In vitro antioxidant activity of mulberry (*Morus indica* L.) leaf extract

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ABSTRACT

The study was aimed to assess the *in vitro* antioxidant activity of mulberry (*Morus indica* L.). Leaf extract. Ethanolic extract of mulberry leaves was tested for the antioxidant activity by *in vitro* assays using butylated hydroxyl toluene as positive control. Erythrocyte membrane of human volunteer was used as peroxidation model system *in vitro*. Mulberry leaf extract displayed scavenging activity against 2,2-diphenyl-1-picrylhydrazyl (DPPH), nitric oxide and superoxide radicals and inhibited $FeSO_4$-induced lipid peroxides and hydroperoxides in erythrocyte membrane in a concentration dependent manner. The extract also exhibited a good reducing power at 250µg/ml. In conclusion, mulberry leaves possess antioxidant properties and the effect *in vitro* is a result of synergistic action of antioxidant phytochemicals-flavonoids, moracins, carotenoids etc. present in the leaves.

Key words: Antioxidant activity, mulberry leaf extract, lipid peroxides, scavenging activity, superoxide radical, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical.

Introduction

Oxygen free radicals such as superoxide, hydroxyl, peroxyl ($RO_2^{\cdot}$), alkoxyl ($RD^{\cdot}$) and hydroperoxyl ($HO_2^{\cdot}$) and nitrogen free radicals i.e. nitrogen oxide and nitrogen dioxide are generally unstable and very reactive [1]. A shift of the balance on the oxidant side may trigger a cascade of reactions leading to the formation of highly reactive cytotoxic compounds such as reactive oxygen metabolites (ROMs). Reactive oxygen species (ROS), reactive nitrogen species (RNS) and reactive chlorine species are produced in animals and humans under physiologic and pathologic conditions [2]. "Oxidative stress"[3] that results due to the improper balance between reactive oxygen metabolites and antioxidant defenses has been one of the factors in the pathogenesis of various diseases such as cardiovascular and neurological disorders, Parkinson's disease, rheumatoid arthritis etc.[4]. Our defenses against oxidative stress are the antioxidants synthesized in our body which include all antioxidant proteins (e.g., transition metal- binding proteins) and various small molecules, some of which are end products of metabolic pathways (eg. bilirubin) and their levels in our body cannot be manipulated by simple means. On the other hand, the levels of antioxidant vitamins taken up in the diet such as ascorbate, α- tocopoherol and β- carotene, non-enzymatic antioxidants i.e. polyphenols and flavonoids can be increased easily by dietary means or by supplementation [5, 6].

Natural antioxidants are preferred because these are considered safe and environmental friendly. There has been a growing interest in replacing commercial antioxidants such as butylated hydroxyl anisole (BHA) and butylated hydroxyl toluene (BHT) with natural ingredients due to possible toxicity of synthetic antioxidants which have restricted use in foods as these are suspected to be carcinogenic [7]. Hence, phytochemicals opened new avenues for the treatment of a no. of diseases. Currently, the use of some naturally occurring antioxidants in preventive and therapeutic medicine is gaining importance. Hence, evaluation of the antioxidative activity of naturally occurring substances has been of interest in recent years.

The leaves of mulberry (*Morus indica* L.) of Moraceae used as a feed for silkworms, are nutritious, palatable and can be eaten as vegetable [8, 9]. In addition to protein, fat, carbohydrates, and dietary fibre, the leaves contain essential elements viz., calcium, potassium, magnesium, iron, sulphur, phosphorus, chromium, zinc, and selenium. Besides, the presence of some vitamins (β carotene, thiamin, riboflavin, niacin, biotin, inositol, choline and vitamin C) the leaves

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also contain certain free amino acids which are physiologically important. Phytochelators such as phytic acid and other substances i.e., flavonoids are also reported in the leaves [10, 11]. Nine flavonoids isolated from the leaves of *M. alba* i.e. Quercetin-3-0-β-D-glucopyanosyl– (1->6)-β- D-glucopyranoside and quercetin etc. were reported to possess antioxidant activity [12]. Sharma et al., [13] reported the presence of three different antioxidative phytoalexins (moracin C, moracin N and chalcomoracin) in mulberry leaves. Review of literature revealed very few reports on the antioxidant potential of *Morus indica* L. leaves, it was therefore thought worthwhile to investigate the free radical scavenging activity and anti-lipidperoxidative potential of *Morus indica* L. leaves.

Materials and Methods

All chemicals and solvents were of analytical grade, obtained from SRL and Merck, Mumbai, India.1,1-diphnyl, 2-picryl hydrazyl (DPPH) and butylated hydroxyl toluene (BHT) were obtained from Sigma Chemicals, USA.

Plant material and extraction

Mulberry (*Morus* indica L.) leaves plucked from healthy plants in bulk from Regional Sericultural Research Station, Anantapur Dist., were thoroughly washed under running tap water, shade dried, powdered and packed in polythene covers to be used for the *in vitro* experiments. Dried powder was weighed and extracted in a Soxhlet extractor using hexane for 6 hrs. for the removal of fatty matter. The defatted material was extracted in Soxhlet extractor with 95% ethanol for 72 hrs. The extract was concentrated in a vacuum evaporator and the residue was stored in a desiccator after noting the yield for subsequent experiments.

Antioxidant assay (in vitro)

DPPH radical scavenging activity

DPPH radical scavenging activity of leaf extract was measured according to the method given by Sreejayan and Rao [14]. To 1ml of various concentrations (50-250 µg/ml) of the extract, 1ml of DPPH (0.1mM) was added. An equal amount methanol and DPPH served as control. After 20 min. incubation in dark, the absorbance of the test mixture was read at 517nm using UV-VIS spectrophotometer. The percentage scavenging activity was calculated by comparing the result of the test with that of control (methanol and 1 ml DPPH) using the formula [15].

$$\text{Scavenging (\%)} = \frac{\text{(Absorbance of control – Absorbance of test)}}{\text{Absorbance of control}} \times 100$$

Nitric oxide radical scavenging activity

Nitric oxide radical scavenging activity was estimated by the method given by Sreejayan and Rao [16] and Marcocci *et al*[17].Nitric oxide (NO) radicals were generated from sodium nitroprusside solution at physiological pH and measured by Griess reaction. To 1ml of sample extract of different concentrations (50-250µg/ml) in phosphate buffer (0.025M, pH7.4), 1 ml of sodium nitroprusside (10mM) was added, inculcated at 25^0C for 150min. followed by the addition of 1 ml of Griess reagent. Butylated hydroxyl toluene treated in a similar way, served as control. Absorbance was read at 546nm and percentage scavenging was calculated using the formula.

Superoxide radical scavenging activity

Superoxide radical scavenging activity was measured according to the method of Robak [18] as given by Mondal *et al* [19]. To 3ml of sample extract of different concentrations (50-250µg/ml) in phosphorus buffer (0.1M, pH7.4), 1ml of NBT (156 µm) and 1 ml of NADH (468 µM) were added. The reaction was started by the addition of 100 µl of PMS (60 µM) and the mixture was then incubated at 25^0C for 5 min followed by measurement of absorbance at 560nm against butylated hydroxy toluene treated in similar way as control and percentage scavenging was calculated using the formula.

Reducing power

The reducing power of the test samples was determined by the method of Yen and Duh [20] as given by Jayaprakash *et al* [21]. Different concentrations of the ethanolic extract (50-250 µg/ml) were mixed with 2.5ml of phosphate buffer (0.2M, pH 6.6) and 2.5ml of 1% potassium ferricyanide in 10ml test tubes. The mixtures were incubated for 20min at 50^0C. At the end of the incubation, 2.5ml of 10% TCA was added to the mixtures, followed by centrifuging at 5000 rpm for 10 min. The upper layer (2.5ml) was mixed with 2.5ml of distilled water and 0.5ml of 0.1% $FeCl_3$ and the absorbance was measured at 700nm against reagent blank containing ethanol instead of extract. Increase in absorbance of the reaction mixture indicates the reducing power of the samples.

Inhibition of lipid peroxidation (malondialdehyde-MDA) and diene conjugates (hydroperoxides) in erythrocyte membrane

The extent of lipid peroxidation and diene conjugates (hydroperoxides) in erythrocyte membrane model were assayed *in vitro* in terms of thiobarbituric acid reactive substances by the method of Buege and Aust [22].

Preparation of extract

The sample was prepared by grinding 1 gm of the dried leaf powder in 2ml of 50% ethanol separately in a pre-chilled mortar and pestle and the extract was centrifuged at 10,000 rpm at 4^0C for 10 min. The supernatant obtained was used within 4 hours of extraction to assess the influence on malondialdehyde and diene conjugates using erythrocyte membrane.

Isolation of RBC membrane

The erythrocyte membrane was isolated according to the method of Dodge *et al*., [23]. Erythrocytes separated from whole blood were washed several times using phosphate buffered saline (PBS) and by centrifuging (10,000 rpm) at 4^0C until clear supernatant and pallet were obtained. The pellet was washed with phosphate buffer (5mM, pH 7.4), finally suspended in 2ml of tris HCl buffer (0.1M, pH 7.4) and used to asses the influence of extract on lipid peroxidation and diene conjugates in erythrocyte membrane.

Lipid peroxidation

One ml of erythrocyte membrane suspension was taken in 3 centrifuge tubes. To the 2nd tube, 1ml of 1mM $FeSO_4$ was added and to the 3rd tube, 1 ml of sample extract was added followed by 1ml of 1mM $FeSO_4$. To all the three tubes, 2 ml of the TCA-TBA-HCl reagent was added, boiled for 15 min. in boiling water bath, cooled and centrifuged at 10,000 rpm to remove precipitate. The absorbance was read at 535 nm against a reagent blank as well as sample blank. The extent of lipid peroxidation in all the 3 tubes was calculated using tetraethoxy propane as standard.

Diene conjugates (hydroperoxides)

One ml of erythrocyte membrane suspension was taken in each of 3 centrifuge tubes. To the 2nd centrifuge tube, 1 ml of 1 m M $FeSO_4$ was added. To the 3rd tube, 1ml of extract was added followed by 1ml of $FeSO_4$ (1mM). To all the three tubes, 5ml of chloroform: methanol (2:1) was added followed by centrifugation at 1,000 rpm for 15 min. to separate the phases. The chloroform layer was placed in test tubes and taken to dryness at 45^0C in a water bath. The lipid residue was dissolved in 1.5ml of cyclohexane and the absorbance was read at 233nm against a cyclohexane blank. The amount of hydroperoxides produced was calculated using molar coefficient of 2.52×10^4 M^{-1}.The values are expressed as mean ± SEM and Student's t test [24] was used for comparison.

Results and Discussion

DPPH radical scavenging activity

Ethanolic extracts of mulberry leaves exhibited DPPH radical scavenging activity in a concentration dependent manner (**Fig 1**). DPPH is relatively a stable free radical. The assay is based on the measurement of the scavenging ability of antioxidants towards the stable radical DPPH. From the present results, it may be postulated that compounds present in mulberry leaves reduced the radical to the corresponding hydrazine when it reacts with hydrogen donors in the antioxidant principles. DPPH radicals react with suitable reducing agents, the electrons become paired off and the solution loses color stochiometrically depending on the number of electrons taken up [25]. In the present study, it was observed that purple color of DPPH was bleached completely and very rapidly by the leaf extract at all concentrations (50-250 µg/ml)in a concentration dependent manner indicating very efficient scavenging activity of mulberry leaf extract (**Fig.1**).

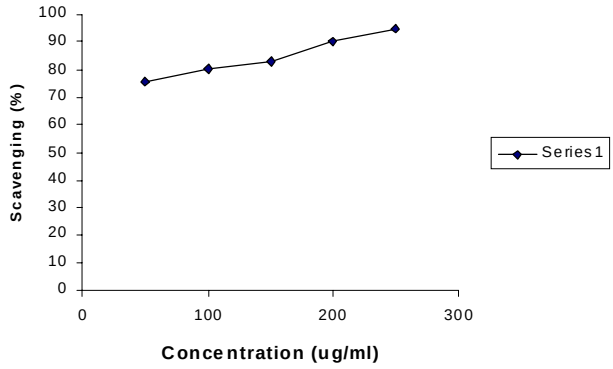

Fig.1 DPPH radical scavenging activity of mulberry leaf extract Values are mean ± SEM of 3 observations

Nitric oxide radical scavenging activity

Nitric oxide radical scavenging activity exhibited by the leaf extract at different concentration is presented in **Fig. 2** which shows that scavenging activity is linearly related to the concentration of the extract. In the present study, the nitrite produced by the incubation of solution of sodium nitroprusside in standard phosphate buffer at 25^0C was reduced by the ethanolic extract of mulberry leaves and is denoted as nitrite radical scavenging activity of the leaves because the antioxidant principles present in the extract, compete with O_2 to react with nitric oxide thereby inhibit the generation of nitrite and is evidenced by lesser colour develpoment with Greiss reagent as compared to that of positive control butylated hydroxylated toluene (BHT). By scavenging nitrite radicals mulberry leaves can protect human volunteers from a number of diseases as excess NO is associated with several diseases [26].

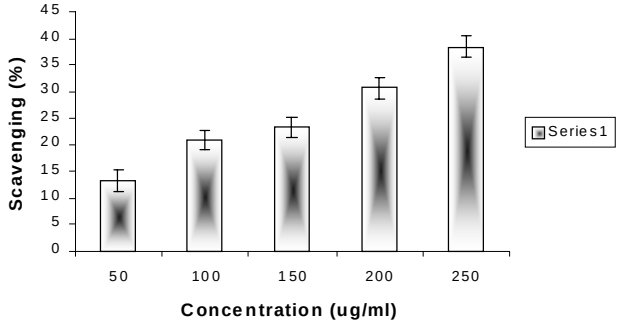

Fig.2 Nitric oxide radical scavenging activity of mulberry leaf extract Values are mean ± SEM of 3 observations

Superoxide radical scavenging activity

Superoxide radical scavenging activity exhibited by the leaf extract at different concentrations in presented in

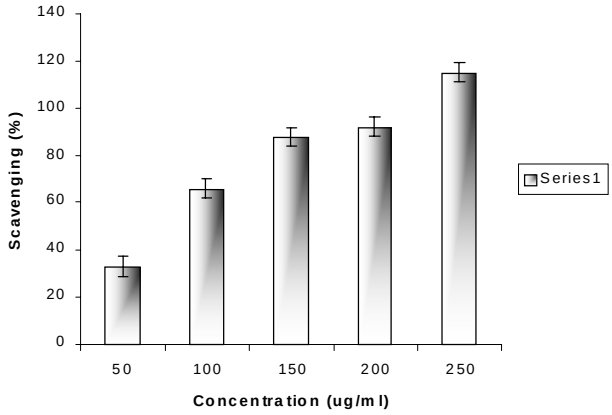

Fig.3 Super oxide radical scavenging activity of mulberry leaf extract Values are mean ± SEM of 3 observations

Fig. 3 which shows the proportionality between concentration of extract and radical scavenging activity of the leaf extract. The probable mechanism of scavenging the superoxide anions may be due to the inhibitory effect of ethanolic extract of the leaves towards generation of superoxides in the reaction mixture. This superoxide radical scavenging activity is evidenced by decreased lipid peroxidation in erythrocyte membrane treated with the leaf extract *in vitro* (**Table 1**).

Table-1

Effect of mulberry leaf extract on lipid peroxidaion and hydroperoxides in erythrocyte membrane model

Experimental Group	TBARS (μ mole/mg protein)	Hydroperoxides (m mole/mg protein)
RBC membrane	0.128 ±0.003	22.01±0.14
RBC membrane + $FeSO_4$	0.240 ±0.001	31.13±0.15
RBC membrane + $FeSO_4$ + mulberry leaf extract	0.131± 0.004**	21.04± 0.011**

Values are the mean ± SD of three observations
Comparison between groups II and III
**p<0.01

Reducing power

The extract exhibited reducing power at different concentrations in a concentration dependent manner as shown in **Fig. 4** which shows a direct relation between the concentration and the extent of reducing capacity of the sample extract. A substance may act as an antioxidant due to its ability to reduce ROS by donating hydrogen atoms [21, 27]. The reducing property of ethanolic extract of the leaves implies that the extract is capable of donating H_2 atoms in a dose dependent manner. The phenolic compounds in the extract may be a contributing factor towards antioxidant activity because the phenolic compounds are known to have antioxidant property due to the presence of hydroxyl groups which can act as H_2 donors [28, 29]. In the present study, reducing power of the leaf extract reflected in controlled LPO and diene conjugates *in vitro* (**Table-1**)

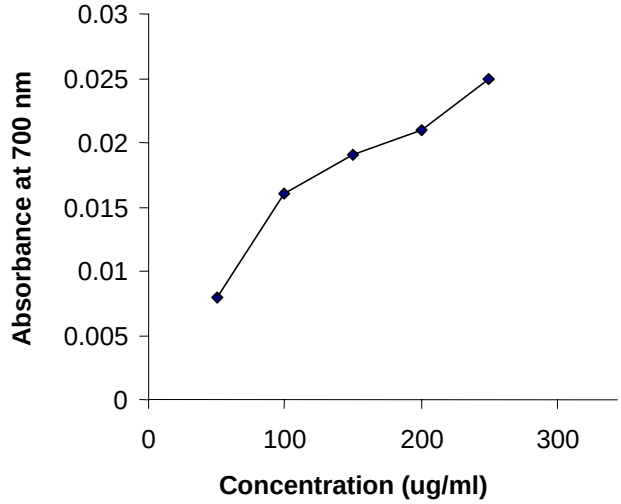

Fig. 4 Reducing power of mulberry leaf extract Values are mean ± SEM of 3 observations

The data presented here indicates that the marked antioxidant activity of the leaf extract seems to be the result of the reducing power. The polyphenols present in the leaves donate electrons and react with free radicals to convert them to more stable products and terminate the free radical chain reaction as reported by Jayaprakash et al., [21] in a study on grape seed extract.

