constructed analytical curves. The dissolution profile of each batch of tablet was then compared with the release of drug from innovator's product (Ditropan XL®).

Calculation of f2 value between ER matrix tablet and innovator's product:

There are several methods for dissolution profile comparison. f_2 is the simplest among those methods. The following equation defines f2. Tt and Rt show the average dissolutions of the test and reference products at the time point (t), respectively, and n is the number of time points at which the average dissolutions are compared [20].

$$f_2 = 50 \text{ X Log } \{[1 + (1/n) \sum_{t=1}^{n} (R_t - T_t)^2]^{-0.5} \text{ X } 100\}$$

Drug Release Kinetics:

The *in vitro* release data obtained from the dissolution studies was fitted into various kinetic models such as, zero order, first order, Higuchi and Korsemeyer-Peppas models to determine the mechanism of release of drug from the optimized ER tablets [21].

Stability Studies:

The stability studies were carried out for the most satisfactory formulation as per ICH guidelines Q1A (R2) to assess the drug and formulation stability. The optimized formulation was sealed in HDPC bottles and

sealed with aluminium foil then capped. The samples were retained in humidity chamber maintained at 25 ºC/ 60 % RH and at 40 ºC/75% RH for two months. At the end of studies, samples were analysed for the post compression parameters and drug content for assessing the stability of the formulation [22].

RESULTS AND DISCUSSION:

The present study was aimed at preparation and evaluation of extended-release matrix formulations of Oxybutynin chloride by wet granulation technique for the treatment of over reactive bladder as an alternative to highly expensive and time taking process OROS technology. The formulation thus developed was compared with the dissolution profile of the innovator's product Ditropan XL (Osmotic Bi Layered Tablet) tablets. The extended-release drug delivery of tablets can be achieved by using the matrix core tablet of Oxybutynin chloride with Klucel HF in core tablet and functional coating with Aquacoat ECD-30 and Hypromellose E5 premium LV. The film coating with given with opadry grey, opadry pink and opadry yellow. Hydroxy propyl cellulose (HPC) is non-ionic water-soluble cellulose ether with a remarkable combination of properties. HPC hydrates rapidly, leading to formation of diffusion controlling gel layer around the tablet core [18]. Aquacoat ECD 30 comprise of EC in the dispersion phase as spherical particles in the size range of 0.1 to 0.3 µm. It is used for the aqueous film coating of solid dosage forms to extend drug release, taste mask, or to protect against moisture [23]. Hypermellose is a well-established polymer for use in extended-release tablets. The combination is used in order to achieve the optimal release characteristics from the tablet. Opadry is an immediate aqueous film coating system protect drug from the effect of moisture and light for an extended period of time without interfering with the release profile of the drug and are preferred materials for producing film coating formulations in terms of performance and global acceptability [24].

API Characterization:

Oxybutynin chloride was subject to organoleptic characteristics such as colour, odour, taste, and appearance by visual observation. The drug was also evaluated for its melting point, loss on drying and solubility characters. The results were found satisfactory and complied with the official standards. The micrometric properties of pure drug of Oxybutynin chloride were evaluated by angle of repose (32.3°C indicated as good flow), bulk density (0.198 g/cc), tapped density (0.353g/cc), Carr's Index (43.75% indicated as very poor flow) and Hausner's Ratio (1.78 indicated as very poor flow properties). The characteristics of the pure drug are tabulated in the table 3.

Table 3: Characterization of Oxybutynin chloride pure drug

Parameter	Characteristics
Odour	Odourless
Taste	Tasteless
Appearance	Crystalline powder
Melting point	128°C.
Loss on drying	1.5% w/w
Solubility	Freely soluble in methanol (0.8 parts per part of solute) and very freely soluble in water (3 parts per part of solute)

Compatibility Studies Using FTIR Spectroscopy:

The FTIR spectra of pure Oxybutynin chloride and formulation blend was performed. Similar characteristic peaks were observed for the drug-excipients mixture, indicating the absence of possible chemical interaction between the drug and excipients. From the figure 1A and1B, it can be seen that the major functional group peaks observed in spectrum of drug with all the polymers remains unchanged as compared with spectra of Oxybutynin chloride. The characteristic peaks and frequency of Oxybutynin chloride and drug-excipients is shown in table 4.

