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Contents

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VIEWS

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Anticancer Activity of Extracts of Various Fractions of *Citrus maxima* (J.Burm.) Merr. on HeLa Cell Line

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ABSTRACT

The present paper deals with, *Citrus maxima* (J. Burm.) Merr. and (Family: Rutaceae) leaf, stem bark and fruit peel extracts screened for anticancer activity. The dried mill powdered leaves, bark and fruit peels were extracted with various solvents-ethanol, acetone and water and their phytochemistry revealed the potency of ethanolic extract being a rich source in extracting the phytochemicals for their antimicrobial and as well as anticancer activity. The anticancer property of plant extracts were analysed using HeLa cell line. The cells were grown in 96 well plates. Ethanol fraction of *Citrus maxima* leaf is chosen to establish the IC_{50} and IC_{50} value is found approximately closer to 50%. Whereas, ethanol fraction of *Citrus maxima* leaf has shown high anticancer property (69.1% dead cells), Ethanol fraction of *C.maxima* bark has shown 15.3% of dead cells, while, acetone and aqueous fractions of *C.maxima* fruit peel have shown 23.3% and 22.1% of dead cells respectively. Hence, these results of leaf and fruit peel extracts of *C.maxima* may helpful to develop nutraceutical product for cancer prevention.

KEY WORDS: *Citrus maxima* (J.Burm.) Merr., anticancer activity, HeLa cell line, DMEM (Dulbecco's modified eagle's medium), Trypsin, Cryopreservation, Thawing, Trypan blue.

INTRODUCTION

Cancer is the name given to any illness resulting from one of our body's own cells growing out of control. Cancer cells are also referred to as tumors or neoplasm. Uncontrolled growth, ability to invade local tissues and ability to spread or metastasize are the characteristics of a cancer cell [1]. There are more than 200 different types of cancers, which are categorized in to carcinoma, sarcoma, lymphoma and leukemia based on the cells from which they arise [2]. The principles involved in treating cancer are either directly destroying the cancer tissue or indirectly destroying them by depriving them of blood supply or their nutrition. But the irony is that whatever conventional treatment is used to destroy cancer cells they do affect to some extent even the normal cells [3]. They may damage skin, liver, intestine, bone marrow and other rapidly multiplying cells [4]. Sometimes the effect of treatment is more adverse than the disease itself. Therefore alternative medicine is the best solution. Alternative medicine includes therapies like herbal remedies, special diets [5].

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The use of plants and plant extracts in the practice of medicine is called as herbalism or phototherapy [6]. Phytomedicinal or herbal approaches can help with symptom management, offer anti-proliferative effects, immune enhancement, and more [7].

A HeLa cell is a cell type in an immortal cell line used in scientific research. These are the human epithelial cells derived from an aggressive glandular cervical carcinoma transformed by human papillomavirus 18 (HPV18) [8]. HeLa cells are adherent cells i.e., they stick to the surface which maintains contact inhibition *in vitro* [9]. Loss of contact inhibition is a classic sign of oncogenic cells, i.e. cells which form tumors in experimental animals [10].

It has the capability of growing both in suspension and as anchorage dependent. This application shows a convenient method for continuous harvesting of large numbers of HeLa cells. Thus these HeLa cells were employed to analyse the anticancerous activity of Citrus species [11].

In the present study, it is much concentrated on the

plant derived products like leaf, stem bark and fruit peel of *C.maxima,* whose phytochemistry reveals various apoptic, anti proliferative, antioxidant and antimicrobial activities which gains clinical significance in the field of drug discovery and therapeutics [12]. Pomelo (*Citrus maxima)* is known worldwide for its delicious taste of fruits and health promoting properties. The fruits of citrus can be consumed mostly fresh and has been used as a herbal medicine or additive or food supplement [13]. *Citrus* is believed to possess bioactivities such as antioxidant, anti-inflammatory, antimicrobial and the supplements of leaf and fruit peel of *Citrus maxima* have been shown highest cytotoxicity can be suggested to prevent cancer and degenerative diseases [14].

MATERIALS AND METHODS

Collection and authentication of material:

The leaves, stem bark and fruit peels of *Citrus maxima* (J. Burm.) Merr. *(Pomelo)* were collected from the local gardens around Devanahalli, Bengaluru, Karnataka, India. Plant species were authenticated by Prof. B.V. Krishnappa, Government First Grade College, Chickballapur, Karnataka, India. And voucher specimens, SBGNSC/O14 were deposited at the herbarium of Botany Department of SBGNS College,Chickballapur, Karnataka, India. The respective materials were washed thoroughly 2-3 times with running tap water. Then the materials were air and shadow dried and mechanically crushed into coarse powder of 40 µm size by using mixer grinder. Powders were stored separately in air tight bottles.

Preparation of extracts:

Extracts were prepared in order to study their antioxidant activity. Ethanolic, acetone and aqueous extracts of each of the leaves, stem bark and fruit peels of *Citrus maxima* were prepared by soaking the material in various solvents for 72h and after every 24h, the mixture was stirred with a sterile glass rod. After the completion of 72h , the extracts were filtered using a Buckner funnel and Whatman No.1 filter paper and concentrated by vacuum drying.

Chemicals and reagents:

DMEM (Dulbecco's modified eagle's medium), Sodium bicarbonate, Trypsin-EDTA (0.25%)-Ethylene diamine tetra acetate, Trypan blue and all other chemicals and solvents were of analytical grade and were obtained from Ranbaxy Fine Chemicals, Mumbai, India.

Cell line used: In this study HeLa cell line is used which were obtained from National center for Cell Sciences, Pune, India.

METHODS:

CELL CULTURE: MEDIA PREPARATION AND STERILIZATION

10.2 gm of powdered DMEM (Dulbecco's modified eagle's medium) was dissolved in 1000 ml of autoclaved double distilled water .To this 1.2 gm of Sodium bicarbonate was added while the media turns pink in color.

Fig. 1 : Media preparation

For the sterilization of the media the filter apparatus was autoclaved and transferred to laminar air flow cabinet. The upper chamber was removed, the membrane filter was placed and upper chamber was replaced and autoclaving was done again for the second time. Then again apparatus was transferred to LAF cabinet and one of the lower chamber's nozzle was attached to a vacuum pump. The media was then poured and pressure was applied to filter the media. The filtered media was collected in the lower chamber.10% or 20% of serum was added according to requirement before use.

TRYPSINIZATION AND SUB CULTURING

Once the cells reach confluency stage sub culturing was done. All the spent media were removed and the cells were washed with saline water. 200 µl of Trypsin-EDTA (0.25%)was added and incubated for 3-4 minutes for the cells to get detached and 1ml of 10% media was added and mixed thoroughly to stop Trypsin action 0.6 ml of the cell suspension was then transferred to a fresh culture dish ,to this 1ml of fresh media was added. The cells were then incubated at 37^0c, 5%CO_2 and 60-70% humidity.

CRYOPRESERVATION

The culture was removed from the dish and collected in a tube and centrifuged at 1500 rpm for 10 minutes. The pellet was dissolved in 0.5 ml of 10% media. Meanwhile freezing mixture was prepared in ice bath. 1.5 ml mixture was added to the cell suspension in the ice bath. The cryovial was kept in the slow cooling device then the sample was transferred to long term storage device.

THAWING

It is the process of transferring cells from frozen state to normal state. The cryo vial was removed from the cryo-can and was made to stand for some time to allow the liquid nitrogen present on the surface of vial to vaporize. Then the vial was warmed in 37ºc water bath. Meanwhile 10 ml of saline was taken in a 15 ml centrifuge tube and the contents of vial were transferred in to the tube and centrifuged at 1500 rpm for 10 minutes. Supernatant was discarded and 0.5-1.0 ml of media was added to the pellet.

Fig-2: Thawing

TRYPAN BLUE EXCLUSION ASSAY FOR CELL VIABILITY/CELL DEATH

This rapid and simple test is used to determine the approximate number of viable cells present in the cell suspension. It is based on the principle that a viable or live cell ,will have an intact cellular membrane and therefore will exclude the dye and have clear cytoplasm whereas a nonviable or dead cell do not have such integrity and so the cytoplasm takes up the blue color.

- The cell suspension obtained after thawing was mixed properly and taken in 5 fresh culture dishes.
- To all 3 culture plates 2 ml of fresh media was added
- To the first dish 20 mg of the acetone extract of Citrus maxima fruit peel was added
- To the second dish 20 mg of ethanol extract of Citrus maxima fruit peel was added
- The third dish was taken as control. No extract was added. It contained untreated cells
- All the plates were kept for 24 hour incubation to test the effect of the sample on the cells
- 10 µl of the cell suspension was then mixed with 20 µl of Trypan Blue dye in an eppendorf tube.
- Meanwhile the surface of haemocytometer was cleaned using ethanol and 10 µl of the prepared mixture was loaded such that the suspension goes in between the cover slip and the surface of haemocytometer.
- And the number of live cells and dead cells were counted inside L_1, L_2, L_3, chambers using a clicking counter and the percentage viability was calculated.

The percentage of cell death was calculated by the formula:

$$\% \text{ cell death} = \frac{\text{Number of dead cells}}{\text{Number of total cells (live + dead)}} \times 100$$

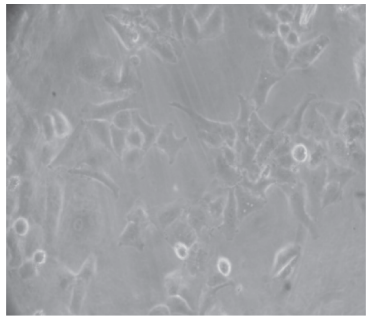

Fig-3:HeLa Cells in control media

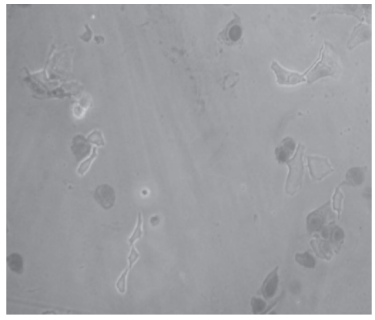

Fig-4:Tryphan blue IC_{50}

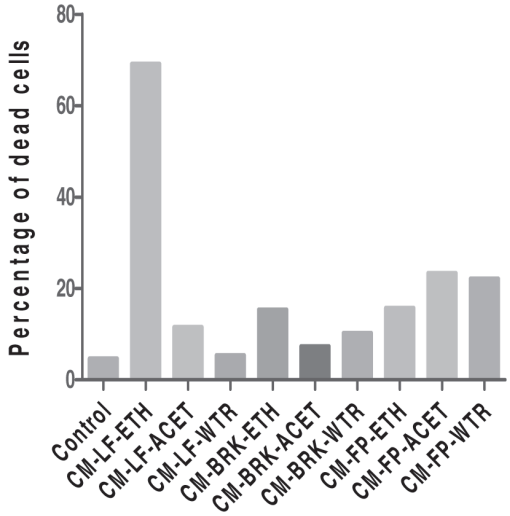

Fig. 5: Effect of *Citrus maxima* on HeLa cell line against by Trypan blue dye exclusion method

RESULT AND DISCUSSION

RESULT:

Table-1:
Effect of *Citrus maxima* on HeLa cell line against by Trypan blue dye exclusion method

Groups	Treatment	Number of Live cells	Number of Dead cells	Total Number of cells	% of Dead cells
Group-I	Control	230	11	241	4.6%
Group-II	CM-LF-ETH (300mg/kg)	68	152	220	69.1%
Group-III	CM-LF-ACET (300mg/kg)	246	32	278	11.5%
Group-IV	CM-LF-WTR (300mg/kg)	232	19	251	5.3%
Group-V	CM-BRK-ETH (300mg/kg)	209	38	247	15.3%
Group-VI	CM-BRK-ACET (300mg/kg)	215	17	232	7.3%
Group-VII	CM-BRK-WTR (300mg/kg)	228	26	254	10.2%
Group-VIII	CM-FP-ETH (300mg/kg)	172	32	204	15.7%
Group-IX	CM-FP-ACET (300mg/kg)	184	56	240	23.3%
Group-X	CM-FP-WTR (300mg/kg)	53	186	239	22.1%

CM-*Citrus maxima, CA-Citrus aurantium,* LF-leaf, BRK-bark, FP-fruit peel, ETH-ethanol, ACET-acetone, WTR-water.

DISCUSSION

The anticancer property of selected plant extracts were analysed using HeLa cell line. The cells were grown in 96 well plates. Among the extracts, ethanol fraction of *Citrus maxima* leaf has shown high percentage of anticancer property as 69.1% of dead cells. At the same time ethanol fractions of Citrus maxima bark and acetone fraction of fruit peel have shown less anticancer property as 15.3% and 23.3% of dead cells respectively, where as, aqueous fraction of *C.maxima* fruit peel has shown 22.1% of dead cells. (Table-1). Further ethanol fraction of *Citrus maxima* leaf is chosen to establish the IC_{50} and $IC_{50\ value}$ is found approximately closer to 50%. (Fig. 4). Thus, it can be said that, the supplements of leaf, and fruit peel of *Citrus maxima* are useful as anticancer agents.

CONCLUSION

In the present investigation it was observed that, the cyto morphological changes of the dead cells include cytoplasmic condensation, formation of vacuoles and membrane protruding, this indicates that cells may have undergone a partial apoptosis on treating with the extracts and also it has been concluded that, ethanol fraction of *Citrus maxima* leaf has shown high percentage of anticancer property as 69.1% of dead cells. Thus, the phytochemicals of those sources might be useful in treating the cancer. Next priority can be given to acetone and aqueous fractions of *Citrus maxima* fruit peel (23.3% and 22.1% of dead cells respectively).

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A Clinical Comparison on Efficacy of Timolol Vs Timolol/ Bimatoprost In Patients With Glaucoma

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ABSTRACT

Objective: To compare the efficacy of timolol 0.5% and timolol 0.5%/Bimatoprost0.03% in lowering the intraocular pressure (IOP) in patients with Glaucoma. **Methods:** In this prospective randomized eight week open label study 30 qualifying patients received either timolol (n=15) or timolol/bimatoprost (n=15). IOP was assessed at the end of 2nd and 8th week of treatment. The primary outcome measure was mean IOP. **Results:** Baseline mean IOP for both group is 23.36±7.112 & 23.93±7.719 and the mean reduction in intraocular pressure from baseline 6.92 mmHg with timolol -Group A (95% Confidence Interval 14.42-18.46) and 7.39 mmHg with timolol/bimatoprost combination - Group B (95% Confidence Interval 14.85-18.23). Both the treatment is safe and effective. **Conclusions:** The fixed combination timolol/bimatoprost demonstrated superior mean IOP-lowering efficacy compared to monotherapy timolol in patients with Glaucoma.