Lipid peroxidation and hydroperoxides

Table 1 shows the effect of mulberry leaf extract on lipid peroxidation and hydroperoxides in human erythrocyte membrane model system. The basal lipid peroxidation of the erythrocyte membrane was enhanced by two fold in the presence of inducer $FeSO_4$. Mulberry leaf extract showed inhibitory effect on lipid peroxidation and diene conjugates / hydroperoxides in erythrocyte membrane. This inhibitory effect of the leaf extract on lipid peroxidation as well as hydroperoxides is a reflection of free radical scavenging activity of the leaves. This is further supported by free radical scavenging activity exhibited by the leaf extract *in vitro* (**Fig.1-3**). The antioxidant capacity of the leaves is probably a result of synergistic action of polyphenols [30], flavonoids i.e quercitin, isoquercitrin [12], moracins [13] and carotenoids [31] present in the leaves which are reported as strong antioxidants.

The results of the study indicate that mulberry leaf extract effectively scavenges nitric oxide, superoxide and

DPPH radicals and exhibits tremendous reducing power. Besides, mulberry leaf extract suppresses $FeSO_4$-induced lipid peroxidation and diene conjugates/ hydroperoxides in erythrocyte membrane very efficiently. This preliminary study warrants a need for further investigations on the analysis of mulberry leaves and molecular mechanism of its protective action.

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Screening of Anti Tumour L-asparaginase Positive Fungal Cultures from Soil

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ABSTACT

An extracellular L- asparaginase producing fungal strains were isolated from soil samples by using a pH and dye –based fast procedure. Fifty fungal strains were isolated. The results are obtained within 96 h when the media was supplemented with L-asparagine. Among the isolates VS-26 found to be highest L-asparaginase activity (3.64 uml^{-1}) and its culture filtrate P^H was 8.78.

KEY WORDS: L-asparaginase, L-asparagine, extracellular, soil samples, fungal strains.

Introduction

L-Asparaginase (L-Asparagine amido hydrolase, EC 3.5.1.1) has received much attention in recent years for its anticarcinogenic potential. This enzyme is also choice for acute

Lymphoblastic leukemia, Lymphosarcoma and in many other clinical experiments relating to tumour therapy in combination with chemotherapy. This treatment brought a major break through in modern oncology, as it induces complete remission in over 90% of children within four weeks [1]. With the development of its new functions, a great demand for L-asparaginase is expected in the forth-coming years.

Ever since *Escherichia coli* L-asparaginase antitumour activity was first demonstrated by Broome [2] and Mashburn and Wriston [3], its production using microbial systems has attracted considerable attention owing to their cost effective and co-friendly nature. A wide variety of microbial strains, such as *E. coli* [4,5], *Erwinia carotovora* [6], *Pseudomonas stutzeri* [7], *Pseudomonas aeruginosa* [8] and *Serratia marcescens* [9], *Mycobacterium pheli* [10], *Staphylococcus 6A* [11], *Streptomyces griseus*[12], *Aspergillus tamari, Aspergillus terreus* [13], were isolated and screened for L-asparaginase-producing potential [14,15].

However, this L-asparaginase from bacterial origin can cause hypersensitivity in the long-term used, leading to allergic reactions, anaphylaxis [16]. The search for other asparaginase sources like eukaryotes, can lead to an enzyme with less adverse effects. It has been observed that eukaryote microorganisms like yeast and filamentous fungi have a potential for asparaginase production [6,17,18]. For example, the mitosporic fungal genera such as *Aspergillus*, *Penicillium* and *Fusarium*, are commonly reported in scientific literature to produce asparaginase [14,19,20,21,22]. In the present investigation, a simple and rapid assay method for the detection of asparaginase producing fungal strains from soil samples using the dye-based method has been used [15]. After an initial screening, strains were subjected for submerged fermentation to determine the enzyme activity.

Material and Methods

Screening

Different soil samples from garden, forest and compost were used in the present study to isolate L-asparaginase producing fungal strains.

The soil samples (1g) of each of the above sample were taken into 100ml sterile distilled water. The suspension was kept on rotary shaker for 30min and kept aside to settle soil matter. One ml of the suspension was serially diluted with

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sterile distilled water and one ml of each of these dilutions was added to 80 ml of sterile modified Czapek Dox's agar medium [23] containing (gl^{-1}): Glucose, 2.0; L- Asparagine, 10.0; KH_2PO_4, 1.52; KCl, 0.52; $MgSO_4.7H_2O$, 0.52; $CuNO_3.3H_2O$, trace; $ZnSO_4.7H_2O$, trace; $FeSO_4.7H_2O$.trace; Agar, 18.0, pH 6.2 and this media was supplemented with phenol red 0.009%(v/v) [15], and anti bacterial agent (Streptomycin) 25µg/ml. The inoculated media were plated into sterile petridishes and incubated at 28°C ± 1 °C, control media were also included in which the substrate L-asparagine was omitted.

Detection of L-Asparaginase positive cultures

The modified Czapek.Dox's agar medium contains 1.0% L-asparagine as sole source of nitrogen and phenol red as pH indicator. L-asparaginase catalyses L-asparagine into aspartic acid and ammonia. This can be easily detected by the change in the pH of the medium using phenol red. The colour change of the medium from yellow to pink is an indication of the extra cellular L-asparaginase production by the colony.

After 48 h of incubation at 28°C±1°C, the fungal colonies with color zones (pink) were picked up and transferred on to the PDA (Potato Dextrose Agar) and Czapek Dox's agar slants.

Estimation of enzyme activity

Quantitative enzyme estimation was carried out in Erlenmeyer flasks (250ml) containing 45ml of modified Czapek Dox's liquid medium (without agar). This medium was inoculated with 5ml of spore suspension of each test organism. The flasks were incubated on rotary shaker at 180 rpm at 28°C±1°C for 96h. Un-inoculated media served as control [15]. At the end of incubation, cultures were harvested by filtration through Whatman No.1 filter paper, the pH and L-asparaginase activity of the culture filtrates was determined.

L-Asparaginase activity was estimated in culture filtrates by Nesserlisation [22]. This activity was read at 450 nm in Elico double beam UV –Visible spectrophotometer (SL 164). Enzyme blank was used as control. A standard curve was prepared with ammonium Sulphate. Enzyme activity expressed in units per ml.

One unit of L-asparaginase activity was defined as that amount of enzyme which catalyses the formation of 1µmol of ammonia per minute under optimal assay conditions.

Results and Discussion

A total of sixty-three fungal colonies showing pink colour zones were isolated from all soil samples and fifty isolates were chosen; out of which those appearing closer to each other were eliminated. The selected fifty fungal isolates were subjected to the L-asparaginase production in modified Czapek Dox's medium (without agar). At the end of incubation, the enzyme activity of the same was estimated. Among all 50 isolates, the isolated fungal strain No.VS-26 exhibited maximum L-asparaginase activity **(**3.64 Uml^{-1}) and pH of the culture filtrate was 8.78. The results are denoted in **table-1**.

Table No. 1

Sl. No.	Culture No.	L-asparaginase activity (Uml^{-1})	pH (Culture filtrates)
1	VS-1	1.46	8.07
2	VS-2	1.99	7.89
3	VS-3	1.53	7.01
4	VS-4	0.92	8.00
5	VS-5	1.89	8.80
6	VS-6	1.86	8.04
7	VS-7	0.89	7.90
8	VS-8	0.73	7.98
9	VS-9	0.83	7.88
10	VS-10	1.89	8.09
11	VS-11	0.96	8.10
12	VS-12	0.73	8.00
13	VS-13	0.83	8.20
14	VS-14	0.86	8.10
15	VS-15	1.43	8.50
16	VS-16	1.53	8.60
17	VS-17	1.29	8.63
18	VS-18	0.79	8.64
19	VS-19	0.73	8.40
20	VS-20	0.86	7.87
21	VS-21	1.81	8.00
22	VS-22	2.06	8.33
23	VS-23	0.99	8.07
24	VS-24	2.20	8.90
25	VS-25	2.32	8.82
26	VS-26	3.64	8.78
27	VS-27	0.03	8.20
28	VS-28	0.08	8.62
29	VS-29	0.09	8.61
30	VS-30	1.00	8.70
31	VS-31	1.01	8.82
32	VS-32	0.99	8.00
33	VS-33	0.89	7.99
34	VS-34	0.12	7.64
35	VS-35	1.02	8.70
36	VS-36	0.02	7.67
37	VS-37	0.08	7.72
38	VS-38	1.08	8.62
39	VS-39	0.15	8.62
40	VS-40	0.91	8.21
41	VS-41	0.28	8.00
42	VS-42	1.70	8.62
43	VS-43	0.14	8.02
44	VS-44	1.02	8.80
45	VS-45	0.07	8.00
46	VS-46	0.09	8.00
47	VS-47	0.11	8.10
48	VS-48	0.12	8.20
49	VS-49	0.05	8.10
50	VS-50	0.07	8.00

A semi-quantitative plate assay was used for screening of L-asparaginase producing fungal strains. The L-asparaginase production accompanied by an increase in the pH of the culture filtrates. The plate assay was devised using this principle by incorporating the pH indicator-phenol red in the medium containing L-asparagine (a sole nitrogen source). Phenol red at acidic pH is yellow at alkaline pH turns pink, thus a pink zone formed around microbial colonies producing L-asparaginase. The plate assay used for screening the isolates to determine their activity (equal to the diameter of the pink zone) also an indication of the amount of L-asparaginase produced by the colony. The diameter of these zones was proportional to L-asparaginase produced by the colony. This revealed that isolate No. VS-26 had the maximum L-asparaginase activity.

Conclusion

This preliminary study indicates that L-asparaginase positive fungal strain VS-26 could be in favor of its possible utilization as a potential source for anti tumour therapeutic agent after extensive anti cancer activity determination. Further extensive studies like, Strain improvement and Optimization of process parameters are underway to enhance enzyme production.

Acknowledgement

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Effect of mucilage of seeds of *Ocimum Basilicum* against cisplatin-induced nephrotoxicity in rats

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ABSTRACT

The present study was focused on the effect of mucilage of Seeds of *Ocimum basilicum* (200 and 400mg/kg, p.o.) on cisplatin-induced (6mg/kg, i.p.,) nephrotoxicity in rats. Nephroprotector activity was assessed by estimating the levels of urea, creatinine in serum, renal functional parameters such as urine to serum creatinine ratio, creatinine clearance, urinary protein excretion and lipid peroxidation in kidney. Administration of cisplatin in animals caused significant increase in blood urea nitrogen, serum creatinine, malondialdehyde levels in kidney. Further, cisplatin deteriorated renal functional parameters characterized by reduction of urine to serum creatinine ratio, creatinine clearance and increased urinary protein excretion. In curative regimen, protection was dose dependent and animals which received 400mg/kg of mucilage significantly reversed the effects caused by cisplatin. In prophylactic regimen, mucilage of Seeds of *Ocimum basilicum* failed to exhibit protection against cisplatin-induced nephrotoxicity.

KEY WORDS: Cisplatin, *Ocimum basilicum*, lipid peroxidation, Nephroprotector activity.

Introduction

Over the past twenty years, the number of persons suffering from renal problems is increasing. The reasons are: exposure to medicines, industrial/environmental pollutants, age, pre-renal disease etc. Cisplatin is widely used as antineoplastic agent against various tumors but its clinical use is limited due to its nephrotoxicity [1]. Although the mechanism of nephrotoxicity is unclear, reports suggest that free radicals play a major role [2]. Antioxidants [3, 4] and plants containing antioxidant principles [5, 6] effectively reduce cisplatin-induced nephrotoxicity.

Ocimum basilicum (*O. basilicum*; Family: Labiatae) is a branching herb found in tropical and warm temperate regions. All parts of the plant are used in traditional medicine but the seeds are of particular interest because they are used as diuretic, diaphoretic, demulcent and to treat nephritis [7, 8]. Traditionally, seeds were used to treat nephritis by soaking them in water till the seeds were fully swollen and taken as such. The present study was focused on the pharmacological validation and justification of the seeds of *O. basilicum* to treat nephritis in the traditional system of medicine.

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Materials and Methods

Cisplatin was obtained as gift sample from Intas Pharmaceuticals, Gujarat, India. Urea, creatinine and serum total proteins were estimated by use of commercial kits (Dr. Reddy's laboratories). All other chemicals used for the study were purchased either from S.D.fine or Merck (India).

Plant material and preparation of mucilage

The seeds of *O. basilicum* were purchased from local market and identified by Dr. Madhava Chetty, Herbarium-Keeper, Dept. of Botany, S.V. University, Tirupati. The seeds were dried under shade and pulverized in a mechanical grinder and stored in a closed container for further use. The mucilage was prepared by mixing the seed powder with required amount of water (25mg/ml).

Evaluation of nephroprotector activity

Animals: Wistar rats of 8-10 weeks of age, weighing 150-200g were used in the study. They were maintained on the standard diet (Gold Mohur,Bangalore) and water was given *ad libitum.* They were housed in polypropylene cages and were acclimatized to laboratory environment for about a week. The study was conducted after obtaining local animal ethical committee clearance. Cisplatin was

administered intraperitoneally (6mg/kg, single dose) and the mucilage was administered orally by gastric intubations.

Animals were divided into 8 groups (n=6) and they were put on the following treatment schedule (Table 1).

Assessment of renal function

Blood urea nitrogen (BUN: Di acetyl monoxime method) [9], serum creatinine (SC: Alkaline picrate method) [9] and serum total proteins (STP: Biuret method) [9] were estimated by using commercial kits. Urine was collected on day 15 (prophylactic regimen), 5 and 16 (curative regimen) for 6 hrs (initiated at 8 am) by keeping the animals in individual metabolic cages and was analyzed for creatinine and protein (Up: sulphosalicylic acid method) [9].

Creatinine clearance was calculated by using formula:

Creatinine clearance = urinary creatinine X urinary volume h^{-1}/ serum creatinine.

Lipid peroxidation in kidney

Lipid peroxidation was evaluated as malondialdehyde (MDA) production as described by Heath and Backer [10]. The animals were sacrificed by decapitation on day 15 or 16. The kidneys were dissected out, immediately placed in ice cold saline to prevent contamination with blood and they were pressed on blotted paper. Kidneys were weighed and homogenized in 1.5% KCl with the help of Teflon homogenizer. To 1 ml of homogenate, 2.5 ml of trichloroaceticacid (TCA, 20%) was added and centrifuged at 3500 rpm for 10 min. The resulting pellet was dissolved in 2.5 ml of 0.05 M H_2SO_4 and then 3 ml of thiobarbituric acid was added and incubated at 37°C for 30 minutes. The contents were then extracted into 4 ml of n-butanol and the absorbance was measured spectrophotometrically at 530 nm.

***In vitro* antioxidant studies**

Nitric oxide scavenging activity

Sodium nitroprusside (5 mM) was mixed with different concentrations of mucilage of seeds of *Ocimum basilicum* (50, 100, 200, 300, 400, 500 µg/ml) and incubated at 25°C for 5 hours. After 5 hours, Griess reagent (2% o-phosphoric acid, 1g of sulphanilamide, 100 mg of N-napthylethylene-diamine made up to 100 ml) was added and absorbance of the chromophore formed was read at 546 nm. Control experiments were also carried out in a similar manner. The experiments were conducted in triplicate. Percentage scavenging effect was calculated [11, 12].

Reducing Power

The reducing power of mucilage of Seeds of *Ocimum basilicum* was determined by Oyaizu method [13]. Different concentrations of mucilage of Seeds of *Ocimum basilicum* (10, 50, 100, 200, 300 µg/ml) was mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide (2.5 ml, 1%). The mixture was incubated at 50°C for 20 minutes. 2.5 ml of 10% TCA was added to the mixture which was then centrifuged at 3000 rpm for 10 minutes. The upper layer (2.5 ml) was diluted with distilled water (2.5 ml) and 0.5 ml ferric chloride solution (0.1%) was added and absorbance was measured at 700 nm. The absorbance of reaction mixture indicates reducing power.

Table-1

Treatment Schedule – Nephroprotector activity

Group	Treatment	Day of biochemical estimation	Purpose
I	————	5, 15, 16	Normal control
II	Cisplatin 1st day Water 6th to15th day5 ,	16	To serve as curative- control
III_a	Cisplatin 1st day Mucilage 6th to15th day (200mg/kg)	5, 16	To assess curative effect
III_b	Cisplatin 1st day Mucilage 6th to15th day (400mg/kg)	5,16	To assess curative effect
IV	Water 1st to 10th day Cisplatin 11th day	15	To serve as prophylactic-control
Va	Mucilage 1st to10th day Cisplatin 11th day (200mg/kg)	15	To assess prophylactic effect
Vb	Mucilage 1st to10th day Cisplatin 11th day (400mg/kg)	15	To assess prophylactic effect
VI	Mucilage 1st to10th day (400mg/kg)	11	To check the effect of mucilage on kidney

Blood and urine was withdrawn on day 5 from groups I, II, IIIa, IIIb to check the induction of nephrotoxicity.

Statistical analysis

The results are expressed as mean ± SEM and the data analysed using one way analysis of variance followed by post hoc Student-Keuls test using SPSS computer software for *in vivo* studies. Student-Keuls test was used for *in vitro* studies. Statistical significance was set at P= 0.05.

Results and Discussion

Animals which received mucilage of seeds of *O.basilicum* alone (group VI) for ten days exhibited no change in serum markers level and urinary functional parameters. Hence, mucilage of seeds of *O.basilicum* did not show any deteriorative effects on kidney. In curative regimen blood and urine were collected from animals of group II, III_a and III_b on day 5 to assess whether cisplatin at the given dose induced nephrotoxicity (Data shown in Table-2 and 3).To assess curative activity the data obtained from the estimation of biochemical parameters of the mucilage treated groups (III_a, III_b) was compared with respective curative control group (II). Similarly, prophylactic activity (V) was assessed in comparison with prophylactic control (group IV).