Table 4: Characteristic peaks of Oxybutynin chloride and drug-excipients:

Characteristic Peaks	Frequency range (cm^{-1})	Frequency of pure drug (cm^{-1})	Frequency of drug-excipients
OH stretching	3600-3500	3500.92	3530.92
OH Bending	1100-1070	1084.03	1064.03
C-H stretching	3200-3100	3095.95	3099.85
C-N stretching	1350-1100	1105.25	1208.33
C=O stretching	1750-1735	1745.61	1732.59
C=C	1600	1599.04	1578.11

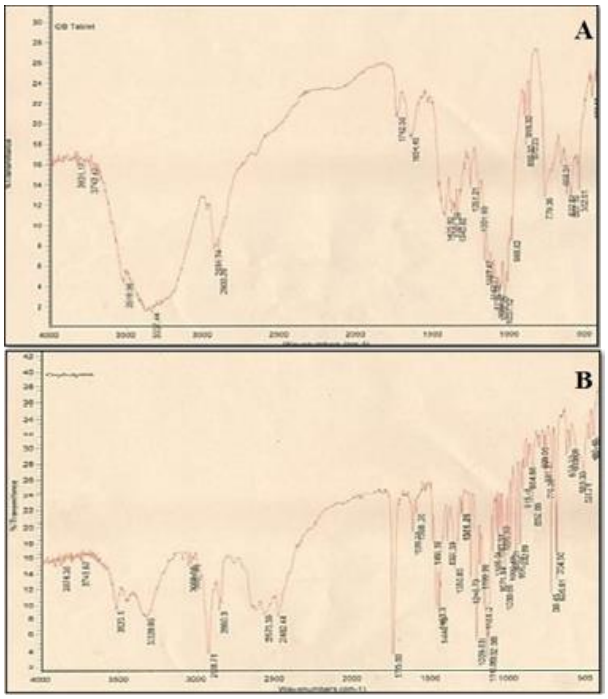

Figure 1: FTIR Spectrum; (A) Oxybutynin Chloride; (B) Formulation blend

Precompression characterization:

The precompression characteristics such as bulk density, tapped density, Carr's index, Hausner's ratio and angle of repose were studied. The granules prepared by wet granulation method were evaluated for various flow properties. The bulk densities of granules of F1 to F6 formulations ranged from 0.55 to 0.614 g/ml. The Tapped density of the powder blends of F1-F11 formulations ranged from 0.611 to 0.647 g/ml. The Carr's index and Hausner's ratio of F1-F11 were between 6.18 -11.29 and 1.04 to 1.12, respectively. Thus flow property of pre-compressed blends were indicated as excellent. The same composition of F7 blend used in core tablet to F8-F10. The results of the pre compression characters are shown in table 5.

Table 5: Physical properties of pre-compressed blend

Formulation Code	Angle of repose (º)	Bulk density (g/mL)	Tapped density (g/mL)	Carr's Index (%)	Hausner's Ratio
F1	32.5	0.607	0.647	6.18	1.066
F2	31.6	0.566	0.626	9.58	1.106
F3	28.4	0.556	0.612	9.15	1.10
F4	27.2	0.55	0.62	11.29	1.127
F5	32.96	0.611	0.639	4.38	1.046
F6	32.06	0.614	0.646	4.95	1.052
F7	31.01	0.601	0.631	10.29	1.067
F8	31.01	0.601	0.631	10.29	1.067
F9	31.01	0.601	0.631	10.29	1.067
F10	31.01	0.601	0.631	10.29	1.067
F11	31.01	0.601	0.631	10.29	1.067

Post-compression Parameters for Oxybutynin Hydrochloride Extend Release tablets:

The post compression parameters of the F1-F11 tablets were evaluated for thickness, hardness, friability, weight uniformity and uniformity content. From F1-F6, the matrix polymer concentration gradually decreased from 35% to 15% in core tablet and with 60:40 ratio of 5% ethyl cellulose: hypromellose E5. From F7-F11, 20% of matrix polymer was used in all core tablets but in coating, various concentration ratios of ethylcellulose and hypromellose E5 were used. The results were tabulated in table 7. The hardness of F1-F11 formulations ranged from 13 to 14 kg/cm^2 and were within the range of desired limits of hardness. Hence all the formulations passed the test for hardness**.** The thickness of F1-F11 formulations were found between 4.5 to 5.2 mm which was according to Pharmacopoeial specification. The weight variation test was performed and the tablets complied with the pharmacopoeial specification for weight variation limit. Friability of the tablets was determined by using Roche friabilator. The friability of all the F1-F11 formulations was determined, and the values were in the range from 0.04 to 0.17 %. Friability values below 1% were an indication of good mechanical resistance of the tablets. The percentage drug content of all the F1-F11 tablets was found to be in the range of 91.29 to 99.06 %. This was within the acceptable limits. The preparation complies with the test if the individual content of tablet is 89 to 101% of the average content.