Keywords: Timolol, Bimatoprost, fixed combination, glaucoma, Intraocular Pressure

Introduction

In India, Glaucoma is the second common cause of blindness in adult population. Experts approximate that 50% of individuals affected by glaucoma may perhaps not know they have it as normally there are no symptoms during the early stages of Glaucoma. Worldwide, glaucoma affects almost 66.7 million people [1]. Glaucoma is called "the sneak thief of sight," because till the permanent visual damage is occurring it often shows no symptoms. Though Glaucoma can be treated, at present there is no cure. Lost vision to glaucoma cannot be recovered, and untreated glaucoma leads to blindness. [2]

Glaucoma is form of slow progressive blindness and takes many years to develop. A person with glaucoma has to lose about 30% of the axons of the optic nerve prior to starts of affecting their vision. This loss is permanent. Consequently, at the beginning stages, the patient with glaucoma may have no vision problems. In the next intermediate and late stages as they lose more axons their peripheral or side vision will be affected. In advanced final stages, the central/reading vision is affected (Fig 1). More frequently than not, the disease in one eye is more advanced than the other.

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Glaucoma is an enduring disease and the principal therapeutic approach is customized to the needs of each individual with glaucoma. The ultimate aim of glaucoma therapy is to reduce the IOP level, as it will slow down the rate of progression of optic nerve damage. Finally, the objective is to allow the patient to maintain functional vision throughout their life, while curtailing the risks of therapy.

The glaucoma are a group of ocular disorders that causes optic neuropathy exemplified by altered optic nerve head (optic disk) that is linked with loss of visual sensitivity and field. Increased intraocular pressure (IOP), a customary diagnostic criterion for glaucoma, is thought to play a vital role in the pathogenesis of glaucoma, but is no longer a diagnostic criterion for it.

Lowering the intraocular pressure (IOP) by early treatment reduces the risk of visual field loss in glaucoma patients.[3,4] It is preferable to control the IOP with monotherapy rather than with multiple drug therapy, as every drug has side effects which will also added up and each drug added will increases the costs and may reduce patient compliance.[5] When a single drug does not effectively reduce IOP, additional hypotensive agents can be added to the therapy[6]. In this situation, a fixed combination preparation may improve both the compliance and the quality of life. [1, 7]

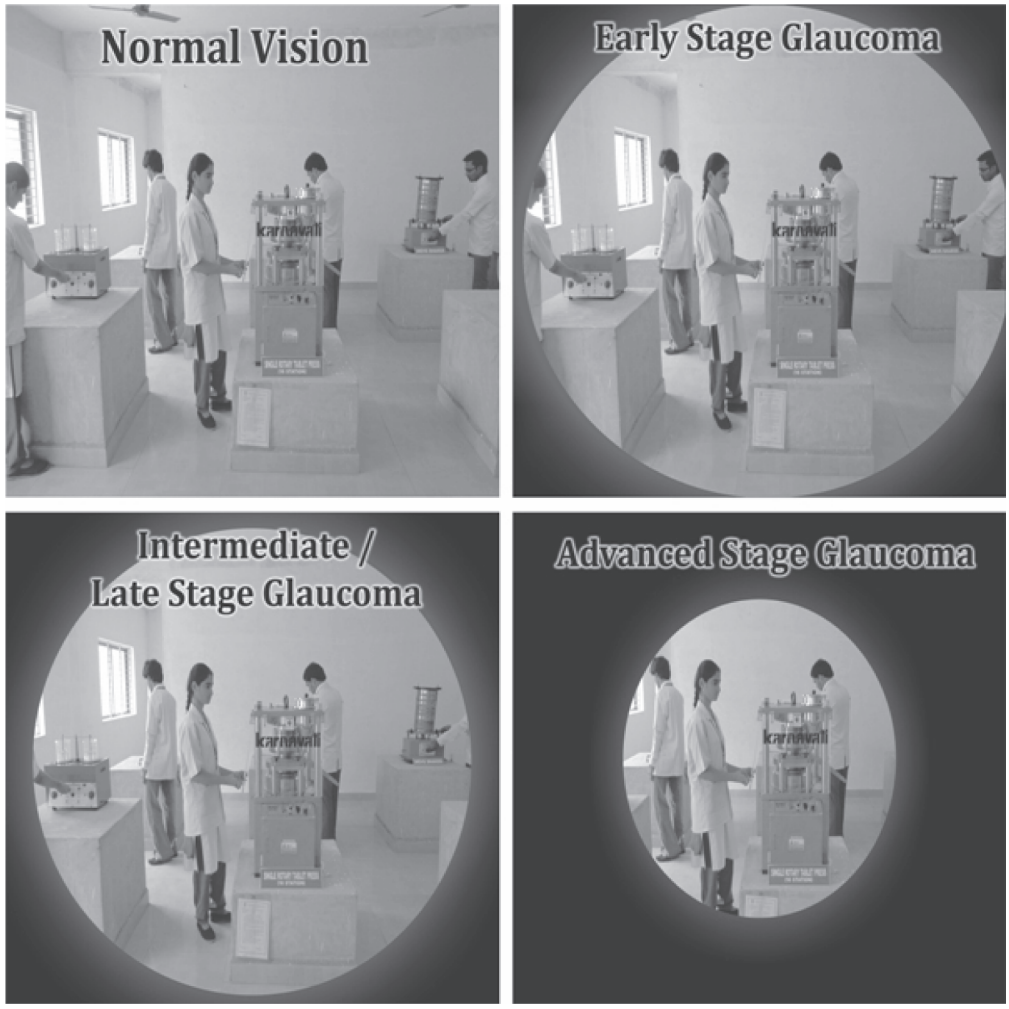

Figure 1: Vision of the Patients seeing same picture at various stages of Glaucoma

Methodology

Study Design

This prospective randomized eight week open label study was conducted in an eye hospital at Chittoor city of Andhra Pradesh state in India. The Study was carried out from August to February 2013. The Protocol was designed and approved by Institutional Ethics Committee (IEC). Informed Consent was obtained from all the patients participating in the study prior to conduct of the study.

Patients

Eligible patients were men or women of at least 21 years of age with a clinical diagnosis of OAG, with or without exfoliation syndrome. The study was conducted on consecutive patients, referred or recruited, attending the outpatient service of the Glaucoma Unit of the study hospital from August to February 2013. Exclusion criteria were Patients with history of incisional ocular surgery or trauma within 6 months before the study, patients with chronic use of ocular medication other than the study medications , pregnant or lactating females, patient with corneal abnormality or any condition that prevented related applanation tonometry, patients with ocular laser treatment or ocular surgery within 3 months, ocular infection and ocular inflammation , dry eyes , advanced cataract or glaucoma and history of renal or hepatic impairment; history of hypoglycemia or uncontrolled diabetes; contraindication to any study medication.

Schedule of visits and assessments

On the basis of the eligibility and exclusion criteria 30 qualifying patients who agreed to participate and gave their consent were selected and randomized into two groups to receive either Timolol - Group A (n=15) or timolol/ bimatoprost – Group B (n=15). At baseline visit their demographics and medical history were collected and reviewed and also the IOP was measured. Initial readings were considered as baseline value. Again IOP was assessed at the end of 2nd and 8th week of treatment which was taken as first review value and second review value.

Data Analysis and Statistics

The primary statistical objective of this study was to observe the IOP lowering efficacy of timolol 0.5% and timolol 0.5%/Bimatoprost0.03%. Hypothesis tests were performed using a repeated measures analysis of variance (ANOVA) and the primary inference was based on the comparisons of mean IOP between the two treatment groups. Statistical significance was achieved with $P < 0.05$.

Results

The Demographics and medical history of the subjects included in this study are given in Table 1. The mean changes in visual acuity, heart rate, papillary size, and blood pressure were insignificant in both Group A & Group B. Gradual reduction of IOP in both the groups was seen and it is shown in Fig 2. Reduction was higher in timolol/ bimatoprost – Group B than that of monotherapy with timolol. 95% CI for both Group A and Group B patients were 14.42-18.46 and 14.85 – 18.23 respectively. Both the treatment was safe and well tolerated. Among the two treatment, timolol/bimatoprost combination shows better efficacy than monotherapy with timolol. (Table 2)

Discussion

In this study, we compared IOP lowering efficacy of monotherapy timolol and fixed combination of timolol/ bimatoprost, in patients with open angle glaucoma. The

Table 1:

Demographics & Medical History (n=30)

Characteristics	Group A (Timolol)	Group B (Timolol/Bimatoprost)
Number of Patients (n)	15	15
Age (Years) Mean ± SD	51.73±12.82	55.73±15.48
Gender		
Male (%)	5 (33.33)	11(73.33)
Female (%)	10 (66.67)	4 (26.67)
Medical History		
Hypertension (HTN)	2 (13.33)	3(20)
Diabetes Mellitus (DM)	3 (20)	4(26.67)
Thyroid disorders	1(6.67)	0
Myocardial Infarction	0	0
DM/HTN	5(33.33)	4(26.67)
Social History		
Smoker or Alcoholics	2 (13.33)	4 (26.67)

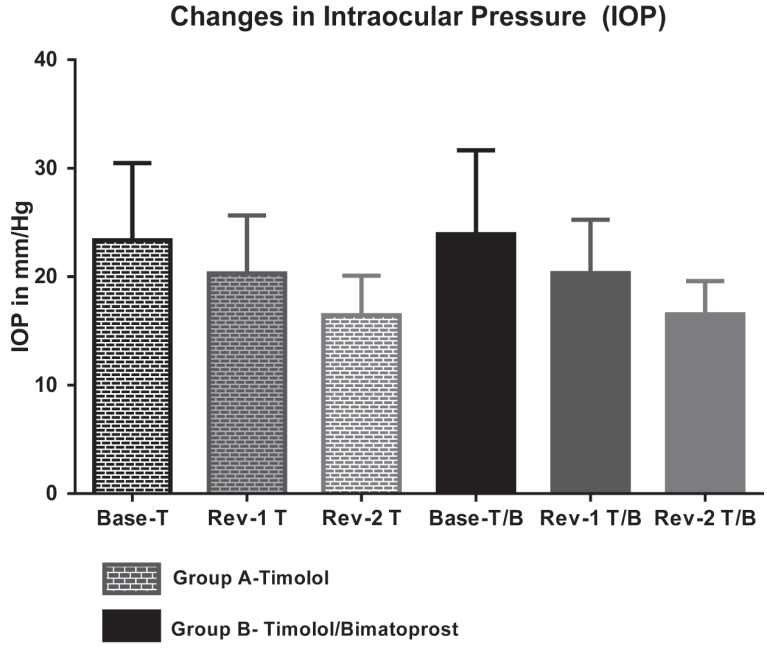

Fig 2 – Mean Changes in Intraocular Pressure (IOP) in Group A & Group B

Table 2:

Changes in Intraocular pressure (IOP)

	ParametersGroup A (Timolol)			Group B (Timolol/Bimatoprost)		
	Baseline	**Review I**	**Review II**	**Baseline**	**Review I**	**Review II**
IOP (Mean±SD)	23.36 ± 7.112	20.30 ± 5.344	16.44 ± 3.655	23.93 ± 7.719	20.33 ± 4.931	16.54 ± 3.057

results obtained were expected to show that timolol/ bimatoprost would have superior to monotherapy timolol, based on previous studies of timolol compared with various other fixed combinations, in which fixed combination of timolol with prostaglandin analogs produced superior IOP lowering effect. However, timolol/bimatoprost combination shows better superior efficacy than monotherapy timolol . Both these regimens had no effect on visual acuity, heart rate, papillary size, and blood pressure. As we already discussed in the introductory part, it is preferable to control IOP with single drug which is cost effective as well as improve the patient compliance compared to combination of more than one drug which is likely to increase the side effect and the cost. But in our study we could find that the fixed combination timolol/bimatoprost shows superior efficacy with similar lesser side effects as with monotherapy indicates the superiority of combination in glaucoma therapy.

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Design and Evaluation of Colon Targeting Aceclofenac Microsponge for Rheumatoid Arthritis

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ABSTRACT

Microsponge having particle size of 5-300µ in diameter, have wide range of entrapment efficiency to load various ingredients in a single microsponges system and release them at desired rates. The objective of this study is to formulate and evaluate a controlled release colon targeting Aceclofenac Microsponge for Rheumatoid arthritis. Microsponge was prepared by Quasi emulsification technique by using polymers like Eudragit, Sodium alginate and HPMC. Compatibility studies are carried out by IR. The prepared Microsponge were characterized for their shape and surface morphology, Drug content, Entrapment efficiency, Production yield, *In vitro* drug release and Release kinetics studies. The *Invitro* drug release for Aceclofenac loaded Microsponge was observed in different dissolution medium at different time interval Simulated Gastric Fluid (SGF) pH 1.2/2hr, duodenal pH 4.5/2hr, Simulated Colonic Fluid (SCF) pH 7/8hr for 12 hrs. The maximum amount of drug release from the Microsponge was found to be in colonic pH. Formulation F4 with highest proportion of polymer show desired cumulative controlled release pattern. According to release kinetics data, formulation F4 obeys zero order release pattern with R^2 value 0.996 and also it shows a best fit for Peppas model where '*n'* value 0.62 confirms that the release pattern follows Non-fickian diffusion mechanism. The reproducible drug release pattern of Aceclofenac colon targeting Microsponge will maintain the plasma drug concentration in controlled manner during the early morning stiffness in Rheumatoid arthritis condition and simultaneously overcome the main side effect of NSAIDs like ulcer.

Keywords: Microsponge, Colon targeting, Aceclofenac, Rheumatoid Arthritis

Introduction

Microsponge are porous Microspheres having a myriad of interconnected voids of particle size range 5-300µ. Microsponge delivery systems are uniform, spherical polymer particles [1]. Microsponge consisting of non-collapsible structures with porous surface through which active ingredients are released in a controlled manner. Depending upon the size the total pore length may range up to 10 ft and pore volume up to 1 ml/g. Their characteristic feature is the capacity to absorb or "load" a high degree of active materials into the particle and on to its surface. [2]

Rheumatoid arthritis is a chronic systemic inflammatory disease predominantly affecting joints and periarticular tissues [3]. The symptoms of Rheumatoid arthritis are pain and early morning stiffness in synovial joints with frequent extra articular manifestations affecting blood vessels, bone marrow, gut, skin, lungs eyes [4].

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Non-steroidal anti- inflammatory drug effectively used for treatment of inflammation and pain caused by rheumatoid arthritis, it will relieve pain and inflammation caused by Rheumatoid arthritis in early morning hours. The gastrointestinal tract (GIT) is the main target of NSAID toxicity. It is the most frequent organ affected by adverse drug reactions and unfortunately ulcer is the most common drug-induced toxicity that can be fatal [5]. NSAID stop cells making prostaglandins. Prostaglandins are chemicals released by injured cells. They cause inflammation and swelling and they sensitize nerve endings, which can lead to pain. If you make less prostaglandin, you have less inflammation and less pain [6].

By stopping cells making prostaglandins, NSAIDs relieve the symptoms of arthritis. They do not stop the inflammation occurring in the future or prevent the disease progressing to joint damage. Targeted delivery into the colon represents an advantageous approach for the treatment of widespread Inflammatory Bowel Disease (IBD) including ulcerative colitis and Crohn's disease [7].