Table-2 lists the effect of oral administration of mucilage of *O.basilicum* on cisplatin-induced elevation of BUN, SC, S_{TP} in rats. The levels of BUN, SC and S_{TP} increased significantly in cisplatin alone treated animals (group II and IV), when compared to normal control animals (group I). The extent of the elevation was reduced significantly in animals treated with mucilage of *O.basilicum* in curative groups, III_a and III_b when compared to the respective control animals (group II).

Table 2

Effect of mucilage of seeds of *O. basilicum* on cisplatin-induced nephrotoxicity

Group	BUN (mg/dl)	SC (mg/dl)	S_{TP} (g/dl)
I	20.3±1.1	0.6±0.04	6.9±0.7
II	75.1±4.5 [a]	1.2±0.08[a]	8.8±0.8[a]
III_a	49.3±1.5 [b]	0.8±0.06 [b]	7.1±0.9[b]
III_b	33.9±3.8 [b,a]	0.7±0.07 [b,a]	6.5±0.5[ba]
IV	133.2±8.0 [a]	2.2±0.09 [a]	9.8±0.5[a]
V_a	130±7.3 [c]	2.2±0.13 [c]	9.5±0.7 [c]
V_b	121.0±5.0[c]	2.0±0.11 [c]	8.8±0.5 [c]
On day 5	93.8±6.4 [a]	2.1±0.08 [a]	9.9±0.4 [a]

Values were expressed mean ± SEM each group containing 6 animals (one-way ANOVA followed by Student's Newman–Keuls post hoc test).

[a]P<0.05 compared to normal control;[b]P<0.05 compared to curative control.
*[c]P<0.05 compared to prophylactic control.

The deterioration of the renal functions induced by cisplatin and the effect of mucilage of *O.basilicum* is given in Table-3. Cisplatin reduced creatinine clearance (Cl_{cr}), urine to serum creatinine ratio (U_{cr}/S_{cr}) and increased excretion of urinary protein in group II and IV animals, when compared to normal control animals. In curative regimen, animals which received 400mg/kg mucilage significantly improved U_{cr}/S_{cr}, Cl_{cr} and reduced urinary protein excretion when compared to group II animals.

Table 3

Effect of mucilage of seeds of *O. basilicum* on cisplatin-induced changes in renal function

Group	U_{cr}/S_{cr}	Cl_{cr} (ml/h/100gBd.Wt)	U_p (mg/24hr)
I	17.7±0.5	18.4±0.8	6.0±0.3
II	9.3±0.7[a]	9.9±0.6[a]	17.9±1.3[a]
III_a	11.0±0.5 [b]	11.5±1.3 [b]	14.2±0.6 [b]
III_b	14.1±1.4 [b]	13.2±1.7[b]	10.9±0.6[b]
IV	7.6±0.2[a]	7.9±0.2[a]	18.5±1.4[a]
V_a	7.2±0.4 [c]	9.9±0.5 [c]	18.5±1.2 [c]
V_b	7.9±0.5[c]	10.0±0.6[c]	18.2±0.3[c]
On day 5	7.4± 0.5 [a]	8.1±0.3 [a]	18.9±1.0 [a]

Values were expressed mean ± SEM each group containing 6 animals (one-way ANOVA followed by Student's Newman–Keuls post hoc test)

[a]P<0.05 compared to normal control;[b]P<0.05 compared to curative control

*[c]P<0.05 compared to prophylactic control.

Induction of nephrotoxicity by cisplatin is assumed to be a rapid process involving reaction with protein in renal tubules, as renal damage occurs with in an hour after administration of cisplatin [14]. It is important that the protective agent be present in sufficient concentration in the renal tubules before injury occurs. This might explain the rationale behind prophylactic treatment. Animals which received mucilage at 200 mg/kg and 400 mg/kg dose in prophylactic regimen failed to protect cisplatin-induced renal damage when compared to group IV animals. At given doses (200 mg/kg and 400 mg/kg) the mucilage of *O. basilicum* was unable to arrest the progress of renal damage caused by cisplatin.

Animals which were treated with cisplatin alone (group II and group IV) exhibited elevated levels of MDA, when compared to normal control animals (group I). Animals belonging to curative groups (III_a and III_b) exhibited decreased levels of MDA, whereas prophylactic group failed to reduce the MDA levels in the kidney (Table-4).

Table No – 4

Effect of mucilage of seeds of *O. basilicum* on Lipid peroxidation in kidney

Group	Percentage inhibition
I	-
II	-
IIIa	9
IIIb	49
IV	-
V_a	2
V_b	8

• Kidneys were isolated from rats on day 15 or16. The percentage of inhibition of MDA was calculated in comparison with respective controls.

Mucilage of seeds of *O. basilicum* was tested for *in vitro* anti oxidant activities like nitric oxide scavenging activity and reducing power at different concentrations i.e., 50, 100, 200, 300, 400µg/ml. As the concentration of the mucilage of seeds of *O. basilicum* increased, the percentage of nitric oxide scavenging activity and reducing power also increased.

The results of our study confirmed the earlier findings that the cisplatin at 6mg/kg produces significant nephrotoxicity characterized by increase in BUN, SC, S_{TP}, urinary excretion of proteins and reduced creatinine clearance and the effects persisted for 2 weeks.

Previous reports on phytochemical studies on *O. basilicum* revealed the presence of terpenoids such as eugenol, methyl chavicol, 1,8-cineole, terpinen-4-ol, gammna-terpene, linalool, methyl cinnamate, alpha-pinene, beta pinene, camphene, myrcene, p-cymene, thymol and methyl-eugenol [15-21], fatty acids like lauric , myristic, Palmitic, stearic , Oleic , linoleic , linolenic and arachidic acid [22]. Further, Methanolic extract of *O. basilicum* reported to show wide range of biological activities such as anti-inflammatory, hepatoprotective, anti-ulcer, antioxidant activities [23-28].

In curative regimen, oral administration of mucilage of seeds of *O. basilicum* showed dose related response on cisplatin-induced effects. The presence of terpenoidal constituents in combination with fatty acids may be responsible for curative activity exhibited by mucilage of seeds of *O. basilicum.* Animals which received mucilage at 200 and 400mg/kg dose in prophylactic regimen failed to protect cisplatin-induced renal damage which may be probably due to the different mechanisms involved in the prophylaxis and cure of renal damage.

Various reports suggest that cisplatin-induced nephrotoxicity occurs partly *via o*xidative stress [29, 30]. Since reports evidenced that the *O. basilicum* possessed anti oxidant activity, the possible mechanism by which the mucilage exhibited nephroprotection could be attributed to its antioxidant property. One of the mechanisms proposed by which cisplatin induces renal damage is by increasing the activity of calcium independent nitric oxide synthase [6]. *In vitro* studies of mucilage of seeds of *O. basilicum* showed concentration dependent nitric oxide scavenging activity. The results of the present study substantiate the use of mucilage of seeds of *O. basilicum* in folklore medicine in the treatment of various renal disorders.

Figure-1

Nitric Oxide Scavenging activity of mucilage *O. basilicum*

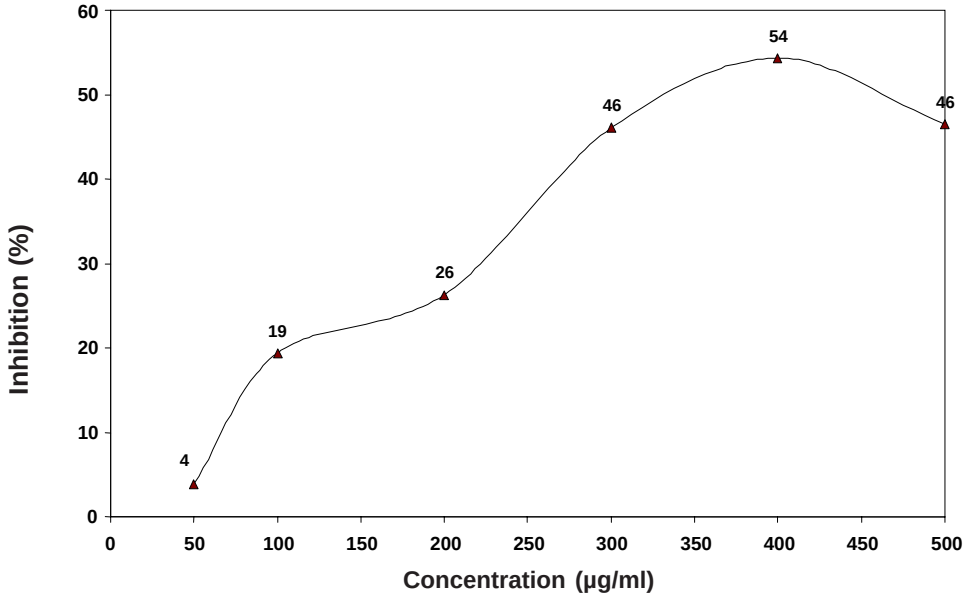
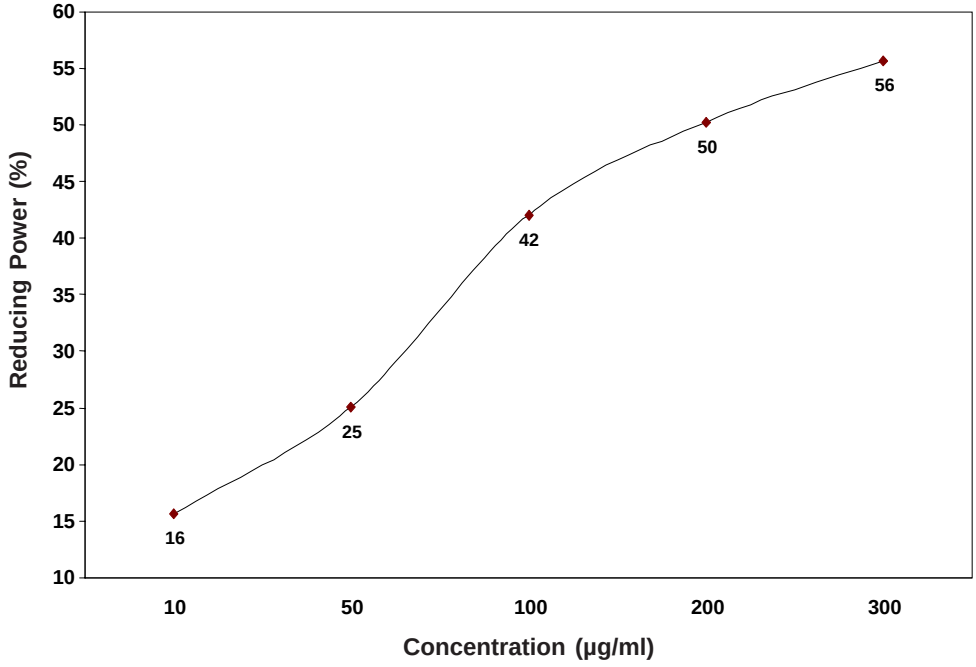


Figure-2

Reducing Power of mucilage of *O. basilicum*



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Potassium dodeca tungstocobaltate trihydrate ($K_5CoW_{12}O_{40}.3H_2O$) catalyzed synthesis of 2,2'-spirobi(4-aryl-7,7-dimethyl-5-oxo-5,6,7,8-tetrahydrochromans

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ABSTRACT

Potassium dodeca tungstocobaltate trihydrate ($K_5CoW_{12}O_{40}.3H_2O$) catalyzed synthesis of 2,2'-Spirobi(4-aryl-7,7-dimethyl-5-oxo-5,6,7,8-tetrahydrochromans) (**3a-d)** in good yield by an application of Michael reaction between 5,5-dimethylcyclohexan-1,3-dione (**1**) and *trans*, *trans*-diarylideneacetones (**2a-d**) at reflux temperature is reported. The catalyst (heterogeneous) exhibited remarkable reusable activity.

Key words: Potassium dodeca tungstocobaltate trihydrate, Michael reaction, heterogeneous, remarkable reusable activity.

Introduction

The use of heterogeneous catalysts in different areas of the organic synthesis has now reached significant levels, not only for the possibility to perform environmentally benign synthesis, but also for the good yields, frequently accompanied by heteropoly acids (HPAs) and related compounds in a field of increasing importance [1-10]. HPAs have several advantages as catalysts, which make them economically and environmentally attractive. On the one hand, HPAs have a very strong Bronsted acidity approaching the super acid region; on the other, they are efficient oxidants, exhibiting fast reversible multielectron redox transformations under mild conditions.

The chemistry of spiroketals has received much attention over the years due to their presence as substructures in the increasing number of medicinally, ecologically important compounds and compounds ocurring in natural products from various sources including insects, microbes, plants, fungi and marine organisms [11-14]. The increasing pharmacological importance of compounds having spiroketal moieties has aroused intense interest in both their synthesis and chemical reactivity. It is evident from the literature that among many strategies, which have evolved for the synthesis of spiroketals, the acid-catalyzed cyclization of dihydroxyketones, or an equivalent thereof, is the predominant ring-forming process. Later many methods were developed for the synthesis of dihydroxyketones precursors. Livant *et al*. reported [15] the synthesis of fused aromatic spiroketal by carrying out the acid-catalyzed reaction of resorcinol with phorone (2,6-dimethyl-2,5-heptadien-4-one) and *trans*, *trans*-dibenzylideneacetone (1,5-diphenyl-1,4-pentadien-3-one) whereby the dihydroxyketone precursor was obtained as Michael 1:2-adduct. This adduct underwent subsequent cyclization yielding the C_2-symmetric spirochroman where the oxygen atoms were derived from two aromatic hydroxy groups. Recently, newer methods have been reported [16-17] for the synthesis of partly and fully fused aromatic spiroketals, which exhibited antifungal and antibiotic activity. Examples of partially reduced aromatic rings or partly unsaturated rings fused with spiroketals ring systems do not appear to be reported in the literature. Very recently, Ahmed *et al*. successfully synthesized and reported [18] spiroketals containing such ring systems using 20 mol% of anhydrous $ZnCl_2$.

Results and Discussion

In the course of our studies [19-22] on the use of heterogeneous catalyst in fine organic chemistry, we have developed a method, which allows the practical route for fused spiroketal skeleton by using inexpensive and reusable potassium dodeca tungstocobaltate trihydrate, $K_5CoW_{12}O_{40}.3H_2O$ (1 mol%) as a heterogeneous catalyst [23-27] in a mixture of boiling toluene and *n*-heptane at reflux temperature (**Scheme-I**). In addition, to the best of our knowledge, there are no reports on the use of potassium dodecatungstocobaltate trihydrate ($K_5CoW_{12}O_{40}.3H_2O$) as a heterogeneous catalyst for this conversion. This fact has prompted us to investigate potassium

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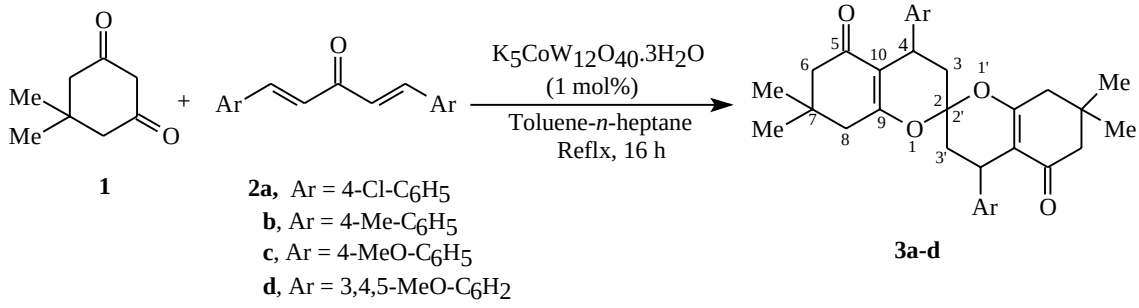
dodecatungstocobaltate trihydrate [$K_5CoW_{12}O_{40}.3H_2O$, (1 mol%)] for the synthesis of 2,2'-Spirobi(4-aryl-7,7-dimethyl-5-oxo-5,6,7,8-tetrahydrochromans) in a facile and practical manner.



1

2a, Ar = 4-Cl-C_6H_5
b, Ar = 4-Me-C_6H_5
c, Ar = 4-MeO-C_6H_5
d, Ar = 3,4,5-MeO-C_6H_2

3a-d

Scheme-I

The significant features of this catalyst are, easy to prepare and handle; inexpensive, recyclable and eco-friendly in nature. $K_5CoW_{12}O_{40}.3H_2O$ catalyzes acetylation and formylation of alcohols [28], esterification and transestrification reactions [29], deprotection of dioxolanes and trimethyl silyl ethers [30], cleavage of oximes [31], Biginelli reaction [32], microwave assisted synthesis of quinoline and dihydroquinoline derivatives [33], a four-component synthesis of *β*-acetamido ketones [34] and synthesis of aryl-14*H*-dibenzo[*a.j*]xanthenes [35].

For our investigation, upon screening with dimedone (**1**) in 2 molar excess and 4-chlorodiarylideneacetone (**2a**) [36-37] in molar proportion in a mixture of boiling toluene and *n*-heptane for 16 h in the presence of $K_5CoW_{12}O_{40}.3H_2O$. After few investigations it was found that 1 mol% $K_5CoW_{12}O_{40}.3H_2O$ is sufficient for this transformation at reflux temperature.

Encouraged by the results obtained for dimedone and 4-chlorodiarylideneacetone, we investigated other substituted diarylideneacetones (**2b-d**) [36-37] to probe their behavior under the current catalytic conditions to get spirochromans (**3a-d**). Products were characterized by comparision of their physical data, ^1H NMR, ^{13}C NMR, IR and mass spectral data with those of authentic samples [18].