***In vitro* Drug Release Studies:**

The results of the *in vitro* release profile of developed formulations F1 to F11 is shown in figure 2A. The formulation F1-F8 and F10 had 15mg of label claim of Oxybutynin chloride. Here the matrix tablets were formulated using 35% HPC & Enteric coated using 10% polymer blend and film coating using Opadry grey which is very viscous in nature. The release of drug depends not only on the nature of matrix but also upon the enteric coating. As the percentage of polymer decreased, the rate of drug release increased.

Among all other formulations, F10 formulation prepared using 15 mg of label claim Oxybutynin chloride and 5% of 50:50 in ratio of Aquacoat ECD-30 and Hypromellose E5cps with opadry pink in 5.25 % film coating exhibited drug release very much comparable at almost all time points to innovator as shown in figure 2B. Hence formulation F10 was used for further evaluations.

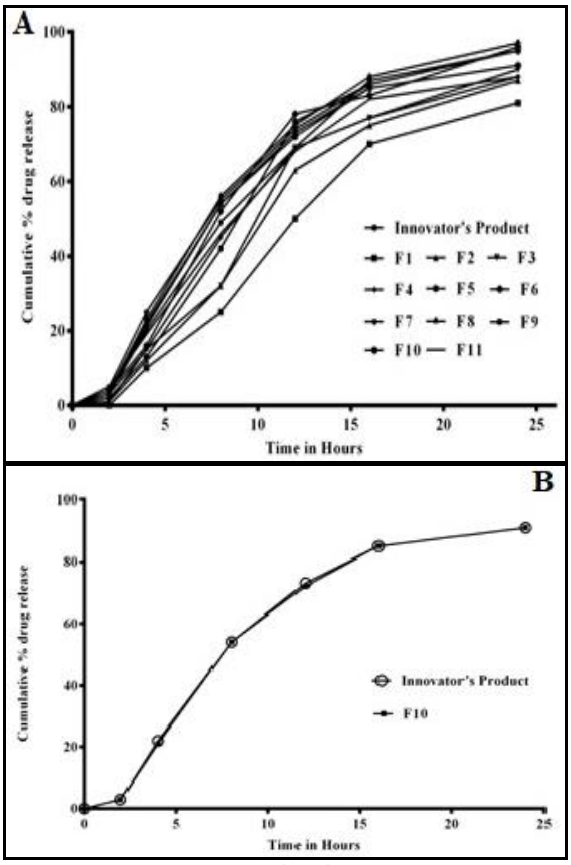

Figure 2: *In vitro* dissolution profile of (A) Formulations F1 to F11 in comparison with Innovator's Product; (B) F10 in comparison with Innovator's Product.

Release kinetics was showed optimized fomulation F10:

The release profile of the optimized formulation of F-10 was applied to the different kinetics equation such as zero order, first order, Higuchi, korsmeyeres peppas in order to define the most plausible mechanism of drug release from the extended release tablet [25]. As shown in figure 3 drug release data was best explained by first order equation, as the plots showed the highest linearity (r^2 = 0.980) indicating that the rate of drug release is concentration dependent., followed by Higuchi's equation (r^2= 0.946). Higuchi's kinetics explains that drug diffuses at a comparatively slower rate as the distance for diffusion increases.