This study going to reveal that, the symptoms of Rheumatoid arthritis are severe in early morning hours, so an attempt has been made to overcome the problem by delaying drug release by formulating colon targeting Aceclofenac Microsponge to maintain peak plasma concentrations in early morning hours and simultaneously to reduce the gastric irritation created by NSAIDs by controlling the release pattern

Materials

Aceclofenac was obtained as a gift sample from Milton laboratories, Pondicherry, India. HPMC, Sodium alginate and Eudragit was purchased from Vellore scientific, Vellore, India. All the chemicals were of analytical grade and double distilled water used throughout the experiment.

Methods

Formulation of Colon targeting Aceclofenac Microsponge:

Microsponges were prepared by the Quasi-emulsion solvent diffusion method by using an external phase containing 200 ml distilled water and 40 mg polyvinyl alcohol (PVA). The internal phase made up of drug, ethyl alcohol, and polymer and to this 20% of triethylcitrate (TEC) was added in order to facilitate the plasticity. At first, the internal phase was prepared at 60°C and added to the external phase at room temperature. After emulsification, the mixture was continuously stirred for 2 hours. Then the mixture was filtered to separate the Microsponge. The product was washed and dried by vacuum oven at 40°C for 24 hours [1]. The compositions of different colon targeting Aceclofenac Microsponge are shown in Table 1.

Evaluation Parameters

Compatibility studies by FTIR:

Infrared spectra of drug and polymer were taken by using KBr pellet technique and were recorded on a Fourier transform IR spectrophotometer (Bruker, Opus software) [8].

Particle Size Determination:

The particle size was measured using an optical microscope. The microparticles were dispersed in water or glycerin, the size of the Microsponge was determined by using calibrated eye piece micrometer. The particle size distribution was plotted and the average size was determined [9].

Surface morphology /Scanning Electron Microscopy (SEM):

The external morphology of the microsponges was studied by scanning electron microscopy. The sample for SEM analysis was prepared by sprinkling the microparticles onto one side of the double adhesive stub. The stubs were then coated with gold using polaran SC 500 sputter coater, to neutralize the electrons and to obtain a clear morphology of the microparticles [10].

Drug entrapment efficiency:

To determine the drug entrapment efficiency, the microsponges were dissolving in 100 ml of Simulated Colonic fluid (pH 7) at 37± 5ºc and then it was stirred at 100 rpm. The samples are centrifuges at 10000 rpm for 30 min .Finally the supernatant solution was assayed by UV Spectrophotometer at 275nm (Analytical technology, India) [11]. The drug entrapment efficiency was calculated by the following formula.

$$\text{Entrapment efficiency (\%)} = \frac{\text{Total amount of drug loaded} - \text{free drug in supernatant}}{\text{Total amount of drug loaded}} \times 100$$

Drug content:

From the formulated Microsponge, 200 mg were randomly picked from the prepared samples and were crushed with help of mortar and pestle. Then it was stirred continuously for 3hr with Simulated Colonic fluid (pH 7). After 3 h, the samples were filtered, suitably diluted and estimated by UV Spectrophotometer at 275nm (Analytical technology, India) [12]. The estimation was done in 3

Table 1:
Formulation proportion for preparation of Aceclofenac Microsponge

Microsponge ingredients	F1	F2	F3	F4	F5
Internal Phase (in Ratio)					
Aceclofenac	1	1	1	1	1
Eudragit RLPO	1	-	-	-	0.5
Sodium alginate	-	1	-	0.5	-
HPMC	-	-	1	0.5	0.5
External Phase (in %)					
PVA	0.5	0.5	0.5	0.5	0.5

replicates to determine the uniformity of drug in microparticles.

Production yield:

The production yield of the microparticles can be determined by calculating accurately the initial weight of the raw materials and the last weight of the microparticles obtained [13].

$$\text{Production yield (Y)} = \frac{\text{Practical mass of Microparticles}}{\text{Theoretical Mass (Polymer+drug)}} \times 100$$

***In vitro* drug release Studies:**

The release of Aceclofenac from Microsponge was investigated using the USP rotating paddle dissolution apparatus at 100 rpm and 37±5°c. The simulation of gastrointestinal transit conditions was achieved by altering the pH of the dissolution medium at various time intervals. The pH of the dissolution medium was kept at 1.2 for 2 h with 0.1 N HCl. Then, 1.7 g of KH_2PO_4 and 2.225 g of Na_2HPO_4 .$2H_2O$ were added, adjusting the pH to 4.5 by adding 1.0 M NaOH. A release rate study was continued for another 2 h. After 4 h, the pH of the dissolution medium was adjusted to 7.0 and maintained for 12 h. The final volume in all cases was 500 ml. The samples were withdrawn from the dissolution medium at various time intervals using a pipette fitted with a micro filter, and the filtrate was subjected to UV analysis at 275nm. All dissolution studies were performed in triplicate [14]**.**

***In vitro* drug release kinetics:**

The release data obtained was fitted into various mathematical models using PCP disso-V2.08 software. The parameters 'n' and time component 'k', the release rate constant and 'R', the regression coefficient were determined by korsmeyer-Peppas equation to understand the release mechanism. To examine the release mechanism of drug from microparticles, the release data was fitted into the Peppa's equation.

$$M_t / M_\infty = Kt^n$$

Where, M_t / M_∞ is the fractional release of drug, 't' denotes the release time, 'K' represents a constant incorporating structural and geometrical characteristics of the device, 'n' is the diffusion exponent and characterize the type of release mechanism during the release process.

Zero order:

$$\% R = kt$$

This model represents an ideal release in order to achieve prolonged pharmacological action. This is applicable to dosage forms like transdermal systems, coated forms, and osmotic systems; as well matrix tablets containing low soluble drugs. If n < 0.5, the polymer relaxation does not affect the molecular transport, hence diffusion is Fickian. If 0.5<n<1.0, the solid transport will be non – Fickian and will be relaxation controlled [15]. (Table-2)

Results and Discussion

The Physicochemical evaluation of Microsponge was shown in Table 3. The mean Particle size analysis of loaded and unloaded Microsponge was performed by Optical microscopy method. The values (*d*50) can be expressed for all formulations as mean size range. Cumulative percentage drug release from Microsponge of different particle size will be plotted against time to study effect of particle size on drug release. Spherical shaped Microsponge was observed by using optical microscope. Among the five formulations of Microsponge, F4 possess small particle size (63.65 ± 4.5µm) and also uniform particle size distribution, with increase in concentration of surfactant.

Scanning electron microscopy (SEM) revealed that the fabricated Microsponge were spherical with uniform smooth surface as shown in Figure 1.Small minute pores on the surface are responsible for the drug release from the Microsponge.

All the formulations show drug entrapment efficiency more than 50% and it was found that entrapment of drug increases with an increase in the amount of the polymer. Formulation F4 shows maximum entrapment of 92.25±2.10 of Aceclofenac with the polymer. The drug content in the Microsponge was found to be in the range of 84.21±3.22 to 91.21±1.65 %. The formulation F4 shows maximum drug content but there is no maximum variation among the batches. Among five formulations three of them show a yield value more than 70%.Results show that percentage yield increases with increase in the concentration of polymer.

Table 2:
Release Kinetics

Release Exponent 'n'	Drug Transport Mechanism	Rate as a function of time
0.5	Fickian Diffusion (Higuchi Matrix)	$t^{n-0.5}$
0.5<n<1.0	Non-Fickian Diffusion	t^{n-1}
1.0	Case – II Transport(Zero Order Release)	Zero Order Release
Higher Release (n>1)	Super Case – II Transport	t^{n-1}

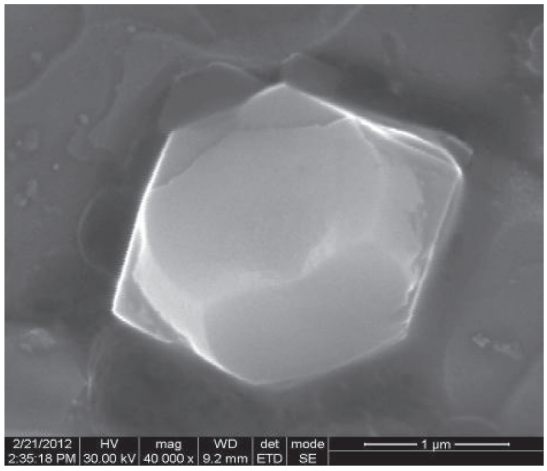

Fig 1: SEM photograph of Colon targeting Aceclofenac Microsponge

The *Invitro* drug release for Aceclofenac loaded Microsponge was observed in different dissolution medium at different time interval (SGF pH 1.2/1hr, duodenal pH 4.5/2hr, SCF pH 7/6hr) for 12 hrs. It was found that the release for all the formulation depend on pH change, different polymer and polymer proportion used in the formulation. It shows that maximum amount of drug is release only in Simulated colonic fluid pH 7,which may confirms that the Microsponge target to colon and release maximum amount of Aceclofenac in controlled manner in colonic pH after 4 hours. The F4 has highest proportion of polymer, showed maximum controlled release pattern as shown in Table 4 and Figure 2.

The data obtained for *in-vitro* release were fitted into equations for the zero-order, first- order and peppas release models .Based on the best cumulative controlled release pattern, the selected formulation F4 with Eudragit RLPO : Sodium alginate also shows a best fit for Peppas model and its release exponent value n is 0.62 i.e. 0.5 <n <1.0, the solid transport or drug release follows Non – Fickian diffusion mechanism and will be relaxation controlled and also the drug release from the Microsponge obeys Zero order release kinetics with regression R^2 value with 0.996 which represents an ideal release in order to achieve prolonged pharmacological action.

Table 3:

Physical evaluation of Aceclofenac Microsponge F1-F5

Formulations code	Particle size (µm)	Production Yield %	Drug content (%w/v)	Drug entrapment efficiency %
F1	72.23 ± 3.00	76.31 ± 3.00	86.60 ± 2.24	87.22 ± 1.80
F2	67.42 ± 6.00	60.01 ± 4.00	84.65 ± 3.25	79.96 ± 1.36
F3	69.32 ± 3.50	65.38 ± 3.40	84.21 ± 3.22	84.26 ± 1.94
F4	63.65 ± 4.50	83.39 ± 4.20	91.21 ± 1.65	92.25 ± 2.10
F5	93.75 ± 2.50	78.96 ± 3.20	89.22 ± 2.68	90.22 ± 1.60

Mean ± SD, n = 3.

Table 4:

***Invitro* drug release of Aceclofenac in different pH condition**

Time (hrs)	F1%	F2%	F3%	F4%	F5%
SGF pH 1.2					
1	0.37±0.40	0.40±0.02	0.34±0.40	0.18±0.44	0.36±0.40
2	0.93±0.24	0.81±0.22	0.59±0.20	0.26±0.82	0.65±0.36
Duodenal pH 4.5					
3	1.23±1.34	1.03±1.52	1.86±1.62	1.34±1.36	1.92±1.34
4	1.43±1.60	1.98±1.42	2.13±1.40	2.13±1.44	2.60±1.76
SCF pH 7					
5	23.29±1.40	26.39±2.46	25.21±2.46	20.97±1.48	23.17±2.48
6	32.45±1.72	36.12±2.32	35.44±2.20	24.24±1.36	27.08±2.34
8	43.10±2.36	46.48±1.30	42.47±2.56	35.73±1.40	41.00±1.42
10	57.99±2.12	58.78±1.72	59.81±1.32	44.10±2.64	59.46±2.72
12	68.07±1.82	71.84±2.04	75.94±1.42	56.63±1.42	65.50±2.12

Mean ± SD, n = 3.(SGF-Simulated gastric fluid, SCF-Simulated Colonic fluid)

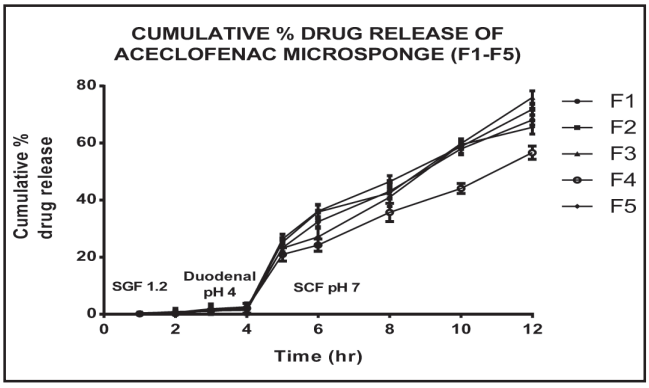

Fig 2: *Invitro* drug release profile - Aceclofenac Microsponge

Conclusion

Research shows that controlled release Colon targeting Aceclofenac Microsponge was an ideal drug delivery system for Rheumatoid arthritis. This was confirmed from the release pattern of drug i.e. maximum in colonic pH. By this delayed drug release of formulated Microsponge, it will maintain peak plasma concentrations in early morning hours especially to treat morning stiffness in RA condition and simultaneously to reduce the gastric irritation created by NSAIDs due to controlled release pattern. It will extend the duration of activity which allows greater patient compliance owing to elimination of frequent dosing regimen.

Acknowledgement

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Tissue Distribution Studies of An Over Dose 6-mercaptopurine In Rats

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ABSTRACT

The purpose of the present study is to determine the toxicokinetic parameters of 6-mercaptopurine (6-mp) and its distribution in various tissues of. 6-mp is an anti-cancer agent and it is used mainly in the treatment regimen of acute lymphoblastic leukemia. The same dose of 6-Mercaptopurine was used to study the toxicokinetics (TK) after multiple injections and the pharmacokinetics (PK) following single administration. Animals were administered with 6-mp in 0.1% CMC and blood samples were collected at respective time points after dosing and assayed by HPLC-RP. The tissue distribution studies of 6-mercaptopurine at an overdose was carried using single dose study in Swiss albino rats. Animals are scarified at a regular time interval of 1, 2, 4 hr. and liver, lung, kidney, heart, spleen and brain were isolated from animals and they were homogenized, centrifuge for sample extraction. The obtained samples were analyzed by HPLC with UV–detection at 322 nm. The present toxicokinetic study concluded that 6-mp was widely distributed throughout body but, liver has highest concentration and brian posses least concentration. As evidenced in this study, due to the increased concentrations of 6-mp in liver, there is relatively an increased incidence of hepato-toxicity and at the least occurrence of neurotoxicity.

Key words: 6-mercaptopurine, Anticancer, Hepatotoxicity, HPLC, Toxicokinetics.

Introduction

6-mercaptopurine is an anticancer drug which belongs to the category of anti-metabolites. It is therapeutically effective in acute lymphoblastic leukemia [1], abortion [2], and tuberculosis [3] and possesses potential anti-oxidant activity [4] as reported in the scientific literature. Since its introduction into clinical use, various adverse effects like bone marrow depression, hepatotoxicity has been reported [5]. Tissue distribution studies were one part of kinetic studies. Knowledge of tissue distribution of a test substance is an important criterion for the identification of target tissues and understanding the mechanisms of metabolism and toxicity in order to get information on the potential for accumulation and persistence of test substance and its metabolite. Analysis of additional tissues at the same time points should be considered for determining the effect of overdose of 6-mercaptopurine and its effect in the target tissues. Toxicity of the drug can be determined using the acute toxicity in accordance to Organisation for Economic Co-operation and Development (OECD) guidelines. Quantification of drug for tissue distribution studies can be performed using organ isolation, homogenization, extraction of test substance and solubilisation, followed by HPLC analysis [6]. To achieve these aim, we developed and validated a highly specific and rapid Reverse Phase –High Performance Liquid Chromatography (RP-HPLC) method for determination of 6-mercaptopurine in bio-samples and applied it for the study of its toxicokinetic characterization after single oral administration of pure 6-mercaptopurine to healthy rats.