Next, we investigated the reusability and recycling of $K_5CoW_{12}O_{40}.3H_2O$. At first, we put dimedone (2.0 mmol), 4-chlorodiarylideneacetone (1.0 mmol) and 1 mol% of $K_5CoW_{12}O_{40}.3H_2O$ in a mixture of boiling toluene and *n*-heptane together, and then the mixture was stirred at reflux temperature for 16 h. When the reaction was completed, the catalyst was separated by simple filtration by diluting with excess toluene and recovered $K_5CoW_{12}O_{40}.3H_2O$ was activated and reused in subsequent reaction without significant decrease in activity even after third run.

In conclusion, we have developed an alternative method for an efficient one-pot synthesis of spirochromans using $K_5CoW_{12}O_{40}.3H_2O$ as a heterogeneous catalyst at reflux temperature. Present methodology offers a simple procedure combined with ease of recovery and reuses of

Table - I

Optimizing the reaction conditions[a]

Entr	Catalyst	Time (h)	Yield [b]
1	$K_5CoW_{12}O_{40}.3H_2O$ (0.01)	16	36
2	$K_5CoW_{12}O_{40}.3H_2O$ (0.005)	16	25
3	$K_5CoW_{12}O_{40}.3H_2O$ (0.1)	16	78
4	$K_5CoW_{12}O_{40}.3H_2O$ (0.15)	18	80

[a] Dimedone/4-chlorodiarylideneacetone/$K_5CoW_{12}O_{40}.3H_2O$ – 2:1:0.1.
[b] Isolated yields.

the catalyst which makes this method economic, benign, and a waste-free chemical process for the synthesis of spiroketals. We believe that this procedure is convenient, economic, and a user-friendly process for the synthesis of spiroketals.

Experimental Section

Typical procedure for 3 : A mixture of dimedone **1** (1.54 g, 11 mmol), *trans*, *trans*-diarylideneacetone **2a-d** (5 mmol) and $K_5CoW_{12}O_{40}.3H_2O$ (640 mg, 1 mol%) in boiling *n*-heptane (20 mL) and toluene (20 mL) was refluxed for 16 h under Dean-Stark attachment. The progress of the reaction was monitored and the purity of product was checked by TLC. The reaction mixture was allowed to cool at RT, diluted with toluene (10 mL) and filtered [to remove the catalyst; removed catalyst was washed with toluene (2 x 5 mL) and activated for recycle]. Filtrate was reduced in volume and neutralized with 5% aq. $NaHCO_3$ solution and then extracted with ether (3 x 20 mL). The ether extract was dried over anhy. Na_2SO_4 and evaporated in *vaccuo*. The crude product was purified by recrystallization from a suitable solvent.

***2,2'-Spirobi(4-chlorophenyl-7,7-dimethyl-5-oxo-5,6,7,8-tetrahydrochroman)* 3a** : Recrystallized from chloroform-pet. ether. Yield 74%; mp 280-283 °C (Lit[6]., mp 285-286 °C dec.); IR (KBr) : ν 1679, 1650, 1491 cm^{-1}. ^1H NMR (200 MHz, $CDCl_3$): δ 1.20 (s, 3H, Me), 1.25 (s, 3H, Me), 1.66 (dd, 1H, H_{ax}-3), 2.02 (dd, 1H, H_{ax}-6), 2.10 (dd, 1H, H_{ax}-8), 2.41 (dd, 1H, H_{eq}-3), 2.32 (dd, 1H, H_{eq}-6),2.51 (br d, 1H, H_{eq}-8), 3.72 (dd, 1H, H_{ax}-4), 7.00 (d, 2H, Ar-H, *J* = 8.0 Hz), 7.26 (d, 2H, Ar-H, *J* = 8.0 Hz). ^{13}C NMR (75 MHz, $CDCl_3$): δ 26.5 (Me), 27.8 (Me), 32.0 (C-7), 33.5 (C-4), 41.6 (C-3), 42.8 (C-8), 51.1 (C-6), 97.5 (C-2), 114.1 (C-10), 128.4 (Ar-C-3', 5'), 126.8 (Ar-C-2', C-6'), 132.8 (Ar-C-4'), 143.1 (Ar-C-1'), 166.0 (C-9), 195.2 (C-5).

***2,2'-Spirobi(4-methylphenyl-7,7-dimethyl-5-oxo-5,6,7,8-tetrahydrochroman)* 3b** : Recrystallized from chloroform-pet. ether. Yield 74%; mp 252-254 °C (Lit[6]. mp 255-256 °C); IR (KBr) : ν 1653, 1625, 1500 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$): δ 1.08 (s, 3H, Me), 1.18 (s, 3H, Me), 1.80 (dd, 1H, H_{ax}-3), 2.15 (dd, 1H, H_{ax}-6), 2.20 (dd, 1H, H_{ax}-8), 2.21 (d, 1H, H_{eq}-3), 2.30 (dd, 1H, H_{eq}-6), 2.42 (br d, 1H, H_{eq}-8), 2.76 (s, 3H, Me), 3.88 (dd, 1H, H_{ax}-4), 6.75 (d, 2H, Ar-H, *J* = 8.5 Hz), 7.00 (d, 2H, Ar-H, *J* = 8.5 Hz). ^{13}C NMR (75 MHz, $CDCl_3$) : δ 21.8 (Me), 23.5 (Me), 26.8 (Me), 32.0 (C-7), 32.5 (C-4), 41.5 (C-3), 42.2 (C-8), 51.3 (C-6), 97.5 (C-2), 113.4 (Ar-C-3', 5'), 114.1 (C-10), 128.0 (Ar-C-2', C-6'), 136.3 (Ar-C-4'), 158.4 (Ar-C-1'), 166.3 (C-9), 196.6 (C-5).

***2,2'-Spirobi(4-methoxyphenyl-7,7-dimethyl-5-oxo-5,6,7,8-tetrahydrochroman)* 3c**: Recrystallized from ethanol. Yield 74%; mp 242-244 °C (Lit[6]. mp 245-247 °C); IR (KBr): ν 1665, 1620, 1505 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$): δ 1.10 (s, 3H, Me), 1.20 (s, 3H, Me), 1.85 (dd, 1H, H_{ax}-3), 2.17 (dd, 1H, H_{ax}-6), 2.18 (dd, 1H, H_{ax}-8), 2.25 (d, 1H, H_{eq}-6), 2.30 (dd, 1H, H_{eq}-3), 2.43 (brd, 1H, H_{eq}-8), 3.81 (s, 3H, OMe), 3.88 (dd, 1H, H_{ax}-4), 6.30 (s, 2H, Ar-H), 7.01 (s, 2H, Ar-H). ^{13}C NMR (75 MHz, $CDCl_3$) : δ 28.2 (Me), 30.0 (Me), 32.2 (C-7), 32.9 (C-4), 42.4 (C-3), 42.5 (C-8), 51.5 (C-6), 55.6 (OMe), 97.9 (C-2), 115.2 (Ar-C-3', 5'), 115.6 (C-10), 127.0 (Ar-C-2', 6'), 139.9 (Ar-C-4'), 153.0 (Ar-C-1'), 166.3 (C-9), 196.2 (C-5).

***2,2'-Spirobi(3,4,5-trimethoxyphenyl-7,7-dimethyl-5-oxo-5,6,7,8-tetrahydrochroman)* 3d** : Recrystallized from ethanol. Yield 65%; mp 132-136 °C; IR (KBr): ν 1663, 1630, 1506 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) : δ 1.10 (s, 3H, Me), 1.20 (s, 3H, Me), 1.85 (dd, 1H, H_{ax}-3), 2.17 (dd, 1H, H_{ax}-6), 2.18 (dd, 1H, H_{ax}-8), 2.25 (d, 1H, H_{eq}-6), 2.30 (dd, 1H, H_{eq}-3), 2.43 (brd, 1H, H_{eq}-8), 3.78 (s, 6H, 2 x OMe), 3.81 (s, 3H, OMe), 3.88 (dd, 1H, H_{ax}-4), 6.30 (s, 2H, Ar-H). ^{13}C NMR (50 MHz, $CDCl_3$) : δ 29.0 (Me), 31.4 (Me), 31.6 (C-7), 31.9 (C-4), 42.0 (C-3), 42.8 (C-8), 51.5 (C-6), 55.8 (2 x OMe), 60.6 (OMe), 97.6 (C-2), 117.7 (Ar-C-3', 5'), 115.0 (C-10), 130.0 (Ar-C-2', 6'), 139.9 (Ar-C-4'), 153.0 (Ar-C-1'), 166.3 (C-9), 196.2 (C-5). Anal. Calcd. for $C_{39}H_{48}O_{10}$: C, 69.21; H, 7.15. Found: C, 69.23; H, 7.18.

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Synthesis of Hexyl oleate using Rhizomucor miehei lipase in organic media : Optimization studies

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ABSTRACT

Immobilized lipase from *Rhizomucor miehei* (Lipase IM -20) was employed in the esterification of oleic acid and hexanol to synthesize hexyl oleate in n-Hexane. Response Surface Methodology(RSM) based on a five level, five- variable central composite rotatable design (CCRD) was used to evaluate the effects of important variables: enzyme concentration (20-40% w/w of acid), acid concentration (0.2-1.0 *M*), incubation period (24-120 h), alcohol concentration (0.25-1.25 *M*)and temperature (30-70 °C) on the esterification of hexyl oleate. Extent of conversion was found to be excellent at all acid and alcohol concentrations employed in the range of 0.2-1.25 *M*, even at low enzyme concentration (20% w/w). The optimum conditions arrived at are as follows: 35% (w/w) enzyme concentration, 1.0 *M* acid concentration, 1.25 *M* alcohol concentration and 120 h incubation period, at 35 °C. Under these conditions, the experimental value was 680 *mM* ester matched very well an experimental value of 678 *mM*.

KEY WORDS: Hexyl oleate, lipase, organic solvent, esterification.

Introduction

Lipases (Triacylglycerol acylases, EC 3.1.1.3) are versatile catalysts, and like many other enzymes, they remain active in non-aqueous media and has got lot of applications for the production of Pharmaceuticals, food flavors, cosmetics, lubricants, detergents etc [1-3]. In recent times the growing interest in this area arises from the potential applications of these systems to carry out unusual reactions to synthesize useful products (esters). The immobilized *Rhizomucor miehei* lipase is extensively used by many researchers to synthesize various esters of commercial importance due its broad substrate specificity (lower to higher molecular weight acids or alcohols). Hexyl oleate is useful as biodegradable lubricant and is used as bare oil lubricant. Recent trends in consumer preference towards 'natural products' indicate that biocatalysts have an advantage over their chemical counterparts as products of biocatalysts may obtain a 'natural' label [4].

Although, there are few reports on the synthesis of oleic acid esters [5-7], they are limited only to feasibility studies. Modeling of these enzyme-catalyzed organic phase reactions received relatively little attention by the scientific community. The purpose of this study is to contribute to the modification of the mechanisms and to obtain the corresponding rate expressions of esterification reactions catalyzed by immobilized lipase in non polar media.

Response surface methodology (RSM) is used to study the effects of several factors influencing the responses by varying them simultaneously by carrying out only a limited number of experiments [8].

Majority of esterification reactions with lipases, has been carried out using water immiscible organic solvents such as hexane, heptane, octane and isooctane etc. Several reaction parameters are known to affect the esterification yields and lipase stability. Of the various reaction parameters, substrate, enzyme concentration, substrate molar ratio, temperature and incubation time are known to affect the esterification yields. Understanding of the factors that control the activity of enzymes in an organic system is essential for optimization of the processes. The esterification activity of lipases mainly depend on availability of water around the enzyme surface, water formed during the reaction which often acts as one of the reactant and the nature of the reaction media used [specificity and activity depends on the solvent used for catalysis] [9].

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The formation of stoichiometric proportions of water in an esterification reaction led to the reversal of the reaction [hydrolysis]. Thus, the equilibrium of a reaction was affected by this excess of water [10]. To achieve high yields, either water has to be removed from the system, or large excess of acid/alcohol has to be used. However, excess alcohol slows the esterification reaction and the presence of higher acid concentration can lower the pH in which the lipase looses activity [11]. A solvent-free system could be used for higher percentage esterification at relatively higher substrate concentrations [12]. Transesterification reactions have also been employed for improved esterification yields [13], since there is no water accumulation and reaction equilibrium can be driven to the product side. Over recent years, many authors have shown that various lipases, either free or immobilized, can catalyze the esterification of acids or alcohols of chain length from C_2-C_4 or C_8-C_{18} [14-18]. The present work focuses on the influence of various reaction parameters affecting the synthesis of hexyl oleate using the lipase from *Rhizomucor miehei*. The removal of water, efficiency of solvent-free system and estimation of kinetic constants has also been investigated.

MATERIALS AND METHODS

Enzyme:

Immobilized Lipase (triacyl glycerol hydrolase, EC 3.1.1.3; Lipozyme **IM** – 20), from *R.Miehei* supported on macroporous weak anionic beads, was obtained from Novo Nordisk (Bagsvaerd, Denmark). Lipase activity was measured by estimating the liberated butyric acid by titrating against 0.04*N* sodium hydroxide using pH stat method of Tietz and Fereick(1966) [19] using Tributyrin emulsion as substrate. The hydrolytic activity of the enzyme was 15,000 U per gram of enzyme at pH 7.0 and 30°C. Water content of the enzyme was estimated to be 8.5% (w/w) gravimetrically. Sodium hydroxide and solvent n-hexane were obtained from S.D Fine Chemicals(Mumbai, India). Substrates oleic acid and hexanol, were from Aldrich chemicals[Milwaukee, WI, USA]. All chemicals and substrates were dried over molecular sieves and the solvents were distilled before use.

Esterification:

Ester synthesis was carried out in a 100ml stoppered conical flask containing appropriate amounts of substrates [alcohol and acid] and appropriate enzyme concentration in 10ml of organic solvent (n-hexane). The flasks were agitated at 150 rpm on a rotary shaker at specified temperature. Samples were withdrawn at regular intervals and assayed by titrimetry and gas chromatography.

Experimental design:

A five-level, five-variable CCRD has been adopted in this study. The factorial design consisted of 16 factorial points, 10 axial points [2 axial points on the axis of each design variable at a distance of 2 from the design center] and 6 center points. The center points are used to estimate the experimental error [20]. For developing the model, the variables and their levels selected for the study are represented in Table 1. For creating response surfaces, the experimental data obtained based on the above design was fitted to a second order polynomial equation of the form:

$$Y = b_o + \sum_{i=1}^{5} b_i x_i + \sum_{i=1}^{5} b_{ii} x_i^2 + \sum_{i=1}^{4} \sum_{j=i+1}^{5} b_{ij} x_i x_j$$

Where Y= ester formed [m*M*]; X_1 = enzyme [% w/w of acid]; X_2 = acid concentration [*M*]; X_3 = incubation time [h]; X_4 = alcohol concentration [*M*]; X_5 = temperature [°C]; b_o = constant; b_{ii} = quadratic term coefficient; b_{ij} = cross product term coefficient.

Regression analysis, statistical significances and response surfaces were done using Corel Quatropro software and Microsoft Excel software [version 5.0; Microsoft Corp., Redmond, WA].

Table 1

Coded and actual level combinations used for model development*.

Test run No.	X_1	X_2	X_3	X_4	X_5	Y_{Exp} (mM)	Y_{pre} (mM)
1	-1(25)	-1(0.4)	-1(48)	-1(0.5)	1(60)	233	203
2	1(35)	-1(0.4)	-1(48)	-1(0.5)	-1(40)	211	190
3	-1(25)	1(0.8)	-1(48)	-1(0.5)	-1(40)	292	277
4	1(35)	1(0.8)	-1(48)	-1(0.5)	1(60)	436	417
5	-1(25)	-1(0.4)	1(96)	-1(0.5)	-1(40)	235	216
6	1(35)	-1(0.4)	1(96)	-1(0.5)	1(60)	360	336
7	-1(25)	1(0.8)	1(96)	-1(0.5)	1(60)	442	425
8	1(35)	1(0.8)	1(96)	-1(0.5)	-1(40)	480	471
9	-1(25)	-1(0.4)	-1(48)	1(1.0)	-1(60)	148	144
10	1(35)	-1(0.4)	-1(48)	1(1.0)	1(60)	276	268
11	-1(25)	1(0.8)	-1(48)	1(1.0)	1(60)	407	406
12	1(35)	1(0.8)	-1(48)	1(1.0)	-1(40)	434	441
13	-1(25)	-1(0.4)	1(96)	1(1.0)	1(60)	265	259
14	1(35)	-1(0.4)	1(96)	1(1.0)	-1(40)	274	276
15	-1(25)	1(0.8)	1(96)	1(1.0)	-1(40)	473	482
16	1(35)	1(0.8)	1(96)	1(1.0)	1(60)	494	499
17	-2(20)	0(0.6)	0(72)	0(0.75)	0(50)	273	296
18	2(40)	0(0.6)	0(72)	0(0.75)	0(50)	403	417
19	0(30)	-2(0.2)	0(72)	0(0.75)	0(50)	107	143
20	0(30)	2(1.0)	0(72)	0(0.75)	0(50)	523	524
21	0(30)	0(0.6)	-2(24)	0(0.75)	0(50)	259	285
22	0(30)	0(0.6)	2(120)	0(0.75)	0(50)	429	446
23	0(30)	0(0.6)	0(72)	-2(0.25)	0(50)	244	302
24	0(30)	0(0.6)	0(72)	2(1.25)	0(50)	383	362
25	0(30)	0(0.6)	0(72)	0(0.75)	-2(30)	262	268
26	0(30)	0(0.6)	0(72)	(0.75)	2(70)	316	347
27	0(30)	0(0.6)	0(72)	0(0.75)	0(50)	371	364

28	0(30)	0(0.6)	0(72)	0(0.75)	0(50)	376	364
29	0(30)	0(0.6)	0(72)	0(0.75)	0(50)	374	364
30	0(30)	0(0.6)	0(72)	0(0.75)	0(50)	350	364
31	0(30)	0(0.6)	0(72)	0(0.75)	0(50)	376	364
32	0(30)	0(0.6)	0(72)	0(0.75)	0(50)	372	364

*Average absolute relative deviation = 5.44% ;Y=ester concentration; X_1 = enzyme [%w/w]; X_2 = acid concentration [*M*]; X_3 = incubation time [h]; X_4 = alcohol concentration [*M*]; X_5 = temperature [°C].