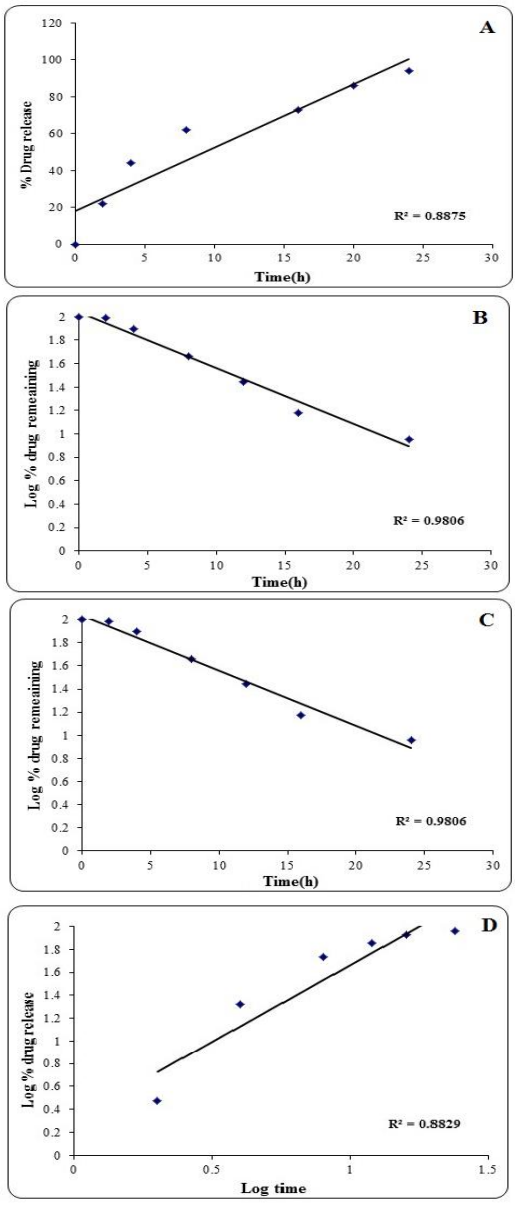

Figure 3: Release kinetics of optimized formulation F10; (a) zero order; (b) First order; (c) Higuchi model; (d) Korsemeyer Peppas model

The corresponding plot (log cumulative % drug release vs. log time) for the Korsmeyer-Peppas equation indicated a good linearity (r^2= 0.882). The diffusion exponent "n" was between 0.45-0.89, which indicates mechanism of release is non-fickian diffusion type. This indicates that the drug release was controlled by more than one process, i.e., both diffusion and dissolution[26].

Determination of Similarity Factor Study:

The similarity factor (f_2) was defined by CDER, FDA, and EMEA as the "logarithmic reciprocal square root transformation of one plus the mean squared difference in percentage dissolved between the test and reference release profiles". The similarity factor (f_2) was calculated to optimized formulation of F-10 dissolution profile with compared to Innovator dissolution profile. The result was found to be 92.47% indicating that as Test and Reference dissolution profile were similar[27,28].

Stability Studies:

The stability studies of optimized formulation F-10 were evaluated after storage period by variouys physicochemical parameters. There were no changes physical appearance and thickness. While the hardness of the tablet gradually decreased, the friability of the tablet did not show any changes up to 2 month. The assay of Formulation 10 showed the drug content to be 99.30% initially, after 1 month it decreased to 98.29% and 99.05%, later it was found to be 97.30% and 98.69% after 2 months at 40°C /75%RH and 25°C /60%RH respectively as shown in figure 4. Thus the product is considered to be stable as it did not show any significant changes in its properties under the tested conditions.

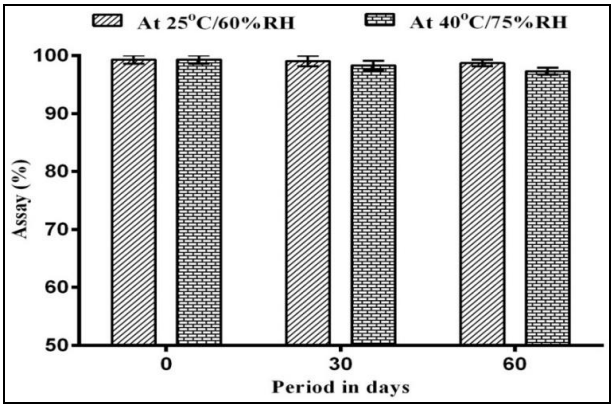

Figure 4: Stability Studies of Optimized Formulation of F10

CONCLUSION:

Extended-release Matrix tablet system is one of the approaches for controlled release systems. This approach was used for the formulation development of controlled release tablets of oxybutynin with a retarding

polymer. The drug release could be achieved for 24 h with a single dose. The developed formulation reduced the frequency of dosing, with minimal side effects and were as effective as osmotic controlled release oral delivery system. It can be conclusively stated that development of extended-release formulation of hydrophilic drugs does not necessitate the inclusion of the hydrophobic polymers to hydrophilic polymers and the desired extended release of hydrophilic drugs is also viable with hydrophilic polymer alone using suitable coating materials. The optimized formulation, F10 has desirable physicochemical properties, release characteristics and stability. Thus the extended release matrix tablets of oxybutynin chloride developed in this study has immense potential to develop into a marketed product following the testing in animals and human volunteers.

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