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Materials and Reagents

All reagents used were of the highest available purity. 6-Mercaptopurine, d,l-dithiothreitol(DTT), Perchloric acid($HCLO_4$), were purchased from Sigma enterprises; Potassium-dihydrogenphosphate (KH_2PO_4), hydrochloric acid (HCl),sodium hydroxide (NaOH) and trifluoroacetic acid (TFA) were obtained from Finar chemicals Pvt. Ltd. (Ahmadabad); and HPLC grade methanol and acetonitrile were obtained from Finar chemical Pvt. Ltd. (Ahmadabad).

Equipments used during the study:

HPLC- [WATERS-2487, PUMP-515, COLUMN-HABIER 250nm, 4.0mm, 2.5µm]

UV-VISIBLE Spectrophotometer – [Schimadzo-1800]

Cooling Centrifuge- [REMI]

$_P$H meter – [Elico]

Minispin – [ependrof of ACG]

Binocular- Microscope- [Olympus]

HPLC conditions

Chromatographic separation was achieved using reversed phase chromatography with isocratic elution. A C_{18} column was used. The mobile phase, consisting of acetonitrile, methanol and KH_2PO_4 (0.02M; pH 2.25) (3:1:96, v/v/v) was filtered and degassed through a 0.45µm filter and pumped at a flow rate of 1ml/min. The column temperature was maintained at 25 æ%C. The HPLC system consisted of a separation module [Model- 515 incorporating solvent degasser and 2-channel UV/vis detector [Model 2487; Waters]. The system was controlled using wu-feng (v 3.0) software. UV detection was carried out at 322nm.

Standard graph of 6-mercaptopurine

To prepare stock solution of 6-mercaptopurine (10mg) was dissolved in an appropriate amount of 0.1M NaOH and then it was diluted with water to 10 ml. From stock solution nine different concentrations as 10, 25, 50,100,250,500 ng and 1, 2.5,5 µg were prepared and calibrated (Fig 1). These concentrations were chosen to match those predicated samples [7].

Animals

Adult Wister albino rats (150 -200 gm) were obtained from (Mahaveer enterprises pvt.ltd). Animals were acclimatized to laboratory conditions for at least one week before using them for experiments and fed with standard rat diet with water *ad libitum*. Ethical clearance for the animal study was obtained from Institutional Animal Ethical Committee (146/1999/CPCSEA). The animals were housed in groups of four in polypropylene cages at an ambient temperature of 25 ± 1ºC and 45-55% relative humidity, with a 12:12 h light/dark cycle.

Acute toxicity studies

6-mercaptopurine suspended in 0.1% CMC was administered orally to group of overnight fasted rats at doses of 5, 50, 300, 2000mg/kg. The starting dose of 300mg/kg was selected based on OECD-423 guidelines (acute toxic class method). The effect of test substance is determined using a stepwise procedure, each step using three animals of a single sex (normally females). Absence or presence of compound-related mortality of the animals dosed at one step will determine the next step. Treatment of animals at the next dose is carried only with the confident evidence of survival of the previously dosed animals. Various parameters like weight, dizziness and LD_{50} were evaluated [8].

Tissue distribution studies

In these studies three groups were taken as 1, 2,4hr respectively. Each group contains four animals. Then each group 400mg/kg is administered orally of 6-mercaptopurine based on OECD-417 guidelines (Toxicokinetics). At respective time intervals the animals were sacrificed. Organs like liver, heart, kidney, brain, spleen, and lung were isolated [9]. The incurred organs were weighed for wet weight and homogenized in saline solution (600 mg/ml).

The obtained tissue homogenates were centrifuged at 9000×*g* for 10 min and stored at "20 æ%C until analysis, to 100µl of tissues samples, 300 µl of methanol was added. The resulting solution was thoroughly vortex-mixed for 10 sec. After centrifugation at 9000×*g* for 10 min, 20µl of the supernatant were injected into the HPLC system for analysis [10].

Results and Discussion:

The results from the current study indicate that after oral administration of 6-mercaptopurine rapidly distributed to various organs. 6-mercaptopurine showed disposition in

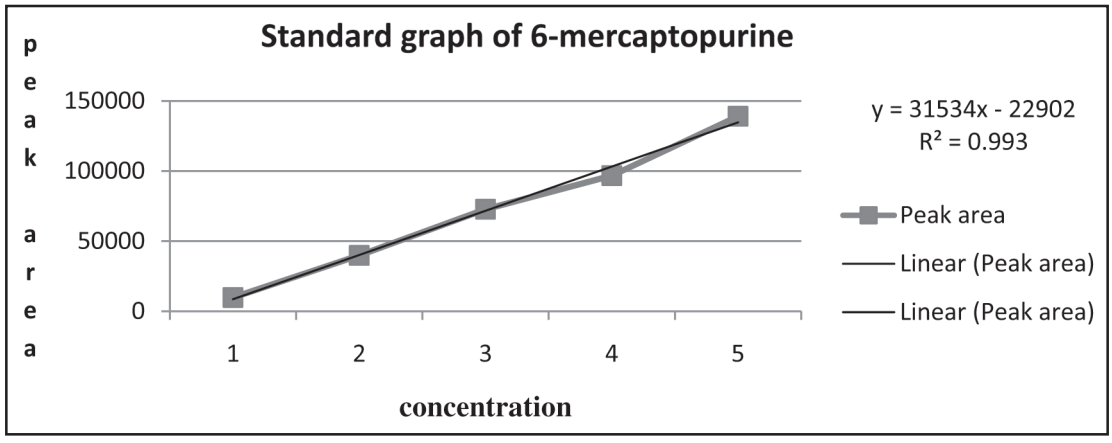

Fig 1: Standard graph of 6-mercaptopurine

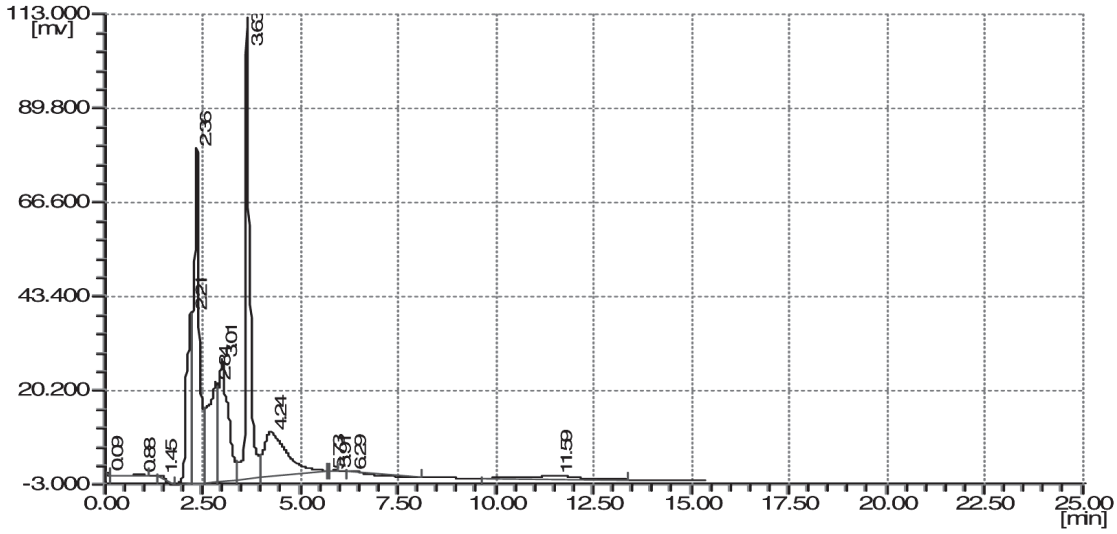

Fig 2: Chromatogram of blank plasma

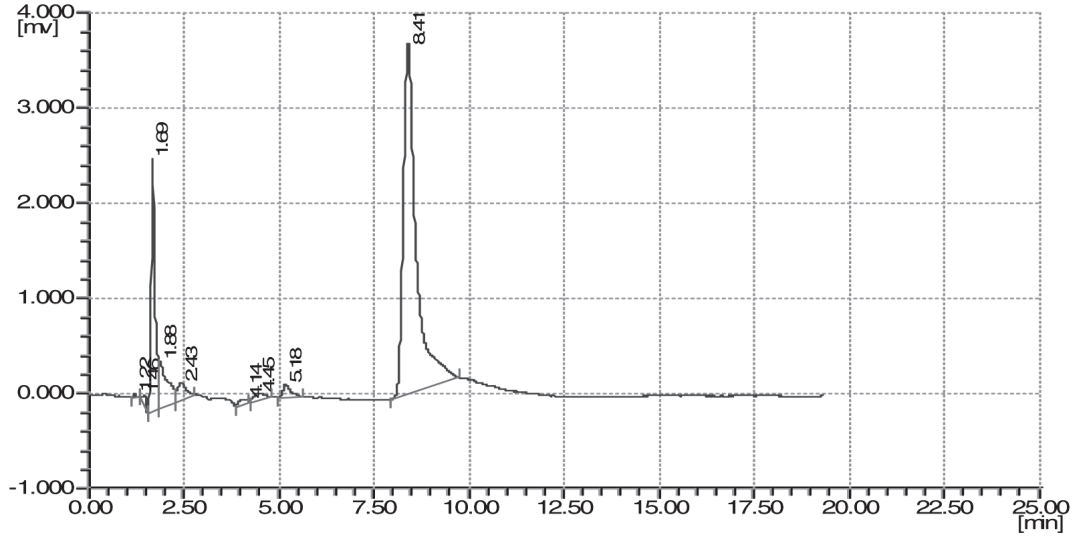

Fig 3: Chromatogram of plasma with drug sample

Table 1:
Concentration of 6-mercaptopurine in various tissues (ug/ml)

Organs at 400 mg/kg	Group-I (1hr) MEAN±STDV	Group-II (2 hr) MEAN±STDV	Group-III (4hr) MEAN±STDV
liver	0.568±0.061	1.155±0.048	0.288±0.036
kidney	0.120±0.187	0.053±0.010	0.028±0.010
lung	0.048±0.010	0.083±0.010	0.015±0.006
brain	0.020±0.008	0.020±0.008	0.038±0.010
heart	0.050±0.008	0.123±0.017	0.048±0.010
spleen	0.020±0.008	0.065±0.013	0.018±0.010

Table 2:

Toxicokinetic parameters after dosing 100, 200, 300 mg/kg of 6-mercaptopurine

Group	Cmax	Tmax	AUC	$t_{1/2}$	MRT	Cl
Group-1	0.77 ±0.13	2.0 ± 0.0	2.48 ± o.48	3.30 ± 1.59	4.98 ± 1.35	3340.83 ± 72.04
Group-2	0.86 ± 0.10	2.0 ± 0.0	3.46± 0.48	2.84± 0.31	4.58± 0.22	2356.22± 309.83
Group-3	1.21± 0.07	2.0± 0.0	4.31± 0.28	3.01± 0.69	4.51± 0.65	1869.63± 120.20

brain, heart, lungs, spleen, kidneys but in liver with greater fraction. The highest levels were evidenced in liver, lungs and kidneys at 2hr, and then it was decreased. The distribution of 6-mercaptopurine was depended on the blood flow or perfusion rate of the organ**.** On the contrary, increasing concentrations of 6-mercaptopurine in kidneys over time indicate that renal excretion might be a major elimination route for 6-mercaptopurine. Meanwhile, 6-mercaptopurine was found in low concentration in brain when compared to other tissues, suggesting that 6-mercaptopurine did not efficiently cross the blood–brain barrier. Nevertheless, the level of 6-mercaptopurine in heart, spleen was much lower than that in above-mentioned tissues, which indicated that 6-mercaptopurine might bind in less proportions with some target proteins in the heart and spleen. The LD_{50} value was determined for 6-mercaptopurine [OECD-417 guidelines] and was found to be 500mg/kg body weight. It was found that AUC, MRT, Cmax were increased as increase in dose. Therefore the maximum concentration of drug in blood was increased rationally with dose. More bioavailability of drug was seen with increase in AUC. $t_{1/2}$ was changed due to decreased metabolism leading to the reduction in clearance rate. Therefore, it might lead to hepatotoxicity and nephrotoxicity.

Conclusion

Data obtained from toxicokinetic and tissue distribution study of 6-mercaptopurine interprets elevated concentration of drug in liver leading to hepatotoxicity. But in brain it has very low concentration, indicating that there is less chance to cause neurotoxicity than other tissues like kidney. These studies will be helpful in determining the toxic effect and therapeutic dose setting of 6-mercaptopurine for clinical studies.

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Design of Tolcapone analogs for Parkinson's disease through binding free energy calculations

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ABSTRACT

Parkinson's disease (PD) is a neurodegenerative disease of relative lack of brain chemical neurotransmitter producing neurons in the pars compacta of the substantia nigra, Dopamine. The most common motor symptoms of the disease are resting tremor, bradykinesia, rigidity and postural instability and non-motor symptoms are sleep problems, depression, urinary problems, dementia and constipation. Catechol O-methyl transferase (COMT) plays an important role in the metabolism of catecholamine neurotransmitters and catechol estrogens. The present paper describes the molecular docking studies of novel drug Tolcapone which inhibits the activity of Catechol O-methyl transferase to slow the progression of PD and possibly improve the person's capabilities. Docking studies of tolcapone have been carried out in the active site using G.O.L.D software. Main objective is to study the effectiveness of tolcapone and its analogs for the treatment of parkinson's disease targeting Catechol O-methyl transferase with molecular docking studies and Binding free energy calculations using G.O.L.D., HYPERCHEM and OPENEYE SOFTWARE, from the molecular docking studies of seven analogues for the active site of COMT it was found that ligand 2 with substitution OCH_3 was the most effective analog in inhibiting the activity of Catechol O-methyl transferase.

Key words: parkinson's disease; catechol O-methyl transferase; tolcapone; molecular docking; binding free energies.

Introduction

Parkinson's disease (PD) is characterized by the unilateral onset of resting tremor in combination with varying degrees of rigidity and bradykinesia, a slowly expanding degeneration of neurons particularly in the mesencephalon. Parkision describes a disease "shaking Palsy", or ans "Paralysis agitans", involuntary tremulous motion, with lessened muscular power. Changes in a person's behavior can be a sign of damage to certain areas of the brain. He made the link between loss of dopamine (Catecholamine neurotransmitter) and PD.[1] The main reason for the PD is decreased levels of dopamine in the substantial nigral cells. The course of Parkinson's disease is unique for every individual, there are many symptoms, and most common motor symptoms of the disease are resting tremor, bradykinesia, rigidity and postural instability due to loss of pigmented cells in the substantia nigra pars compacts in patients with the encephalic form. However, besides the dopaminergic deficit, PD is now believed to be multicentric Neurodegenerative diseases.[2] PD pathology follows a specific sequence, starting in the dorsal motor nucleus of the vagus nerve and the olfactory bulbs and nucleus, followed by the locus coeruleus, less vulnerable nuclei and cortical areas gradually become affected. When PD is suspected, the diagnosis is usually confirmed with behavioral assessments and cognitive tests, often followed by a brain scan if available.