Results and Discussion

Model development:

Response surface methodology consists of an empirical modelization technique, which has been used to evaluate the relation between experimental and observed results. A five-factorial five-level CCRD (Cochran and Cox 1992) was selected to demonstrate the various reaction parameters affecting the synthesis of hexyl oleate ester as a model study. The goodness of fit of the model was checked by the determination of the coefficient [R^2] 0.963, which indicated that only 3.7% of the overall variation was not explained by the model. The values of the coefficients and analysis of variance [ANOVA] are presented in the Table 2.

The ANOVA represents that the model is highly significant as the F_{model} value [14.9] is very high compared to $F_{6,5}$ value [4.1] even at 1% level. These considerations indicate a good adequacy of the second-order polynomial model proposed to explain the observed yields. The coefficients of the response surface model as given by equation [1] were evaluated. Student 't' test indicated that all the linear coefficients, one quadratic term [temperature] and one cross product term [acid and alcohol interaction term] were highly significant [all $P < 0.1$]. However, to minimize the errors, all the coefficients were included in the model. The average absolute relative deviation is 5.44%, which indicates that the model is suitable to adequately represent the real relationships among the selected reaction parameters.

The final predictive equation obtained is given in equation [2]

$$Y = 363.7 + 30.42\,(x_1) + 95.33\,(x_2) + 38.583\,(x_3) + 15\,(x_4) + 19.75\,(x_5) - 1.806\,(x_1^2) - 7.5568\,(x_2^2) - 0.307\,(x_3^2) - 7.932\,(x_4^2) - 14.1\,(x_5^2) - 0.63\,(x_1.x_2) - 5.25\,(x_1.x_3) - 6.25\,(x_1.x_4) - 2\,(x_1.x_5) + 3.375\,(x_2.x_3) + 14.625\,(x_2.x_4) - 10.375\,(x_2.x_5) - 6.5\,(x_3.x_4) - 10.5\,(x_3.x_5) - 8.75\,(x_4.x_5) \quad \ldots.[2]$$

Where Y= ester concentration; X_1 = enzyme [% w/w of acid]; X_2 = acid concentration [*M*] X_3 = incubation time [h]; X_4 = alcohol concentration [*M*]; X_5 = temperature [°C].

In the present study, the range of variables selected based on initial experiments and factors which are known to affect the ester synthesis were only included in the design. The concentration of acid substrate was varied and the ester formation formed after a specified duration was expressed with respect to the esterification of the acid.

Influence of the reaction parameters:

The effect of enzyme concentration *versus* acid concentration at an alcohol concentration of 1.25 *M*, temperature of 30°C and incubation time of 120 h is depicted in Figure 1. Increase in acid concentration resulted in good esterification of all enzyme concentrations. As the enzyme concentration increased, ester formation increased only marginally in all acid concentrations. However, even at the lowest enzyme concentration, maximum ester concentration could be obtained in the acid concentration range 0.2 to 1.0 *M*. For example, at 20% [w/w] enzyme concentration [i.e., 204 mg at 1.0 *M* acid], an ester concentration of 660 m*M* could be achieved, exemplifying that all the enzyme particles were not involved in the reaction and a few of them of them were actively involved in the reaction.

Table - 2

Regression Statistics of the model and ANOVA*

Source	Degrees of Freedom	Sequential Sum–squares	Mean squares	F ratio	Prob. Level
Regression	20	309357	15467.9	14.52	0.000030
Linear	5	290907	58181.5	50.61	0.000000
Quadratic	5	8306.6	1661.3	1.56	0.250120
Cross product	10	10143.2	1014.3	0.95	0.526966
Total Error	11	11719.14	1065.4	—	—
Lack of fit	6	11228.8	1871.5	19.09	0.002653
Pure Error	5	490.3	98.05	—	—

*Coefficient of Determination [R^2]: 0.963; Standard Error: 32.2; Observations: 32; Constant: 363.7. *F*- ratio is the Fisher test ratio (the value to be compared with statistical tabular values [*F*. distribution table] for significances either at P = 0.01 [1%] or P = 0.05 [5%]. (Cochran and Cox 1992).

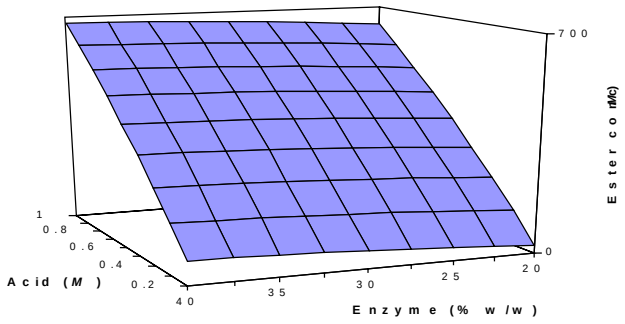

Fig.1: Response surface plot showing the effect of enzyme concentration, acid concentration and their mutual interaction on hexyl oleate synthesis. Other variables (alcohol: 1.25 *M*, incubation time: 120 h and temperature: 30°C) are constant.

Figure 2 depicts the effect of enzyme concentration *versus* incubation time at 1 *M* acid concentration and 1.25 *M* alcohol concentration at 30°C. Increase in enzyme concentration led to marginal increase in ester formation at all incubation times. As incubation time increased, at any given enzyme concentration, an increase in ester formation was observed, the increase being large [380 m*M* to 650 m*M*] at low enzyme and less [450 mM to 680 mM] at high enzyme concentrations, respectively. An increase in enzyme concentration led to a marginal increase in ester formation at all incubation priods. As the incubation time increased, at any given enzyme concentration, an increase in ester formation was observed, the increase being large (380-650mM) at low enzyme and less (450-680 mM) at high enzyme concentrations respectively. Increase in enzyme concentration had only a marginal effect on ester yields, the extent of conversion between 24 and 120 h being 380 to 650 m*M* at 20% [w/w] and 450 to 680 m*M* at 40% [w/w] enzyme concentration. However, an incubation time of 24 h itself gave rise to a maximum ester concentration from 380 m*M* to 450 m*M* in the enzyme concentration range of 20 to 40% [w/w] [i.e., 204 mg to 408 mg] indicating that more than 58% esterification took place within the first 24 h incubation time.

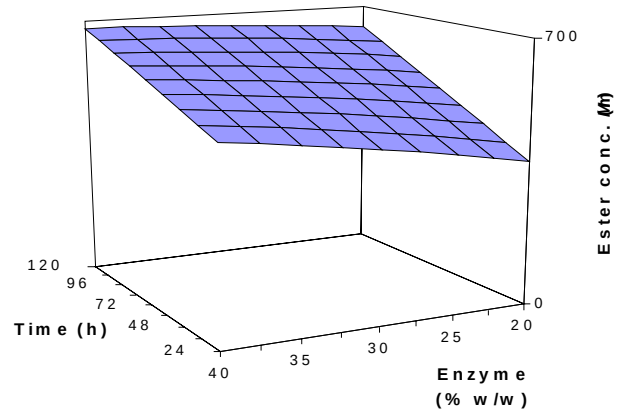

Fig. 2: Response surface plot showing the effect of enzyme concentration, incubation time and their mutual interaction on hexyl oleate synthesis. Other variables (acid: 1.0*M*, alcohol: 1.25*M*, and temperature :30°C) are constant

Figure 3 depicts the effect of temperature *versus* enzyme concentration at fixed concentration of acid [1.0 *M*], alcohol [1.25 *M*], and incubation time [120 h]. There was a decrease in the extent of esterification above 40°C. With an increase in temperature from 40°C to 70°C, esterification decreased at all enzyme concentrations. However, at each temperature, the esterification did not show much variation with increase in enzyme concentration. Lower temperatures [30 – 35°C] favored esterification at all enzyme concentrations employed. Temperature beyond 40°C showed decrease in the yield from 650 to 440 m*M* at 20% [w/w] enzyme and 680 to 500 m*M* at 40% [w/w] enzyme. Maximum ester concentration was detected in the temperature range 30 – 40°C. Although, 500 m*M* ester synthesis was observed at 70°C temperature, the majority of the ester concentration was observed in the early period of the reaction [24 h] indicating that the thermal inactivation of the enzyme was higher at a higher temperature. Even an increase in enzyme concentration had no significant effect at higher temperatures. Hence, the results show the beneficial effect of ambient temperatures in achieving maximum esterification.

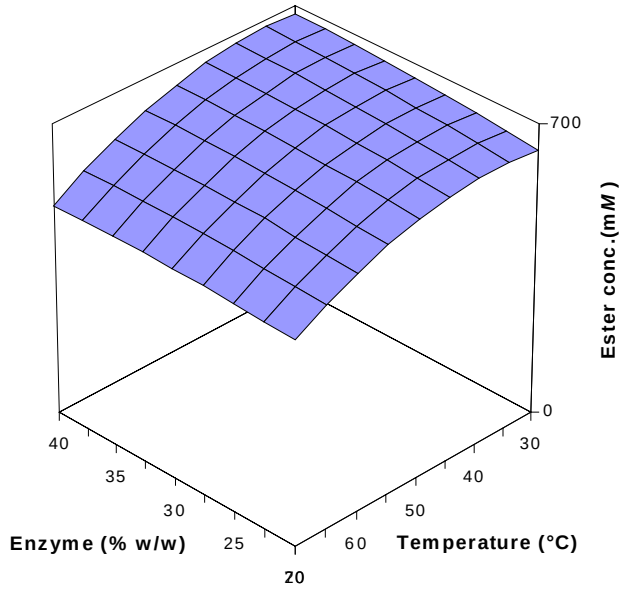

Fig.3: Response surface plot showing the effect of temperature, enzyme concentration and their mutual interaction on hexyl oleate synthesis. Other variables (acid: 1.0*M*, alcohol: 1.25*M*, and incubation time: 120 h) are constant.

The effect of acid concentration *versus* temperature is depicted in Figure 4 at fixed enzyme concentration 40% [w/w], alcohol concentration 1.25 *M* and incubation time 120 h, which exhibits a similar behavior as observed in Figure 3. The surface plot exhibited a very slight curvature with

marginal increase in yields up to 40°C, which decreased again marginally up to 70°C. At all the temperatures, esterification increased with acid concentration. At all acid concentrations, esterification increased with temperature up to 40°C and decreased thereafter. The decrease in ester concentration at a high acid concentration [1 *M*] was found to be 680 m*M* to 506 m*M*. However, at lower acid concentration [0.2 *M*] it was found to be 76.8 m*M* to 68.8 m*M*. This suggests that, enzyme inactivation aggravates at high acid concentrations combined with high temperatures. Therefore, lower temperatures not only allows higher acid concentrations to be used but also results in higher esterification

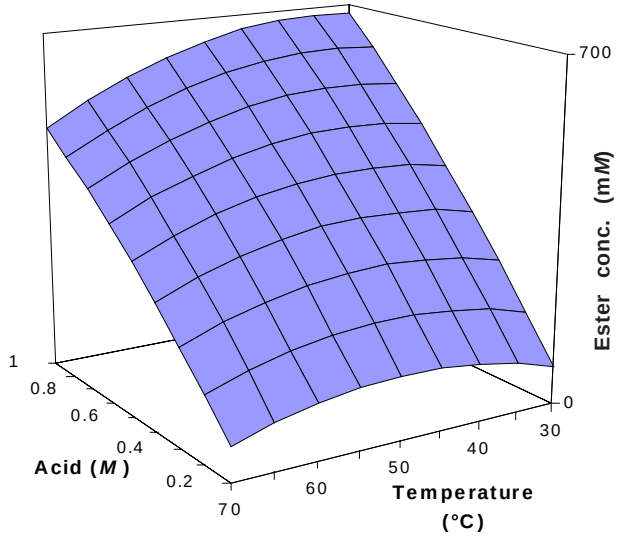

Fig.4: Response surface plot showing the effect of acid concentration, temperature and their mutual interaction on hexyl oleate synthesis. Other variables (enzyme: 40% (w/w), alcohol: 1.25*M* and incubation time: 120 h) are constant.

Figure 5 shows the variation of esterification with incubation time and temperature at 40% [w/w] enzyme concentration, 1.0 *M* acid concentration and at 1.25 *M* alcohol concentration. Only a marginal increase in esterification with increase in incubation time [24 – 120 h] has been observed at higher temperatures [i.e., 504 to 506 m*M*] suggesting that, deactivation of enzyme increased with increasing temperatures. At low temperatures, ester concentration increased with the incubation time [i.e., 509 to 680 m*M*]. However, maximum ester concentration has been achieved within 24h incubation time only.

Optimum Conditions:

To determine optimum conditions Microsoft 'solver' program, with Newton's search method has been used. The most efficient condition or optimum condition would be to use the lowest amount of enzyme to achieve a maximum conversion of the substrate within minimum incubation time and at ambient temperatures. For a given temperature and alcohol concentration, the acid and enzyme

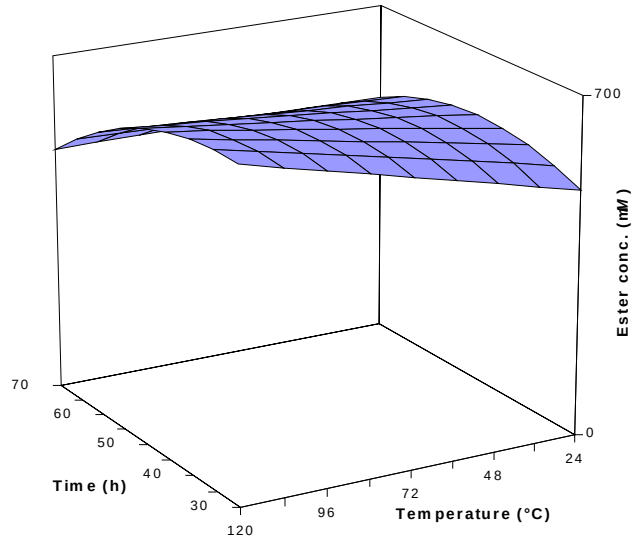

Fig.5: Response surface plot showing the effect of incubation time,temperature and their mutual interaction on hexyl oleate synthesis. Other variables (enzyme: 40% (w/w), acid: 1.0 *M* and alcohol: 1.25*M*) are constant.

concentrations required to attain a known extent of esterification in a given time can be calculated using the equation 2. Contour plots are very useful in visualizing the main effects of various reaction parameters and their mutual interactions. Figure 6 and 7 show the contour plots predicting the extent of esterification for different acid and enzyme concentrations and incubation time, which are quite useful experimentally, to arrive at economical processing conditions to obtain the required yield. From these plots, it can be observed that a desired ester concentration can be attained using specified combinations of enzyme, acid, incubation time and temperature. While several combinations can give the same ester concentration, from an economic viewpoint it is desirable to choose the lowest possible enzyme concentration. For example, 600 m*M* ester could be obtained using about 20% [w/w] enzyme after 105 h, but the same 600 m*M* ester can be achieved using about 40% [w/w] enzyme just after 72 h [Figure 7]. The optimum conditions predicted for synthesizing maximum ester [680 m*M*] under given conditions employed was as follows: enzyme {35% [w/w]}; acid [1.0 *M]*; incubation time [120 h]; alcohol [1.25 *M*] and temperature [35°C]. The actual experimental value obtained was 678 m*M* which was in good agreement with the predicted value.

Validation of the model.

The treatment was repeated for several experimental conditions to establish its repeatability with additional independent variables that were not employed in the generation of the model. Table 3 shows the validation experiments to obtain required yields indicated by the contour plots.

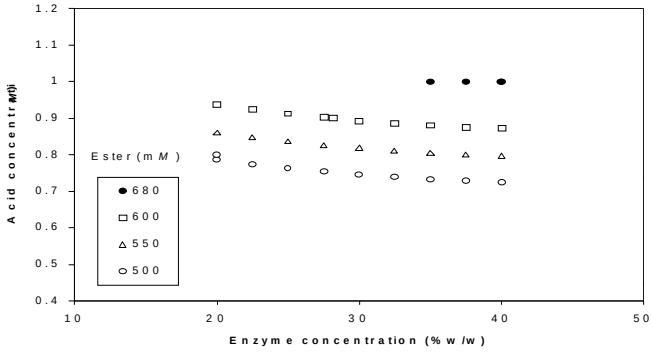

Fig.6: Contour plot showing the range of acid and enzyme concentrations to obtain various ester concentrations. Other variables (alcohol: 1.25 *M* , incubation time: 120 h and temperature: 30°C) are constant.

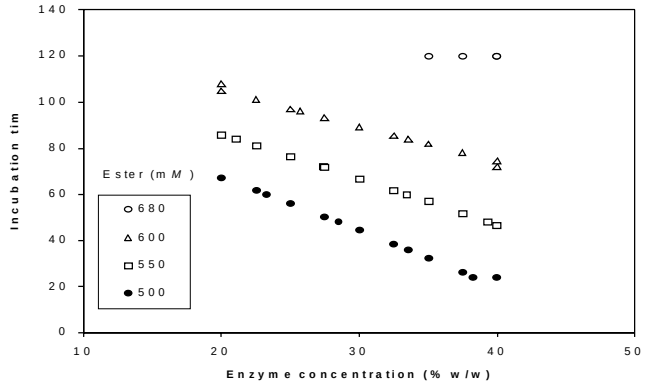

Fig.7: Contour plot showing the range of incubation time and enzyme concentrations to obtain various ester concentrations. Other variables (acid:1.0*M*, alcohol: 1.25*M* and temperature:30°C) are constant.