In this study, the Tolcapone and its analogs were designed and tested for their ability to modulate Catechol-o-Methyltransferase function through docking studies and Binding free energy calculations with the aim of explaining the differences in activity of the Tolcapone analogs as Catechol-o-Methyltransferase inhibitors.[3] Computational chemistry has developed into an important contributor to rational drug design. Quantitative structure activity relationship (QSAR) modeling results in a quantitative correlation between chemical structure and biological activity. QSAR studies have provided valuable insight into the design and development of tolcapone agents.[4]

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Materials and Methods

Determining the Analogs and energy optimization

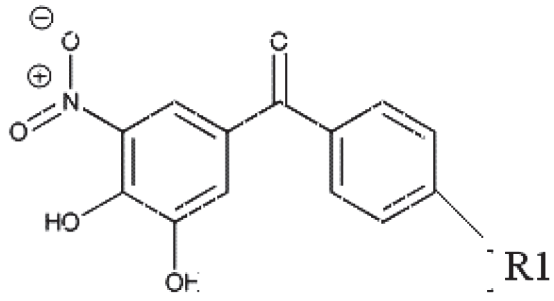
The initial step begins with the selection of analogs, which are then sketched and their energy was optimized to obtain stable structures. Tolcapone analogs are listed in Table 1.

Force –Field Selection Force field selection was done before invoking the Model Builder because it assigns atom types to each atom according to the force field that we specify. The force-field chosen is AMBER2.

Analog selection Tolcapone was the drug selected and modified. Replacing the hydrophilic region on the target molecule with other created six analogs of the drug functional groups (considered at random), the Tolcapone molecule and the six analogs are then studied by performing various energy simulation as well as QSAR calculations.[5] Tolcapone has been considered along with the 6 analogs for various studies as a kind of "Blank", so as to enable the comparative study of the analogs and to help us analyze relative superiority of the analogs to the initial drug itself. The analogs and the Tolcapone molecule itself is initially sketched meticulously using the draw tool in Hyperchem and then the 2D structure was converted into 3D form by clicking.[6]

Table 1

Different Analogs of Tolcapone



3,4-dihydroxy 5-nitro benzophenone

Ligand	R 1
1	CH_3
2	OCH_3
3	OH
4	NH_2
5	CN
6	CF_3
7	CH_2CH_3

Single point energy calculation Single point calculation determines molecular properties, such as energy or spin density, of a defined molecular structure. It gives the measure of the energy of a molecule without altering it.

Geometry Optimization The seven molecules are drawn in their respective 3D structures and the force field has been set, the next step is to optimize the molecule.[8] Energy Minimization or Optimization alters the geometry of the molecule and lowers its energy to yield a molecule of more stable conformation.

Molecular Mechanics Optimization: Parameters Algorithm: Polak- Ribiere (Conjugant gradient); Termination Condition: RMS gradient: 0.1 kcal/ (A^o mol) (Or) Maximum cycles: 600

Molecular Dynamics and QSAR Calculation

The simulations (Molecular Dynamics and Monte Carlo Simulations) are carried out cautiously and the values obtained are carefully documented along with the snapshots. Each simulation is performed starting with the geometrically optimized molecules.[9]

Molecular dynamics calculations Molecular dynamics is carried out to anneal the system to obtain a lower energy minimum. It simulates the evolution of a system over time, producing a trajectory of atomic positions and velocities. The dynamics are run in 3 optional steps like heat, run and cool.

Molecular Dynamics Options: Parameters Times- Heat time: 01 ps, Run time: 0.5 ps, Cool time: 0 ps and Step size: 0.0005 ps; Temperature- Starting temperature: 100 K, Simulation temperature: 300 K and Temperature step: 10 K. This was calculated for different heat times 1, 3, 6, 9, 12, 15, 18, 20.

The averages selected are Kinetic energy (EKIN), Potential energy (EPOT) and Total Energy (ETOT) and Temperature (TEMP). Once the heating phase is finished, the total energy remains constant, and the kinetic energy mirrors the potential energy.[7] When the dynamics run has completed, we can optimize the system to determine a new minimum.

Monte Carlo simulations The Monte Carlo method is used to simulate equilibrium properties. This method samples configurations from a Boltzmann- weighted distribution at a given temperature. At elevated temperatures, this technique may be used to move the molecular system of interest across potential energy barriers.

Monte Carlo Options: **Parameters** Times- Heat time: 0, Run steps: 100, Cool: 0 and Max delta: 0.05; Temperature- Simulation temperature: 300K

QSAR Calculations The QSAR properties like Log P, Volume, Hydration energy, Refractivity, and Polarizability

etc., are calculated for all the molecules. All the QSAR properties of Tolcapone analogs are listed in Table 2.

Optimization of solvent

The molecules are all solvated and then optimized in 4 stages. In the first stage only hydrogen atoms are selected and optimized, followed by water and then the part of the molecules that are reactive and finally the entire molecule it-self in the solvated environment.[10] Each time the optimization is done in two steps: 1) Steepest Descent - Termination condition-RMS gradient: 0.000001 kcal/ (A^o mol) (or) Cycles: 500. 2) Conjugate gradient (Polak-Ribiere) -Termination condition- RMS gradient: 0.000001 kcal/ (A^o mol) (or) Cycles: 2000.

The four stages of optimization are performed in the same order as mentioned and each stage is carried out using the previous stages output as its input. The third step is begun using the optimized structures from step of Analog & energy determination.[11] The atoms involved in hydrogen bonding interactions along with bond length are depicted in Table 3.

Adding periodic box The six molecules being studied are solvated by placing them in a periodic box of water molecules to simulate behavior in an aqueous solution, as in a biological system.

Periodic Box Options: Parameters Smallest Box Enclosing Solute -X: 5.8358555, Y: 2.9186375, Z: 9.2917614; Periodic Box Size- X: 18.70136, Y: 18.70136, Z: 18.70136; Minimum number of water molecules: 216; Minimum distance between solvent and solute atoms: 2.4 A^o

Molecular Mechanics Force field: Parameters

AMBER- 1-4 Scale Factors:
Electrostatic: 0.5 Outer radius: 9.35068
Vander Waals: 0.5 Inner radius: 5.35068

To solvate a system with Hyper Chem we specify a rectangular box or cube of equilibrated water molecules. Now we defined the dimensions of the box, solute was placed in the center, and defined the minimum distance between the solvent and solute atoms.

Optimization by selecting only the Hydrogen's In the first stage, only hydrogen's in the system were allowed to relax. This step relaxes the hydrogen atoms prior to relaxing heavy atoms. It was performed because the hydrogen locations are not specified by the X-ray structure and because adjustments in hydrogen atom locations are necessary to improve hydrogen bond geometries.

Geometry optimization selecting only water molecules In the second stage, only the water molecules were minimized, keeping the inhibitor and the protein fixed.[12] The purpose of this step is to relieve any bad contacts involving water molecules in the initial solvated system.

Optimization selecting active region (Modified hydrophilic region) of Ligand The third stage was performed for all the modified ligand-protein complexes. In this stage, all atoms of the protein were fixed and atoms common to the ligand in the crystal structure complex and the modified ligand were also fixed, while allowing the modified group in the ligand and the solvent to move during optimization. This stage allows for the relaxation of the modified group with respect to the protein and establishes the preferred interactions (e.g., hydrogen bonds).

Optimization of the ligand molecule in its solvated state Now the entire molecule along with the water molecules are selected and optimized. The optimized structure for the solvated system might only be a local minimum.

Energy calculations

Periodic box is removed and Energy is calculated for the ligand molecule and along with it QSAR properties of a compound was calculated.

Table 2
QSAR properties of Tolcapone analogs

S.No	Properties	Ligand						
		R=CH_3	R=OCH_3	R=OH	R=NH_2	R=CN	R=CF_3	R=C_2H_5
1.	Surface Area(grid) A^{02}	442.03	474.39	443.81	448.06	458.65	476.95	482.30
2.	Volume A^{03}	729.41	776.57	721.98	732.05	753.89	781.18	796.37
3.	HydrationEnergy K.Cal/Mol	-21.10	- 23.94	-29.52	-27.47	-26.27	-21.48	-20.35
4.	Log P	-3.39	-4.53	-4.56	-5.26	-3.82	-2.97	-2.99
5.	Refractivity A^{03}	78.36	80.45	75.68	77.63	79.06	79.29	82.96
6.	Polarizability A^{03}	27.42	28.06	26.22	26.94	27.44	27.15	29.25
7.	Mass a.m.u	274.25	290.25	276.23	275.24	285.24	328.22	288.28

Optimization of Protein – Ligand complex

The target for the drug was obtained, and it structure was obtained from PDB (Protein Data Bank). The structure contained 6 chains and using Swiss PDB viewer, the A chain was separated with the ligand bound to it.[13] The ligand was verified and then modified into the various analogs. The ligand protein complex is then subjected to geometric optimization. The H atoms were initially selected, followed by the functional group of the ligand molecules and then the entire protein molecule considered with the ligand. The optimization was carried out in 2 steps:

1) Steepest descent with RMS as 0.000001 or 100 cycles. 2) Polak – Ribere with RMS as 0.000001 or 500 cycles. Then, molecular dynamics of the complex is calculated at 2 ps heat time.

Geometric optimization and Molecular dynamics

Geometric optimization is done to the ligand molecule and the energy of the ligand is noted and along with it molecular dynamics of the ligand is calculated.

Docking

The protein-ligand complex from PDB is extracted. From the protein-ligand complex the protein and ligand molecules are separated and the protein was docked with the ten molecules under scrutiny using GOLD software.[12,23] The results for docking were shown in Table 4.

Preparing input for docking To the protein molecule, hydrogen's are added and optimization was performed till convergence. Similarly, all the ligand molecules are optimized to convergence after hydrogen's are added.[14] The input for docking is now ready. The molecules were docked using GOLD software, which works, on Genetic Algorithm.

Gold Score fitness function Gold Score performs a force field based scoring function and is made up of four components:

1. Protein-ligand hydrogen bond energy (external H-bond)
2. Protein-ligand Vander Waals energy (external vdw)
3. Ligand internal Vander Waals energy (internal vdw)
4. Ligand intramolecular hydrogen bond energy (internal-H- bond).

The external vdw score is multiplied by a factor of 1.375 when the total fitness score is computed. This is an empirical correction to encourage protein-ligand hydrophobic contact. The fitness function has been optimized for the prediction of ligand binding positions .

Gold Score =
S (hb_ext) + S (vdw_ext) + S (hb_int) + S (vdw_int)

Fitness=
S(hb_ext) + 1.3750*S (vdw_ext) + S (hb_int) + 1.0000*S (vdw_int)

Table 3
The atoms involved in hydrogen bonding interactions along with bond lengths

S.No	Molecules	No. of Hydrogen's	Atoms		Bond length(ú)
			COMT residue	atom	
1	**Tolcapone**	1	GLU 18:O	H_{23}	1.795
2	**Analog 2**	4	ASN11:H	O_{28}	2.69
			ASP3:O	O_{12}	2.362
			ASP3 :O	O_{18}	2.634
			LYS36:H	O_{17}	2.377
3	**Analog 3**	2	HIS 12:H	O_{28}	1.907
			LYS 36:H	O_{16}	1.926
4	**Analog 4**	2	ASP30:H	H_{28}	2.242
			LYS36	O_{16}	2.513
5	**Analog 5**	3	ASP 30:O	H_{27}	2.667
			LYS36:H	O_{16}	2.154
			TYR32	O_{16}	2.127
6	**Analog 6**	2	ASP 30: O	H_{27}	2.582
			LYS36:H	O_{16}	2.178
7	**Analog 7**	3	ARG 8: O	H_{28}	2.936
			SER24:H	O_{16}	2.785
			TRY32:H	O_{16}	2.062

Table 4

Docking results

Molecule	Fitness	S(hb_ext)	S(vdw_ext)	S(hb_int)	S(vdw_int)
Tolcapone	63.14	9.49	39.30	0.00	-0.40
Analog 2	61.48	11.12	38.85	0.00	-3.07
Analog 3	61.34	10.00	37.45	0.00	-0.15
Analog 4	59.89	12.13	34.93	0.00	-0.28
Analog 5	61.38	10.83	37.15	0.00	-0.54
Analog 6	64.65	10.24	39.82	0.00	-0.34
Analog 7	60.25	6.44	39.97	0.00	-1.15

Where S (hb_ext) is the protein-ligand hydrogen bond score, S (vdw_ext) is the protein-ligand van der Waals score, S (hb_int) is the score from intramolecular hydrogen bond in the ligand and S (vdw_int) is the score from intramolecular strain in the ligand.

8. Binding Free energy calculations

The values of the binding energies are finally calculated and recorded. These binding energy values determine the activity of the molecules when bound to the protein molecule. [15] Binding free energy calculations shown in Table 5. The minimized structures for all the 10 inhibitors in the complex and solvated states were used for calculating the following energy variables:

$$E_{bind}\ (intra) = E_{com}\ (intra) - E_{sol}\ (intra)$$

$$E_{bind}\ (inter) = E_{com}\ (inter) - E_{sol}\ (inter)$$

Where, E_{bind} (intra) and E_{bind} (inter) are relative intra and intermolecular binding interaction energies of a ligand, respectively, and where E_{com} (intra), E_{com} (inter), E_{sol} (intra), and E_{sol} (inter) are intra and intermolecular interaction energies of a Ligand in the complexed and solvated states, respectively. Relative differences in intra, intermolecular and total binding interaction energies for a pair of Ligand L1 and L2 are given by

$$E_{bind}\ (intra: L1, L2) = E_{bind}\ (intra: L2) - E_{bind}\ (intra: L1)$$

$$E_{bind}\ (inter: L1, L2) = E_{bind}\ (inter: L2) - E_{bind}\ (inter: L1)$$

$$E_{bind}\ (tot: L1, L2) = E_{bind}\ (intra: L1 - L2) + E_{bind}\ (inter: L1 - L2)$$

Where, E_{bind} (tot: L1 L2) is the total relative difference in the binding energies of L1 and L2. Hence, an agreement in the overall trends between the experimental measurements and the energy minimization results were expected. The relative differences in the binding affinities measured experimentally (E_{bind} (expt)) shown in Table 6 and are compared with the relative binding affinities calculated using minimization methods and for all the cases the minimizations results provided qualitative agreement with experimental results.

Docking method

Docking was carried out using G.O.L.D 3.0.1 (Genetic Optimization of Ligand Docking) software which is based on genetic algorithm (GA). This method allows partial

Table 5

Binding free energy calculations

Ligand	x_1	x_2	y_1	y_2	$x_1+x_2=X$	$y_1+y_2=Y$	$Y-X=Z$
Tolcapone	16.24	-29.022	16.377	-48.71	-12.78	-32.33	-19.55
Analog2	16.953	-30.5789	9.001	-50.45	-13.625	-41.44	-27.82
Analog3	15.221	-34.873	16.306	-47.45	-19.652	-31.14	-11.49
Analog4	15.771	-36.169	6.354	-47.06	-20.398	-40.70	-20.31
Analog5	15.807	-36.169	16.021	-47.98	-20.362	-31.95	-11.59
Analog6	10.900	-30.881	6.343	-50.06	-19.981	-43.71	-23.73
Analog7	16.333	-28.794	16.056	-46.41	-12.461	-30.35	-17.89

Table 6

Relative binding free energy calculations

Molecule	Relative binding Free energy
Analog 2	-8.27
Analog 3	8.06
Analog 4	-0.76
Analog 5	7.96
Analog 6	-4.18
Analog 7	1.66

flexibility of protein and full flexibility of ligand. The compounds are docked to the active site of the Catechol-o-Methyltransferase. [16-19] The interaction of these compounds with the active site residues are thoroughly studied using molecular mechanics calculations. The parameters used for GA were population size (100), selection pressure (1.1), number of operations (10,000), number of island (1) and niche size (2). Operator parameters for crossover, mutation and migration were set to 100, 100 and 10 respectively. Default cutoff values of 3.0A° for hydrogen bonds and 6.0A° for Vander Waals were employed. During docking, the default algorithm speed was selected and the ligand binding site was defined within a 10 A° radius. The number of poses for each inhibitor was set 100, and early termination was allowed if the top three bound conformations of a ligand were within 1.5A° RMSD. After docking, the individual binding poses of each ligand were observed and their interactions with the protein were studied. The best and most energetically favorable conformation of each ligand was selected. The results for docking were shown in???????????

Results and Discussion

The crystal structure of catechol O-methyl transferase was taken from the Protein Data Bank (PDB_ID:1GQR). Ligands were removed from the binding site and the chain A was selected for docking studies. Hydrogen atoms were added to the protein. The molecular docking method was performed using the Gold version 3.0.1 program to study the binding orientation of Tolcapone and its analogs into the COMT structure. The docking experiments were performed using the active binding site of catechol O-methyl transferase (Figure.1). The binding site identification was carried out using Cast P server. A new program, CAST, for automatically locating and measuring protein pockets and cavities, based on precise computational geometry methods. CAST identifies and measures pockets and pocket mouth openings, as well as cavities.[20] The program specifies the atoms lining pockets, pocket openings, and buried cavities; the volume and area of pockets and cavities; and the area and circumference of mouth openings.

The geometrical optimization of Tolcapone and its analogs are done using hyperchem and the energy

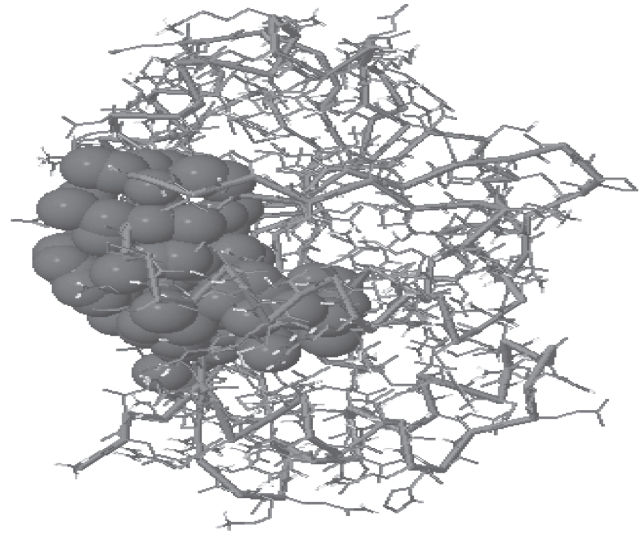


Fig. 1. Active Site of COMT

interactions of the analogs [21] when interacted with the solvent and protein are noted. Along with it the QSAR properties of different analogs are calculated and tabulated in Table 2.

The selected docked conformations of Tolcapone and its analogs are shown in Figure 2 (2A-2G). The location of the docked compounds agreed well with that of activity of Tolcapone and its analogs which would contribute to their inhibitory potency. [22] The docked conformations formed an H-bonding interaction with catechol O-methyl transferase in the active site. In the binding pocket, common H-bonding interactions were formed between all docked ligands and GLU 18. The specific H-bonding interaction with ASN11, ASP3, LYS36, was only found in Ligand 2. In order to explain the binding of these compounds, the H-bonding interactions with the other surrounding residues in the binding pocket were also investigated. In Figure 2A, strong H-bonding interaction between the oxygen atom of GLU18 with the 28th hydrogen atom, The hydrogen atom of ASN11 and 28th oxygen, oxygen atom of ASP3 and 12th oxygen atom , oxygen atom of ASP3 and 18th oxygen atom, hydrogen atom of LYS36 and 17th oxygen atom forms a hydrogen bond with analog 2 when docked with Catechol-o-Methyltransferase. [24-26] The atoms involved in hydrogen bonding interactions along with bond lengths were tabulated in Table 3. The docking results were noted. Results Tabulated in Table 4 suggest that analog 2 has highest fitness (63.32 Kcal/mol) than other tolcapone analogs.

This is further confirmed by using the binding free energy calculations, the solvent intra energy calculations are noted as x_1, Poission-Boltzman solvent mode values are noted as x_2, protein ligand intra energy noted as y_1 and docking inter energy noted as y_2. Then the values of x_1 and x_2 of solvent are added to obtain X and y_1 and y_2 of protein are added to obtain Y. The value of X is subtracted from Y

Fig. 2A-2G: Docked Tolcapone analogs

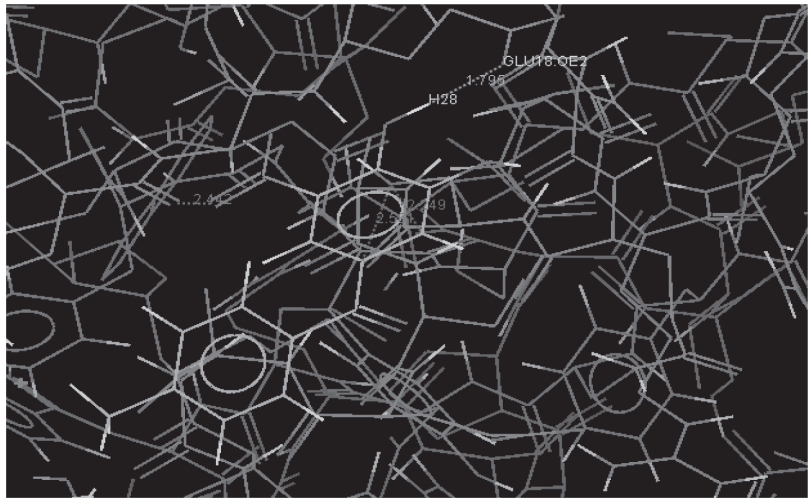


Fig. 2A. Docking of Ligand 1

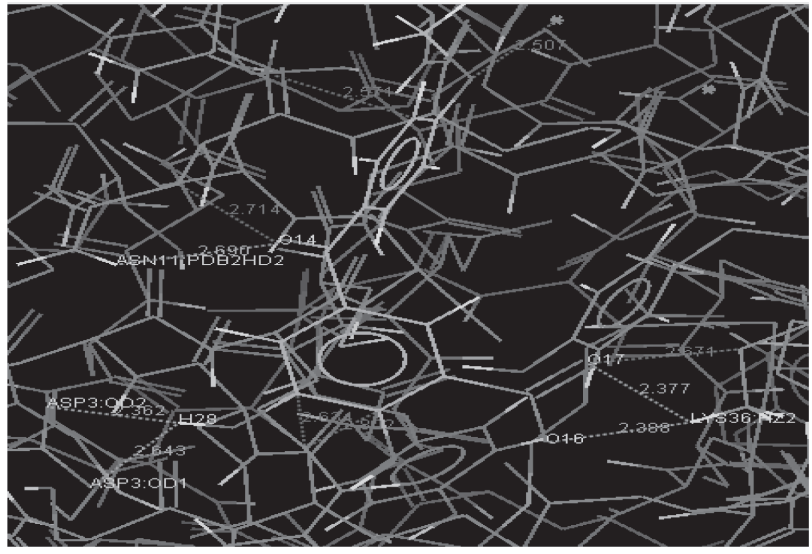


Fig. 2B. Docking of Ligand 2

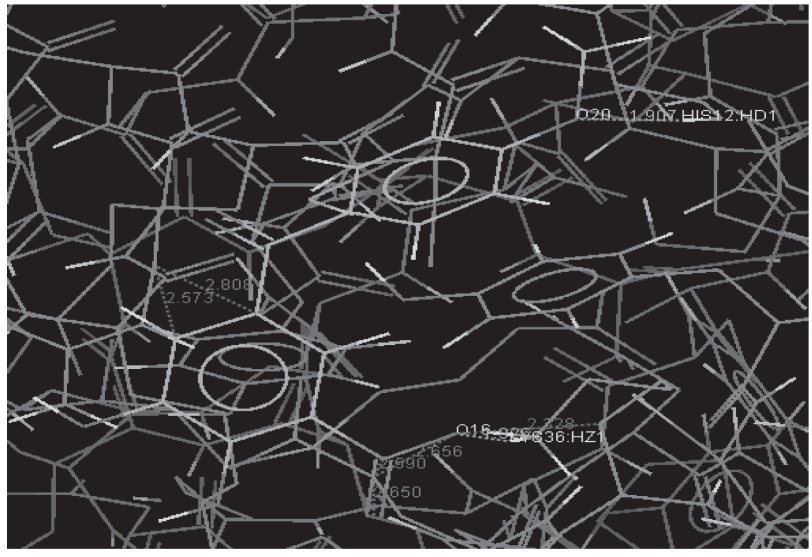


Fig. 2C. Docking of Ligand 3

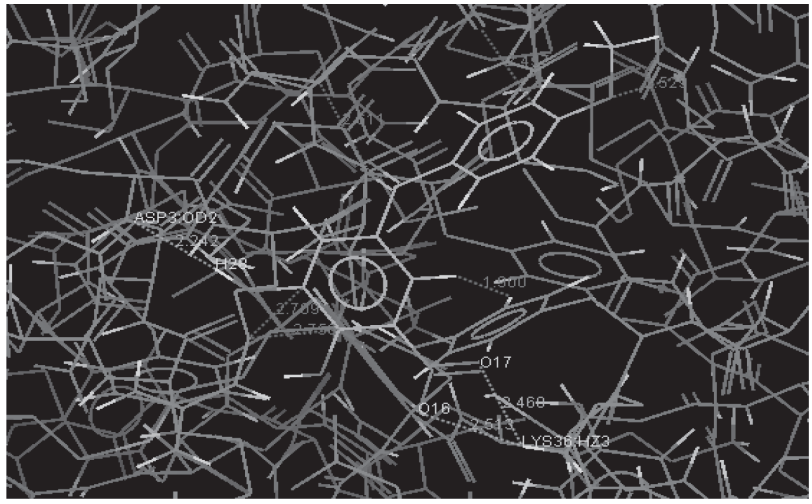


Fig. 2D. Docking of Ligand 4

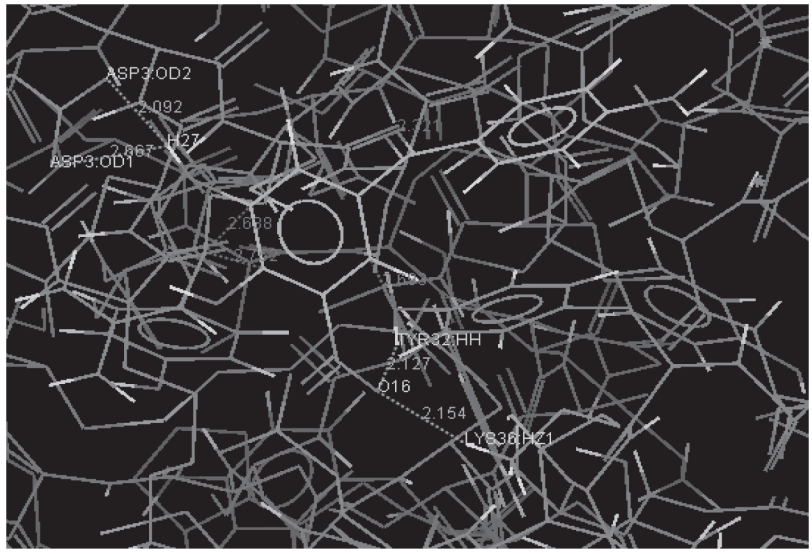


Fig. 2E. Docking of Ligand 5

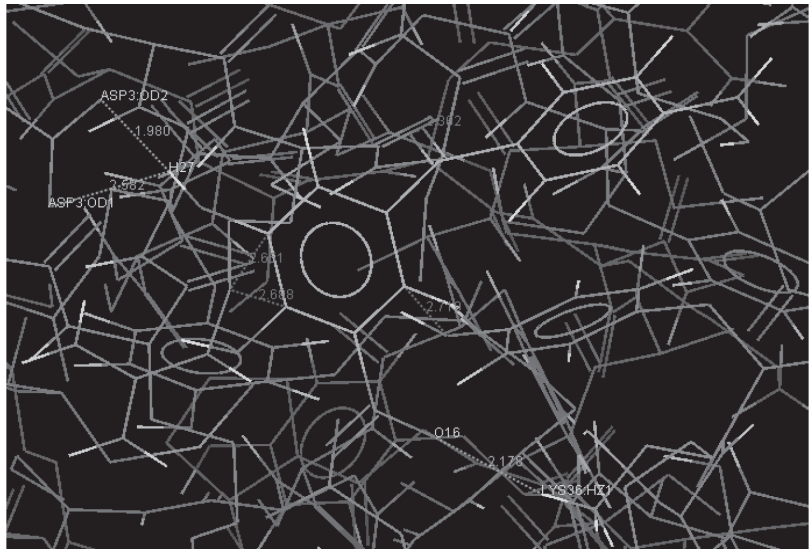


Fig. 2F. Docking of Ligand 6

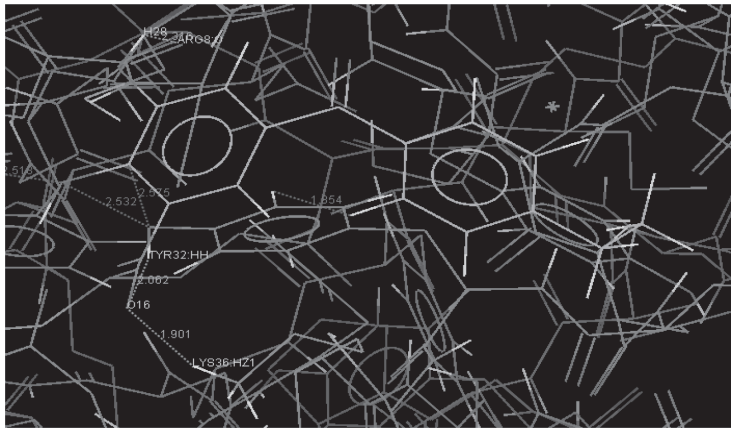


Fig. 2G. Docking of Ligand 7

and noted as Z as shown in Table 5. The Z value of tolcapone is considered as standard and it is subtracted from the Z values of the ligands to obtain the binding free energy values which are tabulated in Table 5. The values obtained from the binding free energy calculations the analog 2 has the more negative value when compared to tolcapone and its analogs suggesting that the reaction is spontaneous.