These optimum conditions have also been confirmed experimentally and it was observed that the experimental and predicted values of ester formation showed good correlation (Table3).

Table - 3
Model validation experiments at different reaction parameters*

X_1	X_2	X_3	X_4	X_5	Y_{Exp} (m*M*)	Y_{Pred} (m*M*)
20	0.937	120	1.25	30	588	600
40	0.873	120	1.25	30	610	600
20	0.861	120	1.25	30	539	550
40	0.797	120	1.25	30	557	550
40	1.0	120	1.25	30	690	680
40	1.0	120	1.25	40	672	680
40	1.0	120	1.25	65	537	550
40	1.0	120	1.25	70	488	500
20	1.0	108	1.25	30	588	600
40	1.0	96	1.25	30	590	600
40	1	24	1.25	30	482	500

*Average absolute relative deviation % = 1.97

In conclusions the present investigation describes the applicability of central composite rotatable design for arriving at the optimum conditions for synthesis of hexyl oleate. The optimum conditions for achieving maximum percentage esterification [67.8%] are as follows: 1.25*M* alcohol concentration,1*M* acid concentration,35%[w/w] enzyme concentration and at 120 h of incubation time and 35°C.

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In Vitro Evaluation Of Antibacterial Activity Of *Actiniopteris Radiata* (Sw.) Link

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ABSTRACT

The whole fern extracts of *Actiniopteris radiata* (Sw.) Link. were prepared separately in hexane, chloroform, ethyl acetate and ethanol. These extracts were assayed for antibacterial property against major pathogens like *S. aureus, K. pneumoniae, P. aeruginosa, S. typhimurium, and E. coli* and *B. subtilis.* Agar well diffusion bioassay was performed for primary screening, followed by MIC and MBC. Ethanol and hexane extracts of *A. radiata* showed maximum antibacterial property against all the bacteria used in the present study followed by ethyl acetate and chloroform extracts respectively. Comparatively, the MBC values were higher than the MIC values in all the extracts. Qualitative analysis of different solvent extract of *A. radiata* revealed the presence of flavonoids, glycosides, phenols, tannins saponins and sterols which may be responsible for the observed antibacterial property of *A. radiata*.

KEY WORDS: *Actiniopteris radiata*, antibacterial activity, DIZ, MIC, MBC, secondary metabolites analysis.

Introduction

According to World Health Organization (WHO), the increase of resistance to antibiotics by bacterial pathogens is a growing problem in both developed and developing countries [1]. This problem has further posed challenges to develop newer and potent drugs against pathogens. Occurrence of multi-drug resistant pathogens is ever increasing, and treatment of such strains has led to the administration of very large doses of antibiotics, resulting in enormous amount of side effects to the patients [2,3]. One of the measures to minimize the increasing rate of resistance in the long run is to have continuous in- depth investigation for new, safe and effective antimicrobials as alternative agents to substitute the existing ones. Natural resources, especially the plants and microorganisms, are the potent candidates for this purpose [4].

Antimicrobial property of several plant products has become the part of the modern science approach to find newer drugs against the pathogenic bacteria. It has been reported that between the years 1983 and 1994, of 93 new antibacterial agents submitted to analysis by the FDA, six were natural products (teicoplanin, mupirocin, miokamycin, carumonam, isepamicin and RV-11). The systematic screening of antibacterial plant extracts represents continuous efforts to find newer compounds with the potential to act against multi-drug-resistant bacteria [5]. The continuous development of antibiotically resistant strains of microbial pathogens such as MRSA (Methicillin resistant *Staphylococcus aureus*), PRSP (Penicillin resistant *Streptococcus pneumoniae*) and VRE (Vancomycin resistant *Enterococci*) is a growing problem, and it is therefore, extremely important to discover and develop new antimicrobial compounds [6]. The screening of plant extracts for antimicrobial activity such as benzoin and emetin has shown that higher plants represent a potential source of new antimicrobial compounds [7,8]. The antimicrobial compounds from plants may inhibit bacteria through different mechanisms than the conventional antibiotics, and could therefore be of clinical value in the treatment of microbial infections [9,10].

Antimicrobial properties have been reported in a wide range of plant extracts [11] and traditional usage of plants in curing various diseases has deep roots in man's history and is an integral part of folklore medicine [12].

Tirumala Hills (Rayalaseema region, Andhra Pradesh, India), which lie geographically in the South Eastern Ghats are known for the rich heritage of flora. A number of plants, which are known to possess medicinal properties, have been in use in the folklore medicine [13]. However, till date there is no clear antimicrobial active principle (s) has been established. One such plant, which is commonly used in

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the folklore medicine, is *Actiniopteris radiata* (Sw.) Link belonging to *Actiniopteridaceae* family is a plant with great medicinal value. According to ayurvedic texts mayurashikhaa is used as astringent, anti-inflammatory, tonic to genitourinary tract, alleviates vitilated blood, indicated in cough, bronchitis, asthma, diarrhoea, dysentery, dysuria, used internally as well as externally for infected wounds and ulcers [14].

Previously the antibacterial activity of aqueous and alcohol-based root, stems and leaf extracts of *Actiniopteris radiata* (Sw.) Link was reported [15]. Since the whole herb is used locally in folklore medicine to treat various ailments, the present study was aimed on phytochemical analysis and evaluation of the antibacterial activity of whole herb of *A. radiata* by employing various organic solvents against the pathogenic bacteria such as *Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Salmonella typhimurium, Pseudomonas aeruginosa* and *Klebsiella pneumoniae*.

Materials and Methods

Plant material

The plant material was collected from Eastern Ghats (Tirumala hills, India), and identified in the herbarium of the Department of Botany, Sri Venkateswara University, Tirupati, India.

Preparation of the extracts

The whole plant was air-dried in the shade and powdered. 100g of the fine air-dried powder was soxhlated with solvents consecutively in the order of their increasing polarity, with hexane (waxy oil), chloroform, ethyl acetate and ethanol respectively, for more than 6 hrs with each solvent; and the extracts were then concentrated *in vacuo to* yield dense residues. The yields of the extracts were 12.5%, 8.9%, 5.0% and 6.5% for ethanol, ethyl acetate, hexane and chloroform respectively. The samples were transferred to glass vials and stored for further studies.

Preparation of test samples

In the studies of the antimicrobial activity, 20mg each of soxhlated plant extract was dissolved in 1ml of DMSO (DiMethylSulfoxide) for bioassay. Solvent control was also included, although no antibacterial activity was observed at the solvent concentration employed. All samples were tested in triplicate and average results were recorded.

Test microorganisms

The soxhlated plant extracts were assayed for antimicrobial activity against six registered bacterial isolates, which were obtained from the NCIM (National Culture Collection, Pune - 411 003, India). The bacteria included two Gram positive bacterial isolates- *Staphylococcus aureus* (NCIM No: 5021, ATCC No: 25923) and *Bacillus subtilis* (NCIM No: 2063, ATCC No: 6633), and four Gram negative bacteria-*Escherichia coli* (NCIM No: 2931, ATCC No: 25922), *Pseudomonas aeruginosa* (NCIM No: 5029, ATCC No: 27853), *Salmonella typhimurium* (NCIM No: 2501, ATCC No: 23564) and *Klebsiella pneumoniae* (NCIM No: 2957). The bacteria were grown and maintained on nutrient agar (Hi-media, Mumbai), and subcultured as and when needed.

Antibacterial bioassay

Agar well diffusion bioassay

For bioassays, a suspension of approximately 1.5x 10^8 bacterial cells/ml in sterile normal saline was prepared as described by Forbes *et al* [16]. About 1.5 ml of it was uniformly spread on nutrient agar media (Hi-Media) in 12x 1.2 cm glass Petri dishes. Kept aside for 15 min and excess of suspension was then drained and discarded properly. Wells of 6 mm in diameter and about 2 cm apart were punctured in the agar culture medium using sterile cork borer. Respective concentrations were administered to fullness in each well. Culture plates were incubated at 37^{o}C for 48h. Bioactivity was determined by measuring Diameter of the Inhibition Zone (DIZ) in mm. The plant extracts concentrations were taken from 25 and 50mg/ml were evaluated for well method. Each was done in triplicates and mean of the DIZ was calculated. Controls included the use of solvent without test sample, although no antibacterial activity noted at the solvent concentration employed for the test [17]. The standard drugs taken were Ciprofloxacin and Chloramphenicol to compare with the soxhlated solvent extracts.

Determination of MIC and MBC

Minimum Inhibitory Concentration (MIC) and Minimum Bacterial Concentration (MBC) were determined for the extracts that showed total growth inhibition using the protocol described below. The minimum concentration, at which there was no visually detectable bacterial growth, was taken as MIC. Extract concentration of 0.1mg to 7.5mg/ml in steps of 0.1mg /ml were evaluated. Specifically 0.1ml of standardized inoculum (1-2 x 10^7 CFU/ml) was added to each test tube. The tubes were incubated aerobically at 37^{o}C for 18-24hrs. Two control**s** were maintained for each test sample. The lowest concentration (highest dilution) of the extract that produced no visible signs of bacterial growth (no turbidity) when compared with the control tubes were regarded as MIC. However, the MBC was determined by subculturing the test dilution on to a fresh drug-free solid medium and incubated further for 18-24hrs. The highest dilution that yielded no single bacterial colony on a solid medium was taken as MBC [17].

Screening for Secondary Metabolites

Secondary metabolites are identified in the extracts of *Actiniopteris radiata* by using standard methods. 500 mg of each extract was dissolved in 100 ml of the respective solvent and filtered through Whattman filter paper No.1.

Thus, the filtrates obtained were used as test solutions for the screening.

Qualitative analysis

The details for the qualitative analysis [18,19,20,21,22] were described in the below.

Test for alkaloids

*Iodine test***:** 1ml of KI in Iodine solution was added to the 2 ml of test solution. A brown precipitate formation indicated the presence of alkaloids.

*Dragendorff's reagent***:** 2 ml of Dragendorff's reagent and 2 ml of diluted HCl were added to the test solution. An orange-red coloured precipitate indicates the presence of alkaloids.

Wagner's test: 2 ml of Wagner's reagent was added to 2 ml of test solution. The formation of reddish brown precipitate indicates the presence of alkaloids.

*Mayer's test***:** To a little of test solution add a few drops of Mayer's reagent. White precipitate formed indicates the presence of alkaloids. Some alkaloids are soluble in excess of the reagent. If no precipitate forms with the addition of few drops more reagent is to be added.

Test for flavonoids

Pew's test (Zn/HCl): A pinch of zinc powder and about 5 drops of 5N HCl were added to the test solution. It results deep purple red or cherry red colour.

Shinoda test (Mg/HCl): A pinch of magnesium powder and 5 N HCl were added to the test solution and a deep red or magenta colour is formed.

*NaOH test***:** 1 ml of 1N NaOH solution was added to the 1 ml of test solution formation of yellow colour indicated the presence of flavonoids.

Test for glycosides

Keller-Killiani test: 1 ml of glacial acetic acid was carefully added to 2 ml of test solution of the extract and mixed well. Further, 2 drops of ferric chloride solution was added after cooling. These contents were transferred carefully to a test tube containing 2 ml of concentrated H_2SO_4. A reddish brown ring was observed at the junction of two layers.

*Concentrated H_2SO_4 test***:** 1 ml of concentrated H_2SO_4 was added to 1 ml of test solution and is allowed to stand for 2 minutes. The formation of reddish colour indicates the presence of glycosides.

*Molisch's test***:** A mixture of Molisch's reagent and concentrated H_2SO_4 (1:1) was added to the test solution. Formation of reddish-violet coloured ring at the junction of two liquids at the presence of glycosides.

Test for phenols

Ellagic acid test: The test solution was treated with few drops of 5 % (V/V) glacial acetic acid and 5 % (W/V) $NaNO_2$ solution. The solution turns muddy yellow, olive brown, niger brown, deep chocolate colours depending on the amount of ellagic acid present.

*Phenol test***:** When 0.5 ml of $FeCl_3$ (W/V) solution was added to 2 ml of test solution, formation of an intense colour indicates the presence of phenols.

Test for saponins

*Foam test***:** 0.1g of crude extract was shaken vigorously in 2 ml distilled water. Formation of honeycomb like froth persists for a few minutes indicate the presence of saponins.

Test for sterols

*Libermann-Burchard test***:** A green colour was formed, when the Libermann-Burchard reagent is added to the test solution, indicate the presence of sterols.

*Salkowski test***:** A wine red colour was developed when chloroform and concentrated H_2SO_4 were added to the test solution; indicate the presence of steroidal nucleus.

Test for tannins:

Gelatin test: The test solution was evaporated to dryness and the resulted residue was dissolved in 1 % (W/V) liquefied gelatin. To this 10 % (W/V) NaCl solution was added. A white precipitate was obtained which indicate the presence of tannins.

Results

In order to understand the antibacterial property of *Actiniopteris radiata,* agar well diffusion method was carried out against all selected bacteria. All extracts of *A. radiata* showed antibacterial activity against selected bacteria, viz., *E. coli*, *P. aeruginosa*, *S. typhimurium*, *S. aureus*, *K. pneumoniae* and *B. subtilis.*

The highest zone of inhibition was obtained with ethanol and hexane extracts at a concentration of 50mg/ml against all the tested bacterial strains. However, average zone of inhibition was observed with ethyl acetate extract at all concentrations (25 and 50mg/ml). The chloroform extract showed least zone of inhibition against Gram negative bacteria at low concentrations (25mg/ml). However, higher concentrations of chloroform extract (50mg/ml) showed distinct antibacterial property against both Gram positive and Gram negative bacteria (Table 1).

The MBC values correlated with MIC values. Comparatively, MBC values were higher than the MIC values. The lowest MIC values were observed in ethanol and hexane extract, moderate MIC values were obtained in ethyl acetate extract, and higher values were obtained in

Table. 1

DIZ (mm) of various solvent extracts of *Actiniopteris radiata* against different bacteria.

Bacteria	5µg/disc	30µg/disc	25mg/ml				50mg/ml			
	Cf	Ch	H	C	Ea	E	H	C	Ea	E
E. coli	26.9±0.01	22.0±0.0	14.0±0.1	10.0±0.0	12.7±0.20	15.1±0.01	16.1±0.10	13.0±0.00	14.2±0.02	16.4±0.02
P. aeruginosa	24.5±0.03	23.5±2.0	13.5±0.7	11.0±0.0	12.0±0.05	14.2±0.20	16.0±0.30	14.0±0.50	15.0±0.0	17.5±0.03
S. typhimurium	22.6±0.06	22.5±0.01	14.8±0.01	12.0±0.0	14.3±0.02	16.3±0.40	16.0±0.10	15.0±0.60	16.0±0.0	18.2±0.02
S. aureus	23.0±0.01	25.0±0.02	12.9±0.02	10.7±0.0	12.1±0.4	14.5±0.01	14.6±0.04	13.2±0.70	14.2±0.01	16.0±0.0
K. pneumoniae	24.0±0.04	30.0±0.04	13.5±0.10	10.0±0.0	11.0±0.0	13.8±0.02	16.2±0.07	12.1±0.10	14.0±0.0	16.8±0.01
B. subtilis	11.9±0.03	26.0±0.05	14.4±0.40	12.3±0.1	12.7±0.01	16.3±0.03	16.0±0.0	14.1±1.02	15.1±0.05	18.2±0.20

Cf =Ciprofloxacillin; Ch = Chloramphenicol; H=Hexane; C=Chloroform; Ea=Ethyl acetate; E=Ethanol.

Table. 2

MIC and MBC (mg/ml) of various solvent extracts against different bacteria.

Bacteria	MIC				MBC			
	H	C	Ea	E	H	C	Ea	E
E. coli	0.560±0.44	2.1±0.05	1.86±0.08	0.510±0.05	1.22±0.11	3.8±0.23	3.36±0.12	1.10±0.02
P. aeruginosa	0.540±0.12	2.96±0.06	2.74±0.03	0.520±0.23	1.45±0.04	5.76±0.02	5.11±0.01	1.20±0.04
S. typhimurium	0.630±0.09	3.06±0.09	2.72±0.1	0.610±0.13	1.55±0.23	6.1±0.09	5.88±0.12	1.32±0.08
S. aureus	0.450±0.10	2.75±0.07	2.4±0.10	0.440±0.15	0.810±0.33	5.23±0.14	4.95±0.42	0.790±0.14
K. pneumoniae	0.490±0.14	3.0±0.06	2.83±0.12	0.460±0.07	1.10±0.41	6.08±0.05	5.76±0.14	0.820±0.09
B. subtilis	0.550±0.21	3.1±0.05	2.78±0.10	0.530±0.11	1.20±0.11	5.16±0.08	4.97±0.09	0.910±0.16

E=ethanol; Ea=ethyl acetate; H=hexane; C=chloroform.

chloroform extract. The ethanol extract showed least MIC and MBC values against *S. aureus* (0.440 and 0.790 mg/ml) and highest MIC values against *S. typhimurium* (0.610 and 1.32 mg/ml) as shown in Table 2.