Conclusion

A comparison of the calculated binding affinities for structurally similar Inhibitors, TOLCAPONE and its analogues, to CATECHOL-O-METHYLTRANSFERASE, indicates that the molecular mechanics methods gave suitable analogues. These results clearly indicate that one can use molecular mechanics based methods for qualitative assessment of relative binding affinities for speeding up drug discovery process by eliminating less potent compounds from synthesis.

A comparison of QSAR properties of the different analogs is done and from the results it is observed that log P value of the analog 4 with the constituent NH_2 is more where as the log P value is less for the analog 6 with the constituent CF_3 along with it the refractivity and polarisability values are higher in the case of analog 7 and lowest value is observed with the analog 3.

In case of docking scores which was calculated using the G.O.L.D software it is observed that the analog 2 with the constituent OCH_3 has the highest fitness score when compared to the other ligands where as the least fitness score is observed with the analog 3 with the constituent OH. From the above studies of binding free energy calculations, docking method and GOLD it was concluded that ligand 2 (OCH_3) was found to be most effective analog of tolcapone for binding to catechol-o-methyl transferase.

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Synthesis and Characterization of 2-acetylthiophene benzoylhydrazone and its applications for the Spectrophotometric determination of mercury and copper in environmental, soil, biological, green leafy vegetable and medicinal samples

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ABSTRACT

The analytical properties of 2-acetylthiophene benzoylhydrazone (ATBH) are described for the first time. The reagent has been synthesized and chararacterized by IR, NMR, Mass Spectral data. ATBH complexes with mercury (II) and copper (II) in acidic medium (pH 6.0 Sodium acetate–acetic acid buffer) to form deep yellow coloured, 1:2 complexes with the molar absorptivities of mercury and copper complexes are 1.5 x 10^4, and 2.0 x 10^4 $Lmol^{-1}$ cm^{-1} respectively. These colour reactions have been investigated for the spectrophotometric determination of mercury(II) and copper (II) in aqueous medium and a number of environmental, biological, medicinal and soil samples. The results of the proposed method in the analysis of the samples were comparable with those obtained by dithizone method.

Key words : Spectrophotometric determination, benzoylhydrazone, mercury(II), copper(II) various samples.

Introduction

The non analytical applications of physiologically active hydrazones are in the treatment of several diseases such as tuberculosis, leprosy and mental disorder. The tuberculostatic[1,2] activity of aroylhydrazones (R-CH=N-NH-CO-R[1]) is due to formation of stable chelates with transitional metals present in the cells. Thus many vital enzymatic reactions catalyzed by these metal ions cannot take place[3-5] in presence of hydrazones. Hydrazones are also act as herbicides, insecticides, nemotocides, redenticides, plant growth regulators etc,. On the other hand they are also act as good analytical reagents[6] in the detection, determination and isolation of compounds containing the carbonyl group. Although hydrazones are used in the spectrophotometric determination of metal ions, hetero cyclic hydrazones are not exploited much. It will be of interest to investigate the analytical properties of hydrazones derived from sulphur containing heterocyclic compounds.

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It is well known that among the trace metals mercury is one of the most hazardous pollutants in the marine environment. All though all forms of mercury (e.g. elemental inorganic and organic[7]) can have adverse health effects at sufficient high doses, their toxicities differ depending on the form. Compared with other mercury methyl mercury (MMHg) has been observed in marine life and humans in minimata, japan[8,] in marine birds in Sweden[9], and in human in Iraq[10]. Consumption of marine fish and sea food products is the principal pathway by which human are exposed to very toxic organomercurial, MMHg[11,12].

Copper is an important element in biological, industrial and environmental chemistry. Copper plays a key role in biological processes (e.g. Synthesis of haemoglobin, oxidases, enzymes, etc.). It is an important constituent of proteins and enzymes. It is essential for mammals in the synthesis of hemoglobin. Though copper is an essential element, it become hazardous when present in excess. Discharge of copper containing waste into environment leads to the natural imbalance of the ecosystem. Elevated level of copper in soil also affects the growth and

metabolism of plants[13]. The excessive accumulation of copper in liver, kidney, brain and cornea[14] leads to failure of liver, mall function of kidney and various neurological abnormalities (Wilson's disease symptoms). For these reasons the analytical monitoring of mercury and copper in environmental, biological, industrial, and leafy vegetable and food samples is extremely important.

This paper describes the non-extractive spectrophotometric determination of mercury (II) and copper (II) as their ATBH complexes in aqueous medium. A close literature survey reveals that ATBH has so far not been employed for the analytical determination of mercury (II) and copper (II).

Materials and Methods

Preparation of reagent

The reagent (ATBH) was prepared by simple condensation of 1 mole of 2-acetylthiophene and 1 mole of benzoylhydrazide in a 250-ml Erlenmeyer flask, a hot methonolic solution of 2-aecetylthiophene (0.04069 mol, 4.7 ml dissolved in 15 ml of methanol and benzoylhydrazide 0.04069 mol, 6 gram of substance dissolved in 20 ml of hot distilled water and 5 ml of sodium hydroxide) were mixed and heated under reflux for 5 hours. Shiny pale yellow crystals were separated out on cooling the reaction mixture. The crystals were collected by filtration and washed with hot water and with 1: 1 cold methanol. The compound was recrystallized and dried in vacuuo. Yield 82 percent, M.P. 186 – 189^0 C.

Characterization of ATBH

The compound was chararacterized by IR, NMR, Mass and UV spectral analysis.

Infrared spectrum of ATBH shows strong bands at 3216(s), 3036(m), 2924 (m), 1650 m), **1590 (s)**, 1577(m), 1511 (m), 1484 (m), 1272(s), cm^{-1} respectively corresponding to υ(NH) secondary, υ (C-H) aromatic stretch(phenyl), υ (C-H) aliphatic stretching, υ(C=O) hydrazine, **υ(C=N) azomethine,** υ(C-C) aromatic ring, υ(C-C) aromatic ring, υ(C-C) aromatic ring, υ(C-N) stretch vibrations respectively.

^{1}H-NMR spectrum of ATBH ($CDCl_3$+DMSO-d_6) showed signals at 2.36 (3H, s), 7.0-7.9 (8H, m) and 8.9(1H, s) due to CH_3, furan and phenyl protons, -NH(imino) groups of hydrazone respectively.

Mass spectrum of ATBH shows molecular ion peak is observed at *m/z* value of 245. This corresponds to molecular ion peak. Other peaks due to the loss of methyl radical, thiophene radical and C_6H_5CO radical are observed at *m/z* values of 228, 161 and 140 respectively.

The pKa values of ATBH

The pKa values of ATBH were determined by recording the UV-Visible spectra of micro molar (4 x 10^{-6} M) solution of the reagent at various pH values and by taking the arithmetic means of the values obtained from the measurements at different wavelengths determined spectrophotometrically using Phillip and Merritt method[15]. The values of deprotonation of ATBH are 5.6 (pK_1) and 8.5 (pK_2) corresponding to the formation of enol form and conjugated mono anion form respectively.

The reagent solution (0.01M) was prepared by dissolving 120 mg of the compound in 50 ml of DMF and it is stable for more than 2 hours.

0.2M sodium acetate – 0.2M acetic acid (pH 4.6) was used in the present study. The standard Hg(II), Cu(II) solutions (1 x 10^{-2}M) were prepared by using analytical reagent grade $HgCl_2$ and $Cu(CH_3COO)_2$ H_2O respectively. The stock solution of mercury(II) and copper(II) were standardized using titrimetric and gravimetric methods respectively.

Shimadzu 160A UV visible spectrophotometer equipped with 1.0 cm quartz cells and Elico Model LI-120 pH meter were used in present study.

The reactions of some important metal ions were tested at different pH values. The samples were prepared in 25-ml volumetric flask by mixing 10ml buffer, 1ml of metal ion and 1.5ml of 0.001M ATBH solution. The reaction mixture was diluted to mark with distilled water. The absorbance was measured in 350-600 nm range against the reagent blank.

An aliquot of metal ion in the Beer's law validity range (0.40-4.01 µg/ml of Hg(II), 0.127-1.27 µg/ml of Cu(II)),10ml of buffer solution (pH 6.0 for Hg and pH 6.0 for Cu) 2.5ml of DMF and i.5ml of 1x10^{-2}M ATBH solution were taken in 25-ml standard flask and diluted to the mark with distilled water. The absorbance of the coloured solution as measured at corresponding wavelength (415nm for Hg, 415nm for Cu) against reagent blank. Calibration graph were prepared. The measured absorbance values were used to compute the amount of mercury and copper present in the unknown solution.

Determination of mercury, copper in various water, soil, biological and leafy vegetable samples

The present method is applied for the determination of Hg (II) and Cu(II) in water samples, soil samples and biological samples.

Water samples[15,20] Each filtered (with whattman No. 40) water sample (250 ml) was mixed with 10 ml of concentrated nitric acid in a 500 ml distillation flask. The sample was digested in the presence of an excess potassium permanganate solution according to the method recommended by Fifield *et al.*,[16]. The solution was cooled and neutralized with dilute NH_4OH solution. The digest was transferred into a 25-ml calibrated flask and diluted upto the mark with deionized water. The results were given in Table 2.

Recommended procedure

A known aliquot of the sample solution was taken in a 25 ml standard flask containing buffer solution of pH 6.0 and 6.0, and reagent [ATBH; 1.5ml 1 x 10^{-2}M] solution and made upto the mark with distilled water. Absorbance of the solution was measured at $ë_{max}$ against the reagent blank. The absorbance values were referred to the predetermined calibration plot to compute the amount of mercury.

Soils samples[16,20] A 2 g weight of soil, 5 – 7 ml of concentrated H_2SO_4 and an excess of $KMnO_4$ are mixed in a conical flask equipped with a reflux condenser. The crystals of $KMnO_4$ are added slowly in small portions, while stirring. It is heated until vapours of SO_3 are evolved. After cooling down, 10 ml of distilled water are added. The excess of $KMnO_4$ and manganese oxides are eliminated by adding H_2O_2. Iron is isolated by precipitation as hydroxide. After filtration, the solution is transferred into 25-ml standard flask and the volume is brought to the mark with distilled water. Aliquots of this solution were taken for analysis by following procedure given above. The results were given in Table 2.

Biological samples[17,20]A 2 – 5 g of dried fish and sheep liver samples were taken in a 250 ml beaker. A 6 ml of concentrated nitric acid was added and gently heated for half-an-hour. After the disappearance of the froth, 6 ml of 1: 1 nitric acid and perchloric acid were added. The contents were digested for one hour and repeatedly treated with 6 ml portions of nitric acid and perchloric acid mixture until the solution becomes colourless. The acid solution was evaporated to dryness and the resulting white residue was dissolved in minimum volume of 1M nitric acid and made upto the volume in a 50-ml volumetric flask[18]. Aliquots of this solution were taken for analysis by following recommended procedure given above. The results were given in Table 2, 3.

Vegetable samples[19,20] Dry ashing method was used in the analysis of organic samples. A 10 g of dried leafy vegetable sample was taken in a silica dish. The sample was heated over a low burner until the material chars. The charred mass was moistened with 1 : 1 HNO_3. Occasionally a 20 percent solution of magnesium nitrate was used for this purpose, particularly if the ash content is very low. Again evaporated to dryness, and transferred to a muffle furnace. The temperature to about 500^0C was reached in the course of about 3 hours. Heating was continued until the ash becomes white. The dish was cooled and the ash was dissolved in a 5 ml portion of 1: 1 HCl. Distilled water was added amounting to about twice the volume of acid added. The solution was filtered to remove any insoluble residue and washed with 1: 4 HCl. The solution was diluted to 50 ml in a standard flask. Aliquots of this sample were taken for the determination of copper by following procedure given above. The results were given in Table 3.

Results and Discussion

The reagent 2-acetylthiophene benzoylhydrazone is easily prepared under reflux conditions. A 0.001M solution of ATBH is stable for more than two hours.

In buffer medium (pH 6.0), the ligand presumably exists in enolic form and coordinates the divalent metal ion as mono anion. The reagent gives intense colour reaction only with mercury and copper and show maximum absorbance at 415 and 415 nm respectively. The reagent (ATBH) is considered as potential reagent for selective spectrophotometric determination of mercury(II) and copper(II). The structure of ATBH is given in Fig 1.

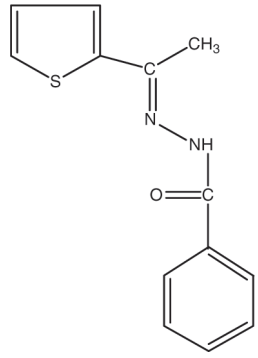


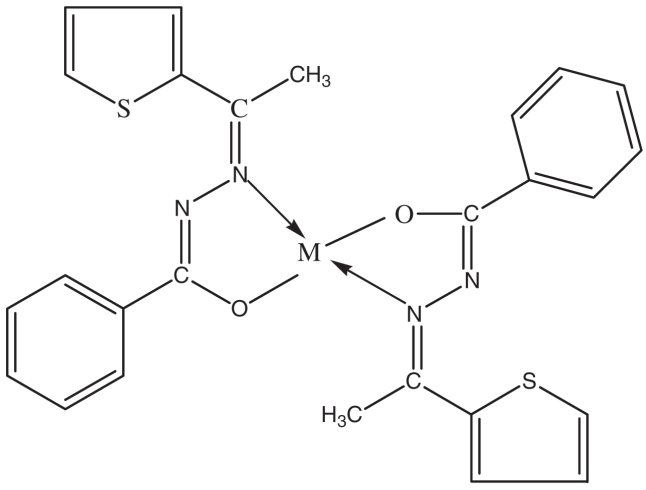
Fig 1. Structure of ATBH

Determination of mercury(II) and copper(II)

Mercury(II) and copper(II) reacts with ATBH in acidic pH (6.0) to give coloured complexes. The colour reaction is instantaneous even at room temperature. The order of addition of reagent, metal ion, buffer, 2.5 ml of DMF. The absorbance of the coloured complex remains constant for more than 2 hours. A 10-fold molar excess of the reagent is adequate for full colour development. Addition of excess of reagent has no adverse effect on the absorbance of the complexes.

The system obeys, Beer's law in the concentration range 0.40 – 4.01 µg/ml of mercury and 0.12 – 1.27 µg/ml of copper. The molar absorptivity and Sandell's sensitivity of the methods for Hg(II) and Cu(II) are found to be 1.5 x 10^4 and 2.4 x 10^4 $Lmol^{-1}$ cm^{-1} and 0.134, 0.0032 µg/cm^2 respectively. The specific absorptivity of the system 0.075 and 0.315 ml/$g^{-1}cm^{-1}$ Hg(II) and Cu(II) respectively. The relative standard deviation for ten replicate analysis of Hg(II) and Cu(II) are 0.64 and 0.9 percent respectively.

Job's and Molar ratio methods gave the composition of the Hg(II) and Cu(II) complexes as 1:2, 1:2 (M : L) respectively. The stability constants of Hg(II) and Cu(II) complexes calculated by Job's method are found to be 2.1 x 10^{13} and 8.3 x 10^9 respectively. The structure of Hg/Cu - ATBH complex is in Figure 2.



M = Hg or Cu

Fig 2. The structure of Hg/Cu – $(ATBH)_2$ complex

Interferences

The effect of some of the ions which often accompany mercury and copper has been studied by adding different amounts of anions and cations to 2.0, 1.52 (ppm) µg/ml of mercury and copper in solution. The colour developed as described in the recommended procedure. An error of ± 2 percent in the absorbance reading is considered tolerable. The results are given in Table 1.

Applications

The present methods (ATBH) were applied for determination of mercury (II) and copper (II) when present alone and present in various water, soil, biological, green leafy vegetable and medicinal samples, and the results were comparable with those obtained by dithizone[20] method.

Table – 1

Tolerance limitsof foreign ions in the determination of 0.80 and 0.6354 µg/ml of Hg(II) and Cu(II) resepectivley

Ion added	Tolerance limit (¼g/ml) Hg	Tolerance limit (¼g/ml) Cu	Ion added	Tolerance limit (¼g/ml) Hg	Tolerance limit (¼g/ml) Cu
Citrate	544.2	1088	U(VI)	95.2	95.2
Tartrate	296	592.3	W (VI)	59	367.6
Iodide	504.4	507.6	Sn (II)	47.5	-
phosphate	392	392	Cd (II)	27	27
Sulphate	192	384	Mn (II)	22	132
oxalate	352	352	Cr (VI)	12.5	10.4
Bromide	160	318	Pb(II)	8.2	2.5
Nitrate	248	248	Zr (IV)	7.3	-
Chloride	142	240	Ag(I)	4.1	5.3
Carbonate	120	240	Co (II)	2.4	11.78
Thiourea	31	154	Ti(II)	2.4	-
acetate	94	94	V (V)	2.0	2.2
Fluoride	78	7.8	Pt(IV)	2.0	-
			Zn(II)	1.3	-
			Zn (II)	1.3	13.0
			Fe (III)	1.1	22.34
			Pd (II)	0.90	10.25
			Au(III)	0.40	0.39
			Ni (II)	0.23	11.73
			Fe (II)	0.12	11.2
			Cu (II)	0.12	-
			Mo(VI)	-	24
			Hg (II)	-	0.40

Table. 2

Determination of mercury in some environmental water, soil, medicinal and biological samples

Name of the samples	Amount of mercury[a] found (µg/ml)	
	ATBH method	Dithizone method
Drain water[e]	3.29	3.29
Laboratory water[d]	1.93	1.85
Sea water[c]	1.06	1.00
Tap water [f]	0.65	0.69
Well water [g]	0.92	0.84
River water[b]	0.62	0.61
Urban soil	0.98	0.98
Agricultural soil	0.48	0.41
Road side soil	1.68	1.62
Brain damage patient	0.895	0.863
Kidney damage patient	0.912	0.879
Paralysis patient	1.475	1.469
Fish liver	1.28	0.84
Sheep liver	0. 83	0.86
Ayurvedic medicinal samples		
Siddamakaradhwaj	0.443	0.445
Panchabhanaras	1.590	1.588
Vasanthakusumakaram	0.964	0.968

[a]. Average of three determinations. [b]. Tungabhadra river water, (Kurnool) [c]. Bay of Bengal, (Chennai) [d]. Laboratory water, (Dept. of Chemistry, S.K.U. Anantapur) [e]. Anantapur town, Drain Water. [f]. Anantapur municipal storage tank water.

Table 3

Determination of copper in some leafy vegetable and liver samples

Name of the samples	Amount of copper[a] found (µg/ml in dried leaves)	
	ATBH method	Dithizone method
Thotakura(*Amaranthus gangeticus*)	0.261	0.265
Chukkaku(Irumex vesicarius)	0.261	0.236
Tutikura(*Ipomoea reptuns*)	0.305	0.290
Cauliflower green(*brassica deraceavar botntis*)	0.279	0.273
Medicinal leaves		
Vepaku(*Azadirachta indica*)	0.291	0.275
Gaddi chamanti(*Tridax procombens L*)	0.123	0.121
Fish liver	1.299	1.203
Sheep liver	1.259	1.280

[a]. Average of three determinations.

Conclusions

The study reveals that the mercury and copper content can be determined in ppm level in water, soil, biological green leafy vegetable and medicinal samples using present method. All these findings cause great concern regarding public health (Especially in taking marine fish etc.,) demanding an accurate determination of these metal ions at trace levels the present study may provide awareness among the public.

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Evaluation of Antiepileptic Activity of Aqueous extract of barringtonia racemosa in mice

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ABSTRACT

The aim of the present study was to investigate antiepileptic activity of aqueous extract of *Barringtonia racemosa* (AEBR) in mice. The antiepileptic activity of AEBR at 30, 100 and 300 mg/Kg, p.o. was evaluated by the convulsions induced in mice by maximum electroshock (MES), Pentylenetetrazole (PTZ) and Isoniazid (INH). Statistical analysis was carried out by one-way analysis of variance (ANOVA) followed by Dunnett's test. In MES method, AEBR (30, 100 and 300 mg/Kg) inhibited convulsions significantly potent than Diazepam. In PTZ method, AEBR inhibited convulsions potent than Phenobarbitone sodium (PS). In INH method, AEBR delayed the onset of convulsions less potent than Diazepam. In Present investigation, AEBR showed significant dose dependent antiepileptic effect potent than Diazepam and PS.

Keywords: Epilepsy, *Barringtonia racemosa*, Pentylenetetrazole, Isoniazid.

Introduction

The term epilepsy is of Greek origin that originally was known as "falling sickness" and means "Seizure" or "Seized" [1]. Epilepsy is a major neurological disorder and upto 5% of the world population have epilepsy in their lifetime. It affects an estimated 7 million people in India and 50 million worldwide, approximately 40% of them are women. In developed countries where drugs are easily available, epilepsy responds to treatment in up to 70% of the patients. However, in developing countries 75% of people with epilepsy do not receive effective treatment. It is estimated that up to 5% of people suffer at least one seizure in their lifetime.

Epilepsy is a chronic and often progressive disorder characterized by the periodic and unpredictable occurrence of epileptic seizures, i.e., involuntary contraction of striated muscle repeatedly. Convulsion arises due to sudden excessive and rapid discharge of cerebral neurons in the grey matter of the brain [2]. All the currently available AED have potential for adverse effects on cognition and behavior [3]. Search for anti-epileptic agents has made man turn to alternative sources, indigenous system of medicine.

Barringtonia racemosa is a shrub or a tree and is a member of the Lecythidaceae. Traditionally, it is used to treat epilepsy [4], cancer, pain including head ache, tooth ache and muscle pain [5,6]. The aim of the present study is to evaluate the potential of aqueous extract of leaves of *Barringtonia racemosa* (AEBR) to protect the mice from convulsions.

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Materials and Methods

Drugs and chemicals

Pentylenetetrazole (Sigma Aldrich Chemical Co.), Isoniazid (s.d. Fine-Chem. LTD), Diazepam (Ranbaxy) and Phenobarbitone sodium (Bayer AG).

Plant Collection

The leaves of *Barringtonia racemosa* were collected from Tirupati, Andhra Pradesh, authenticated by Prof. C. Madhava Chetty, department of Botany, Sri Venkateswara University and voucher specimen has been deposited.

Preparation of extract

The fresh leaves of *Barringtonia racemosa* were collected and washed under running tap water. They were shade dried at room temperature and the dried leaves were made in to coarse powder. The powder was passed through a 60 No mesh sieve. The grounded powder was macerated with ethanol at room temperature. The solvent was then removed by filtration and fresh solvent was added to the plant material. The extraction process was twice repeated. The combined filtrates were then evaporated under reduced pressure [7].

Animals

Swiss Albino mice of either sex weighing 18-22 g were used. They were housed in standard polypropylene cages

and kept under controlled room temperature (24±2^{0}C; relative humidity 60-70%) in a 12h light – dark cycle. The mice were given a standard laboratory diet and water *ad libitum.*

Qualitative analysis

The crude aqueous extract was distilled water and subjected to preliminary phytochemical screening. The study was carried out by using standard procedure [8].

Acute Toxicity Study

Acute toxicity study will be performed for the extracts to ascertain safe dose by acute oral toxic class method of Organization of Economic Co-operation and Development, as per 420 guidelines (OECD) [9].

Antiepileptic Activity

Maximum electroshock (MES) in mice

Five groups of six male Swiss albino mice (25 – 30) were used. The test was started one hour after oral treatment with the test compound (AEBR 30, 100, 300 mg/Kg, p.o.) or the vehicle or the standard (Diazepam 3 mg/Kg, p.o.). An apparatus with corneal electrodes was used to deliver the stimuli. The intensity of the stimulus is dependent on the apparatus, eg: 30mA, 50Hz for 0.2 sec has been used. The onset and the duration of tonic himb extension was recorded and percentage of inhibition of seizures relative to controls was calculated [10].

Pentylenetetrazole (PTZ) induced convulsions in mice

Control group received vehicle, test group received AEBR (30, 100 and 300 mg/Kg, p.o.) and standard group received Phenobarbitone sodium, (40 mg/Kg, i.p.). Convulsions were induced by administering PTZ (75 mg/Kg, i.p.), 1hr after AEBR and 15 min after PS administration. The onset and the duration of convulsions were recorded and percentage inhibition was calculated [11].

Isoniazid (INH) induced convulsions in mice

Control group received vehicle, test group received AEBR (30, 100 and 300 mg/Kg, p.o.) and standard group received Diazepam, (4 mg/Kg, i.p.). Convulsions were induced by administering INH (300 mg/Kg, s.c.), 1hr after drug administration. The onset time of convulsions was recorded [12].

Statistical analysis:

The data was analyzed by using one-way analysis of variance (ANOVA), followed by Dunnett's test. $P < 0.05$ was considered as statistically significant. The data are expressed as mean ± Standard deviation.

Results

Qualitative analysis

The preliminary phytochemical screening of the aqueous extract reveals the presence of steroids, alkaloids, glycosides, tannins, saponins and proteins as major compounds tabulated in Table 1.

Table 1:

Phytochemical constituents of aqueous extract of *Barringtonia racemosa*

Phytochemical constituents	Aqueous extract of *Barringtonia racemosa* (AEBR)
Steroids	+
Alkaloids	+
Flavonoids	-
Glycosides	+
Tannins	+
Lipids	-
Saponins	+
Proteins	+

(+): Present (-): Absent

Acute Toxicity Study

In acute toxicity study, mortality was found at 2 gm/Kg, p.o. So, doses of 30, 100 and 300 mg/Kg, p.o. were selected for the study.

Antiepileptic Activity

Maximum electroshock (MES) in mice

In MES method, AEBR increased the onset time and decreased the duration of tonic hind limb extension when compared to control group, which are shown in Table 2. AEBR exhibited significant dose dependent antiepileptic activity. AEBR at 300 mg/Kg exhibited antiepileptic activity potent than Diazepam.

Pentylenetetrazole (PTZ) induced convulsions in mice

In PTZ method also, AEBR increased the onset time and decreased the duration of convulsions when compared to control group presented in Table 3. AEBR exhibited significant dose dependent antiepileptic activity. AEBR at all three doses exhibited antiepileptic activity potent than PS.

Isoniazid (INH) induced convulsions in mice

AEBR at all three doses exhibited significant dose dependent delay in onset of convulsions when compared to control but less potent than Diazepam shown in Table 4.

Discussion

The convulsions induced by MES, PTZ and INH were effectively controlled by AEBR. Normally, antiepileptic drugs may act by modifying excitatory and inhibitory neurotransmission through effects on voltage gated ion channels, GABA(A) receptors and glutamate mediated excitatory neurotransmission [13]. Here PTZ act as GABA

Table 2

Effect of aqueous extract of *Barringtonia racemosa* on maximal electroshock induced convulsions in mice

Treatment	Dose	Onset time (s)	Duration of tonic hind limb extension (s)	Percentage of Inhibition (%)
Vehicle	1 ml	1.52±0.05	93.56±0.33	-
AEBR	30 mg/Kg	2.22±0.10**	45.17±0.31**	51.72**
AEBR	100 mg/Kg	2.92±0.06**	40.71±0.47**	56.49**
AEBR	300 mg/Kg	3.64±0.21**	36.93±0.47**	60.53**
Diazepam	3 mg/Kg	3.53±0.25**	39.59±0.35**	57.69**

AEBR: Aqueous extract of *Barringtonia racemosa*; Values are mean ± SD (n = 6). Statistical significance was determined by ANOVA, followed by Dunnett's t test (n=6); $^{**}p < 0.01$.

Table 3

Effect of aqueous extract of *Barringtonia racemosa* on PTZ-induced convulsions in mice

Treatment	Dose	Onset time (min)	Duration of convulsions (min)	Percentage ofInhibition (%)
Vehicle	1 ml	4.06±0.32	20.12±0.44	-
AEBR	30 mg/Kg	4.42±0.19**	7.02±0.39**	65.13**
AEBR	100 mg/Kg	5.52±0.06**	5.37±0.42**	73.29**
AEBR	300 mg/Kg	6.55±0.02**	4.20±0.40**	79.10**
PS	40 mg/Kg	6.32±0.36**	10.06±0.39**	50**

AEBR: Aqueous extract of *Barringtonia racemosa*; Values are mean ± SD (n = 6). Statistical significance was determined by ANOVA, followed by Dunnett's t test (n=6); $^{**}p < 0.01$.

Table 4:

Effect of aqueous extract of *Barringtonia racemosa* on INH-induced convulsions in mice

Treatment	Dose	Onset of convulsions (min)
Vehicle	1 ml	25.08±0.26
AEBR	30 mg/Kg	35.33±0.26
AEBR	100 mg/Kg	41.39±0.39
AEBR	300 mg/Kg	46.22±0.42
Diazepam	4 mg/Kg	54.24±0.52

AEBR: Aqueous extract of *Barringtonia racemosa*; Values are mean ± SD (n = 6). Statistical significance was determined by ANOVA, followed by Dunnett's t test (n=6); $^{**}p < 0.01$.

antagonist and INH act as GABA synthesis inhibitor [10]. Maximum protection of mice from convulsions was observed in PTZ model. So, AEBR may be a GABA agonist and the antiepileptic effect may be due to enhanced GABA ergic neurotransmission.

Conclusion

In the present investigation, AEBR showed significant dose dependent antiepileptic effect potent than Diazepam and PS. The antiepileptic activity may be due to the phytoconstituents present in the extract which are responsible for the enhanced GABAergic neurotransmission. Further studies are necessary to identify and reveal the active phytoconstituent responsible for the antiepileptic effect.

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