Table. 3

Screening tests for secondary metabolites in solvent extract of *Actiniopteris radiata.*

Secondary metabolites	Name of the test	Result (+/-)			
		H	C	Ea	E
Alkaloids	Iodine	-	-	-	-
	Dragendorff's	-	-	-	-
	Wagner's	-	-	-	-
	Mayer's	-	-	-	-
Flavonoids	Pews	-	+	+	+
	Shinda	-	+	+	+
	NaOH	-	+	+	+
Glycosides	Keller-Kiliani	+	-	+	+
	Conc.H_2SO_4	+	-	+	+
	Molisch	+	-	+	+
Phenols	Ellagic acid	-	-	-	+
	Phenols	-	-	-	+
Saponins	Foam	+	-	-	-
Sterols	LibermanBuchard	-	+	-	-
	Salkowski	-	-	-	-
Tannins	Gelatin	-	-	-	+

H=Hexane; C=Chloroform; Ea=Ethyl acetate and E=Ethanol.

The plant extracts were also screened for qualitative analysis to know the relative distribution of the secondary metabolites which may be responsible for the potent antibacterial activity. The chloroform extract of *A. radiata* showed flavonoids and sterols while hexane extract contained wax, glycosides and saponins. On the other hand, ethylacetate extract contained flavonoids and glycosides, and ethanol extract contained flavonoids, glycosides, phenols, tannins and alkaloids (Table 3).

Discussion

Pathogenic bacteria have developed resistance in the long run that are resistant to almost all antibiotics today. Particularly worrisome are wounds and other hospital cross infections caused by *Staphylococci.* On the other hand *Enterococci*, which causes pneumonia and other fatal infections. The problem has become more serious by the emergence of MDR strains of these organisms, so called super bugs, that are resistant to Vancomycin, a drug widely recognized as the last line of defense in many Gram positive bacterial infections. Most classes of antibiotics were discovered in the 1940's, and are directed at a few specific aspects of bacterial physiology, mainly the inhibition of the biosynthesis of the cell wall, DNA and proteins. It is believed that wide spread drug resistance among bacterial pathogens is due to *in vivo* transposition, and is mostly due to plasmids [23]. The main thrust in the post-genomic and proteomic *era* is to develop newer rational drugs against these MDR strains. Uses of plants and their products as anti microbial agent's date back to the start of human civilization. One such plant, which is commonly used in the south Eastern Ghats of India in the Tirumala hills, is *A. radiata*.

In India, mortality rate due to infections in the hospitals is largely due to *S. aureus, P. aeruginosa, K. pneumoniae* and *E. coli* [24]. With the identification of various MDR strains in *S. aureus*, the treatment and management of infections caused by these strains has become very difficult, therefore, the challenge to discover newer and potent drugs is ever increasing. It is very much clear now that horizontal gene transfer in *S. aureus* resulted in the occurrence of various drug resistant strains [25,26,27]. Therefore, studies were undertaken to test the extracts of *A. radiata* against these pathogens. The highest activity was observed in ethanol extract followed by hexane, ethyl acetate and chloroform. When these extracts were analyzed for secondary metabolites obtained results were supporting the MIC and MBC findings.

The chloroform extracts of *A. radiata* showed flavonoids and sterols, while hexane extract contained wax, largely glycosides and saponins. On the other hand, ethylacetate extract contained both flavonoids and glycosides. Ethanol extract of *A. radiata* contained flavonoids, glycosides and phenols. The highest activity of ethanol extract may be due to the presence of flavonoids, glycosides and phenols. The secondary metabolites of various chemical types present in *A. radiata* are known to possess antimicrobial activities.

Flavonoids are found to be effective antimicrobial substances against a wide range of microorganisms, probably due to their ability to complex with extra cellular and soluble proteins and to complex with bacterial cell wall; more lipophilic flavonoids may also disrupt microbial membrane [28]. Phenolics and polyphenols present in the plants are known to be toxic to micro-organisms [29]. Antibacterial activity of tannins may be related to their ability to inactivate microbial adhesins, enzymes and cell envelope transport proteins, they also complex with polysaccharides [30]. The broad spectrum antibacterial activity exhibited by *A. radiata* may be attributed to the various active constituents presents in it which either due to their individual or combined action. Hence the present findings provide a scientific base for some of the medicinal claims of *A. radiata*. Further, studies are in progress in our laboratory to isolate and characterize these active compounds.

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Hepatoprotective activity of stem bark of *Madhuca longifolia* against carbon tetrachloride-induced liver damage in rats

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ABSTRACT

The objective of the present investigation was to study the hepatoprotective activity of ethyl acetate fraction and methanolic extract of stem bark of *Madhuca longifolia,* using carbon tetrachloride induced liver damage model in rats. Liver damage in rats was produced by carbon tetrachloride (0.5 ml/mg.b.w.i.p) once daily for seven days. Ethylacetate fraction and methanolic extract of *M.longifolia* (100mg/kg, 200mg/kg.b.w.p.o.), were administered respectively, Silymarin (25mg/kg.b.w.p.o.) was given as reference standard. The stem bark extracts were effective in protecting the liver against the injury induced by CCl_4 in animals.This was evident from significant reduction in serum enzymes, Serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase, Alkaline phosphatase (ALP) and Total bilirubin (TB).Various pathological changes like centribular necrosis and vacuolization were observed in liver of CCL_4 treated rats, which were significantly reduced in groups treated with ethylacetate fraction and methanolic extract *M.longifolia* and silymarin. It was concluded from this study that ethyl acetate fraction and methanolic extract of *M.longifolia* possess hepatoprotective activity against CCl_4 induced hepatotoxicity in rats**.**

KEY WORDS: Hepatoprotective, *Madhuca longifolia*, silymarin, Carbon tetrachloride.

Introduction

The plant *Madhuca longifolia (*Koenig*)* (Syn, *Bassia longifolia* Koenig) Sapotaceae called as South Indian Mahua, is a tree widely distributed throught India [1] The previous phytochemical studies on this plant have revealed the presence of Mi-Saponins A, B [2] and C [3]. Four new oleanane type triterpene saponins madlongisides (A to D) [4] protobassic acid [5] were isolated from seeds of this plant. Traditionally flowers are used as stimulant and diuretic, bark is astringent, emollient and useful in inflammation [6]. The evaluation of the stem bark of *Madhuca longifolia* in the treatment of liver disease has not been reported in the laboratory animals. The objective of present study was to study the effect of both ethyl acetate fraction and methanolic extract of stem bark of *Madhuca longifolia* on hepatoprotective activity in rats against carbon tetrachloride as hepatotoxin to prove its claim in the folklore practices against liver disorders.

Materials and Methods

Collection of the plant

The stem bark of *Madhuca longifolia* was collected in and around Andhra university,Visakhapatnam, in the month of July 2006.The authentication of the plant was done by Dr. M. Venkaiah, Associate Professor, Department of Botany, Andhra University, Visakhapatnam. A voucher specimen (No.TSNDOP7/2006) has been preserved in the herbarium of the department.

CCl_4 was obtained from Poona Chemical Laboratory, Pune, India. Silymarin-Microlab,Bangalore, Karnataka,India. Estimation kits-Span Diagnostics, Surat, India. All other chemicals were obtained from local sources (Sai chemicals, Visakhapatnam) and were of analytical grade.

Extraction procedure

Freshly collected plant material was shade dried at room temperature and coarsely powdered in Wielly mill.The powder stem bark plant (1kg) was subjected to extraction with methanol in soxhlet extractor apparatus. The extract was concentrated to dryness till free from solvents in rotary evaporator. Yield of total 80 gm was methanolic extract obtained.The methanolic extract was fractionated with hexane (10×250ml) and ethyl acetate (10×250ml). All solubles were subsequently concentrated under reduced pressure at controlled temperature, stored in a desiccators, the

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percentage yield of hexane soluble fraction was 2gms, ethyl acetate soluble fraction was 28.5 gms and remaining methanol soluble extract was 46.5 gms. On preliminary phytochemical screening the ethyl acetate fraction revealed presence of steroids, triterpinoids and methanolic extract revealed presence of saponins and tannins [7].

Animal Used

Wistar albino rats of either sex weighing between 200-250 gm were obtained from M/s. Mahavir Enterprises, Hyderabad. The animals were housed under standard environmental conditions (temperature of 22 ± 1°C with an alternating 12 h light – dark cycle and relative humidity of 60 ± 5%), one week before the start and also during the experiment as per the rules and regulations of the Institutional Ethics Committee and by animal regulatory body of the government (Regd: No: 516/01/A/CPCSEA). They were fed with standard laboratory diet supplied by M/s. Rayans biotechnologies Pvt. Ltd., Hyderabad, and water *ad libitum*.

Determination of Acute Toxicity [8]

Acute toxicity studies were performed for extracts according to the toxic classic method as per OECD guidelines. Female albino rats were used for acute toxicity study. The animals were kept fasting overnight providing only water, after which the extracts were administered orally at the dose of 300 mg/kg and observed for 14 days. If mortality was observed in 2 out of 3 animals, then the dose administered was assigned as toxic dose. If the mortality was observed in 1 animal, then the same dose was repeated again to confirm the toxic dose. If the mortality was not observed, the procedure was repeated for further higher dose i.e.,2000 mg/kg, 2500mg/kg, 3000mg/kg.

Carbon tetrachloride-induced hepatotoxicity

The animals were divided into seven groups of six animals each. Group-I served as normal control received 5% acacia mucilage (1 ml/kg.b.w.p.o) daily once for 7 days. Group-II served as toxic control received CCl_4 (0.5 ml/kg.b.w. i.p) daily once for 7 days [9]. Group-III was treated with the reference drug Silymarin (25mg/kg.b.w.p.o) daily once for 7 days [10]. Groups IV-V were treated with ethyl acetate fraction of *M.longifolia* stem bark at doses of 100 and 200 mg/kg p.o. followed by CCl_4 (0.5 ml/kg.b.w. i.p) daily once for 7 days. Groups VI-VII were treated with methanolic soluble extract of *M.longifolia* stem bark at doses of 100 and 200 mg/kg, *p.o.* followed by CCl_4 (0.5 ml/kg.b.w. i.p) *daily* once for 7 days.

Collection of blood samples

All the animals were sacrificed on 7th day under light ether anesthesia.The blood samples were collected separately in sterilized dry centrifuge tubes by puncture of retro-orbital plexus and allowed to coagulate for 30 min at 37 °C.The clear serum was separated at 2500 rpm for 10min and subjected to biochemical investigation viz..,serum glutamic oxaloacetate trans aminase (SGOT), serum glutamic Pyruvate trans aminase (SGPT), Alkaline phosphatase (ALP) and Total Bilirubin (TB).

Assessment of liver function

The Serum glutamic oxaloacetic transaminase (SGOT) and serum glutamic pyruvic transaminase (SGPT) were estimated by UV kinetic method in which both SGOT and SGPT were assayed based on enzyme-coupled system, where keto acid formed by the aminotransaminase reacts in a system using NADH.The coenzyme is oxidized to NAD and the decrease in absorbance at 340 nm for SGOT malate dehydrogenase (MDH) reduces to malate with simultaneous oxidation of NADH to NAD.The rate of oxidation of NADH is measured, whereas SGPT[11] the pyruvate formed in the reaction is converted to lactate by lactate dehydrogenase. Estimation of Alkaline phosphate (ALKP) [12] involves hydrolysis of P-nitrophenyl phosphate by alkaline phosphatase to give P- nitrophenol, which gives yellow color in alkaline solution.The increase in absorbance due to its formation is directly proportional to ALKP activity. Estimation of total bilirubin (TB) [13] involved the reaction of bilirubin with diazotized sulphanic acid to form an azocompound, the color of which is measured at 546 nm. All the estimations were carriedout using standard kits in semi auto analyzer Screen Master 3000.

Statistical analysis

Results of biochemical estimation were reported as Mean ±SEM for determination of significant inter group difference was analysed separately and one-way analysis of variance (ANOVA)was carried out [14]. Dunnet's test was used for indidual comparisons [15].

Results

The LD_{50} of ethyl acetate and methanol stem bark extracts were found to be 2000mg/kg.b.w.p.o 1/10th, 1/20th these doses 200mg/kg.b.w.p.o and 100mg/kg.b.w.p.o were selected for the evaluation of hepatoprotective activity. Effect of ethyl acetate fraction (200mg/kg.p.o. and 100mg/kg.p.o) and methanol extracts (200mg/kg.p.o. and 100mg/kg.p.o.) of *Madhuca longifolia* stem bark on CCl_4 induced liver damage in rats with reference to biochemical changes in serum is shown in (Table-1). Percentage decrease or increase was calculated by considering the enzyme level difference between hepatotoxin treated and control rats as 100% of level of reduction and recorded in (Table- 2)

The comparative efficacy of the extracts tested for their hepatoprotective activity were depicted in the form of a bar diagram fig (1)

Histopathology of liver tissues: In the histological studies, normal control animals showed normal hepatic

Table-1

Effect of *Madhuca longifolia* stem bark on carbon tetrachloride -induced toxicity in rats

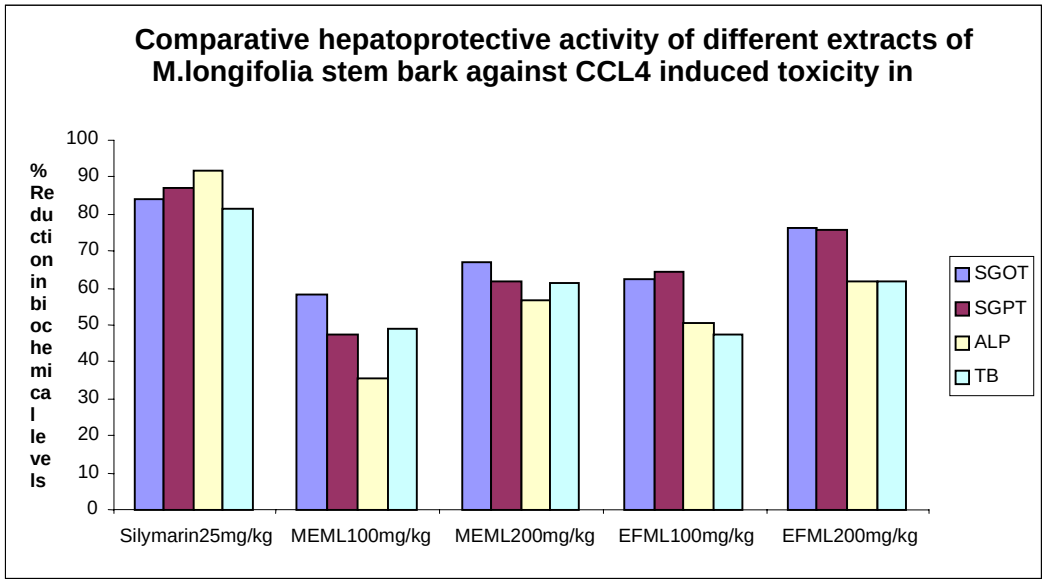
Treatment with	SGOT (mg/dl)	SGPT (IU/L)	ALP (IU/L)	TB (IU/L)
Control(5% Gum ACACIA.P.O)	123.83±1.70	82.66±1.54	58.33±2.20	1.06±0.08
CCL_4 (0.5 ML/KG.I.P)	997.66±22.74	736.0±17.57	488.48±8.23	4.48±0.17
SILYMARIN (25MG/KG.P.O)	223.16±1.40	164.50±1.60	85.16±2.80	1.7±0.13
EFML (100MG/KG.P.O)	52.83±1.64	315.83±0.94	282.16±2.18	2.48±0.18
EFML (200MG/KG.P.O)	332.5±1.82	242.16±1.30	243.83±1.07	2.00±0.15
MEML (100MG/KG.P.O)	486.5±2.45	424.16±1.35	332.5±1.17	3.13±0.17
MEML (200MG/KG.P.O.)	409.33±1.02	332.66±1.62	262.66±1.35	2.38±0.12

EFML – Ethyl acetate fraction of *Madhuca longifolia*, MEML – Methanol extract of *Madhuca longifolia.*, n=6, *P=0.01. Data analysed by ANOVA followed by Dunnett's test. All groups compared with carbon tetrachloride (0.5 ml/kg) alone. All values are Mean ±SEM.

Table-2

Percentage decrease in levels of biochemical parameters due to treatment with different extracts of stem bark of *Madhuca longifolia*

Treated with	% Decrease Biochemical Levels			
	SGOT	SGPT	ALP	TB
Silymarin 25mg/kg	83.63	87.47	91.87	81.28
EEML 100mg/kg	62.34	64.31	50.37	47.36
EEML 200mg/kg	76.12	75.58	61.98	61.98
MEML 100mg/kg	58.49	47.73	35.28	48.83
MEML 200mg/kg	67.32	61.73	56.28	61.40



architecture (Fig-I), the toxic group animals exhibited intense centribular necrosis (N), Vacuolization and macro vesicular fatty changes (F) (Fig-2).Silymarin treated animals showed a normal hepatic architecture (Fig-3), Moderate accumulation of fatty lobules and cellular necrosis (Fig-4, 5) were observed in the animals treated with methanolic extract. However, the Ethyl acetate fraction treated animals exhibited significant liver protection against CCl_4 induced liver damage ,as evident by the presence of normal hepatic cords, absence of necrosis and fatty infilteration (Fig-6,7).

Discussion

The carbon tetrachloride mechanism begins with the trichloromethyl radical ($\cdot CCL_3$) and chlorine($\cdot Cl$) by the action of the mixed function of cytochrome P-450 oxygenase system. This free radical, which is initially formed as unreactive, reacts very rapidly with oxygen to yield a highly reactive trichloromethyl peroxy radical ($\cdot OOCCL_3$). Both radicals are capable of binding to proteins or lipids or abstracting a hydrogen atom an unsaturated lipid, thus initiating lipid peroxidation. This process of lipid peroxidation can significantly damage hepatic plasma membranes [16].The increased levels of SGOT, SGPT, ALP and TB are conventional indicators of liver injury [17]. The ability of hepatoprotective drug to reduce the injurious effect or to preserve the normal hepatic physiological mechanisms that have been disturbed by a hepatotoxin is the index of its protective effect [18]. Hepatocellular necrosis leads to evaluation of the serum marker enzymes, which are released from the liver in blood [19].

The present study revealed a significant increase in the activities of SGOT, SGPT, ALP and TB levels on exposure to CCl_4 indicating considerable hepatocellular injury. Among the two extracts tested ethyl acetate fraction and methanolic extracts of stem bark of *M.longifolia* found to possess significant($P<0.01$) protective effect same as the silymarin significant protective effect ($P<0.01$) against hepatotoxicity induced by carbon tetrachloride.But Group VII (ethyl acetate fraction 200mg/kg.b.w.p.o) is more effective

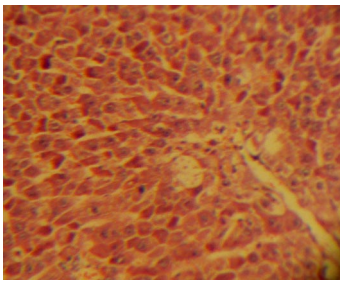


Fig.1

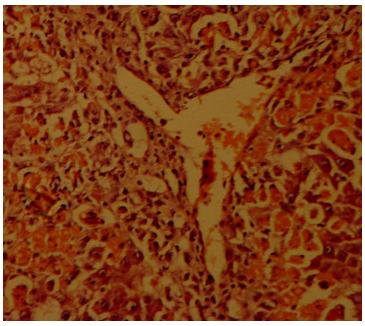


Fig.2

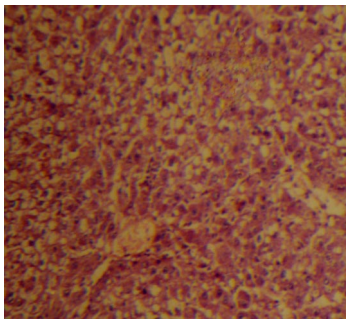


Fig.3

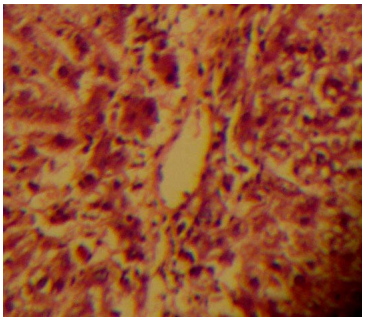


Fig.4

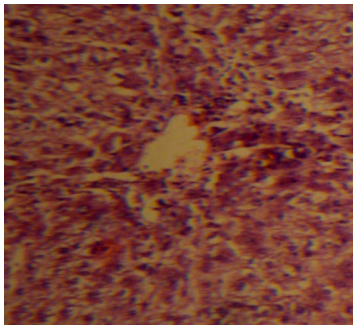


Fig.5

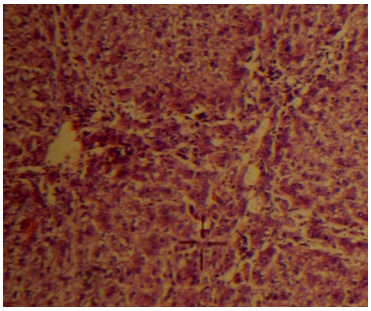


Fig.6

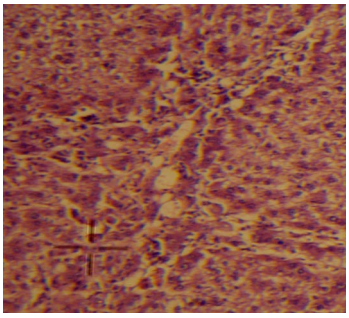


Fig.7

Fig.1: Representative photographs of histopathalogical changes showing effect of the test material on the rats intoxicated with carbon tetrachloride.1.Normal control, 2.Carbontetrachloride 0.5 ml/kg. 3.silymarin 25mg/kg. 4, 5.Methanolic extract (100mg/kg, 200mg/kg.), 6, 7.Ethylacetate fraction (100mg/kg, 200mg/kg.)

than all other groups and it may be hypothesized that rich content of active constituents.

The hepatoprotective effect of the drugs was further concluded by the histopathalogical examinations of the liver sections which reveales that the normal liver architecture was disturbed by hepatotoxin intoxication. In the liver sections of the rats treated with ethylacetate fraction and methanolic extract and intoxicated with CCl_4 the normal cellular architecture was retained as compared to silymarin, there by confirming the protective effect of the extracts of *M.longifolia*..

Accordance with these results, ethyal acetate fraction and methanol extract at two different dose levels offer dose dependent hepatoprotective activity. Which may be attributed to the individual or combined activity of phytoconstituents present in it. The hepatoprotective activity of these drugs might be due to stabilization of the membrane inhibiting effect on lipid peroxidation or due to their stimulatory effects on hepatic regeneration. The protective action may be due to scavenging effect of free radicals The components of the extract responsible for this effect however was not investigated. Further investigations are needed for identification of the active compounds responsible for hepatoprotective activity. In conclusion this study confirms the therapeutic potential of *M.longifolia*.

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In Vitro Studies on Adsorption of Omeprazole by Activated Charcoal

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ABSTRACT

In vitro experimental studies were performed to determine the extent of adsorption of Omeprazole onto activate charcoal in solution of varying pH (1.2, 4.0, 8.0 and 9.2 using different buffer solutions) and temperatures (37.5^{O}C, 40^{O}C and 43^{O}C). Amount of drug adsorption for varied amount (mg/ml) of drug in buffer solution (1.2, 1.84, 2.45, 3.07 and 3.68) and for varied amounts (mg) of charcoal (50,100, 200, 300 and 400) are studied. The results of studies shows that the amount of drug adsorption was more at pH of 4.0 and was more at lower temperature studied (37.5^{O}C). The adsorption studies were compared with that of prepared charcoal tablets and found that the adsorption was more with the activated powdered charcoal. Heat of adsorption is fond to be -15.917kcal/gm mole. Hence it was concluded that physical adsorption is involved.

KEY WORDS**:** Omeprazole, Drug Adsorption, Activated Charcoal, Isotherms

Introduction

Frequent drug poisonings that occur may be accidental or intentional, are due to acute drug ingestion (overdose). Generally, the drug poisonings are due to self medication or suicide attempts.

Activated charcoal is one of the most widely used micro crystalline porous material used in the emergency treatment of oral drug poisoning because of its wide spectrum capability for efficient adsorbing capacity. It is being used for pharmaceutical and medical puposes because of its extreme surface area (300-1500m^2/g) and high degree of porosity (10-60 A^o). Activated charcoal given orally as slurry can effectively adsorb and hold many drugs. The activated charcoal is widely used as antidote in emergency treatment of drug poisoning. Many investigators [1-8] have studied the ability of activated charcoal in in-vitro adsorption of various drugs from solutions at varied conditions of pH and temperatures. In-vitro and in-vivo experiments were reported to make reasonable predictions concerning the relative antidotal efficiency of activated charcoal in man on the basis of appropriate invitro adsorption studies[9].

The present study focuses on in-vitro adsorption of Omeprazole by activated charcoal as adsorbent. Omeprazole is one of the known antacids used in the treatment of Gastric ulcer, duodenal ulcer,hert burns, erosive esophagatis etc. Large single oral dose up to 160mg and introvaneous single dose upto 80mg have been tolerated without adverse effect. However an overdose more than 160mg may lead to blood and lymph disorders, hyper sensivity reactions, nervous system disorders, eye disorders, ear disorders, gastrointenstinal disorders, skin and bone disorders and even it may lead to permanent kidney damage. The objective of the present work is to study the physiochemical parameters responsible for Omeprazole adsorption on to activated charcoal and to evaluate the thermodynamic parameters for the adsorption process[10].

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Materials And Methods

Materials

Omeprazole the selected drug 99% pure procured from M/S Teena laboratories Pvt Limited, Hyderabad and Activated charcoal powder (-8 + 20 ASTM mesh, average particle size of 1.61mm) from the local market, Standard buffer solutions are prepared in the laboratory by standard procedures. Other chemicals used were of analytical grade.

Assay of Omeprazole

1.1 g of Omeprazole drug dissolved in a mixture of 10 ml of distilled water and 40 ml of ethyl alcohol. Titrate potentiometrically with 0.5M NaOH solustion. Each ml of 0.5M NaOH corresponds to 0.1727g of Omeprazole.

Experimental Method

In vitro adsorption studies of Omeprazole were carried out as per the following procedure:

1) **Effect of Adsorption Time** at constant pH(4.0) , drug concentration (2.45mg/ml), and temperature of 37 .5 °C

Omeprazole solution was prepared by dissolving 100mg in 40 ml of pH 4.0 buffer solution (Citrate phosphate buffer). Varied amounts(50, 100, 200, 400 mg) of charcoal powder were added to the Omeprazole solution and shaken in a water bath shaker (NSW India 133) at 135rpm for different time intervals (30,60,90,120 minutes) at a temperature of 37 .5 °C. With inbuilt thermostatic temperature controller, the water bath shaker temperature was maintained on average and was observed with digital display. Periodically samples were taken, filtered by using Whatman 40 filter paper and assayed.

2) **Effect of pH** at constant temperature 37 .5° C and amount of charcoal powder (200mg) Omeprazole solutions of different concentrations (1.23, 1.84, 2.45, 3.07, and 3.68) were prepared in a buffer solution for different pH (Hydrochloric acid buffer pH 1.2; Citrate phosphate buffer pH 4.0; Borate buffer pH 8.0; and Borate buffer pH 9.2)

3) **Effect of Temperature** at constant pH (4.0) and constant amount of charcoal (200mg) Different concentrations of drug solutions(1.2, 1.84, 2.45, 3.07, 3.68 mg/ml) were prepared. Adsorption studies were carried out at different temperatures (37.5, 40.0, 43.05°C) for different time intervals (30, 60, 90, 120 minutes)

4) **Effect of drug concentration** at constant temperature (37 .5° C) and pH(4.0).

Different concentrations of drug solutions (1.2, 1.84, 2.45, 3.07, 3.68mg/ml) were prepared in buffer solution of pH 4.0 and added different amounts of charcoal (150, 200mg). Samples were assayed at different time intervals (30, 60, 90, 120 minutes)

5) **Comparative studies of percentage adsorption of drug onto the Charcoal tablets and charcoal powder** at constant pH(4.0), temperature 37 .5 °C),and amount of charcoal tablet (200mg)

Different concentrations of drug solutions (1.2, 1.84, 2.45, 3.07, 3.68mg/ml) were prepared in buffer solution of pH 4.0. Adsorption studies were carried out by adding charcoal tablet (200mg) at 37 .5 ° C.

Results And Discusion

Effect of Adsorption Time: Adsorption of Omeprazole drug was increased with the increasing amount of activated charcoal and the time required to reach equilibrium was decreased markedly.

Table - 1

Effect of Time on Adsorption

Time (min)	Percentage of Adsorption				
	50mg	100mg	200mg	300mg	400mg
30	13.76	20.79	38.40	56.00	77.12
60	34.88	38.40	56.00	77.12	77.12
90	45.44	56.00	77.12	77.12	77.12
120	52.48	56.00	77.12	77.12	77.12
150	56.00	56.00	77.12	77.12	77.12

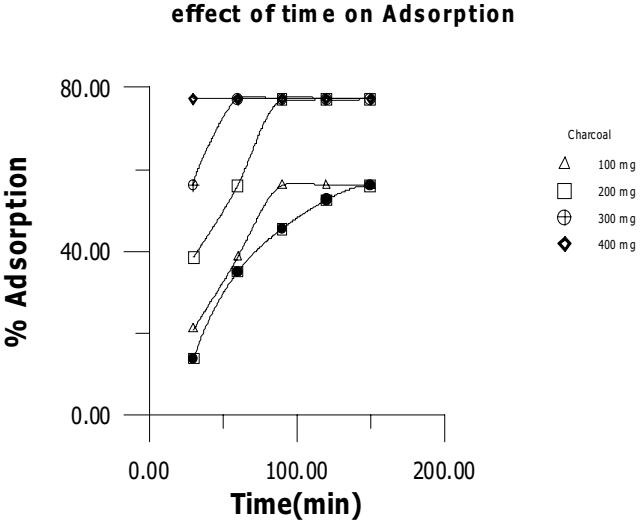

Fig.1: Percentage Adsorption of Omeprazole onto Actvated Charcoal against Time

Effect of pH: Omeprazole drug adsorption is higher at pH of 4.0 for all concentrations of drug studied at 37.5 °C.

Table - 2

Effect of pH on adsorption

	Omeprazole drug concentration (mg/ml)				
	1.23	1.84	2.45	3.07	3.68
pH	**Percentage drug adsorption**				
1.2	47.2	50.72	70.64	78.88	79.71
4	68.32	74.19	77.12	82.51	85.05
8	40.16	41.34	47.2	61.98	61.28
9.2	19.04	27.25	33.12	42.27	42.5

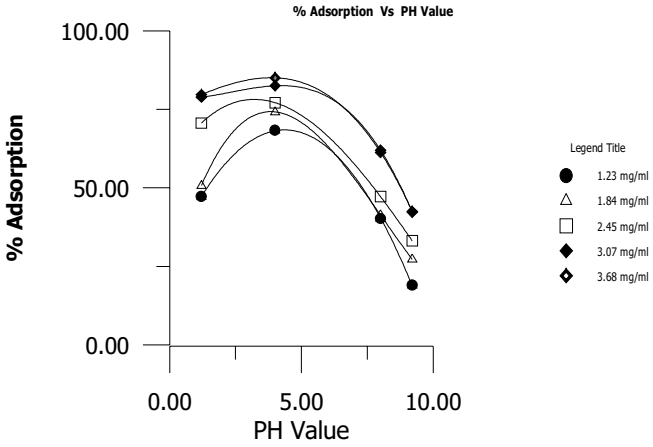

Fig.2: Percentage Adsorption of Omeprazole onto Actvated Charcoal against pH

Effect of Temperature: Percentage of drug adsorption was observed decrease as the temperature increased and it also noticed that drug adsorption increased with the initial drug concentration.

Table - 3

Effect of Temparature on adsorption

Initial Drug concn. (mg/ml)	Temperature °C		
	37.5	40.0	43.0
	Percentage drug adsorption		
1.23	68.32	47.2	44.56
1.84	74.19	60.1	57.06
2.45	78.64	66.04	64.13
3.07	82.51	69.62	68.36
3.68	85.05	70.67	66.03

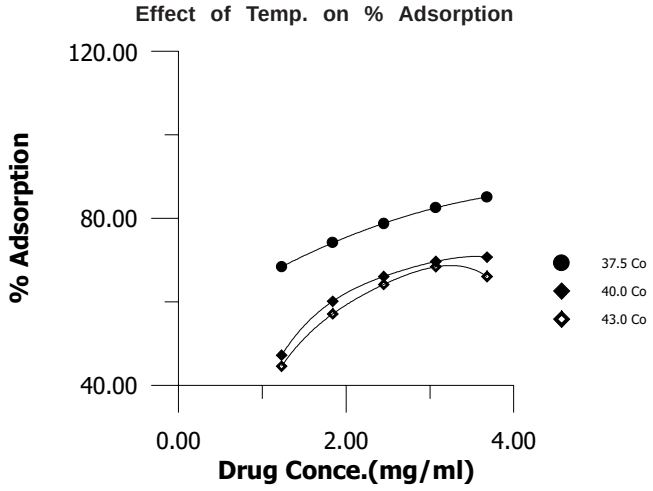

Fig.3: Percentage Adsorption of Omeprazole onto Actvated Charcoal against Drug Concentration

Effect drug concentration : The activated charcoal has shown increased drug adsorption with increased amount of initial concentration drug and the similar increase was also obtained with increased adsorbent activated charcoal.

Table - 4

Effect of concentration on adsorption

Drug concn. (mg/ml)	Activated charcoal	
	150 mg	200 mg
	Percent Drug Adsorption	
1.23	47.2	68.32
1.84	55.41	74.19
2.45	59.52	77.12
3.07	74.65	82.51
3.68	74.18	85.05

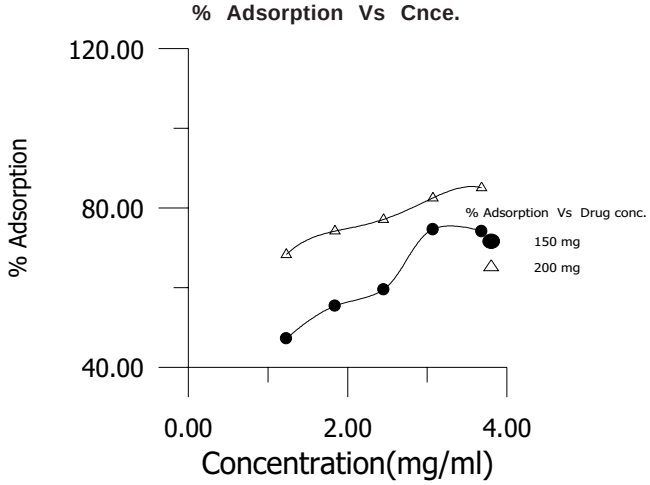

Fig.4: Percentage Adsorption of Omeprazole onto Actvated Charcoal against Drug Concentration

Comparative studies of percentage adsorption of drug onto the Charcoal tablets and charcoal powder : Comparision was made for charcoal tablet with the powder form for the drug adsorption and it was found that there was considerable fall of drug adsorption with charcoal tablet.

Table - 5

Comparision of drug adsorption from charcoal powder and Tablet

Initial drug Concn (mg/ml)	Percent drug adsorption	
	Charcoal Tablet	Charcoal Powder
1.23	26.08	68.32
1.84	36.64	74.19
2.45	45.44	77.12
3.07	50.72	82.51
3.68	51.89	85.05

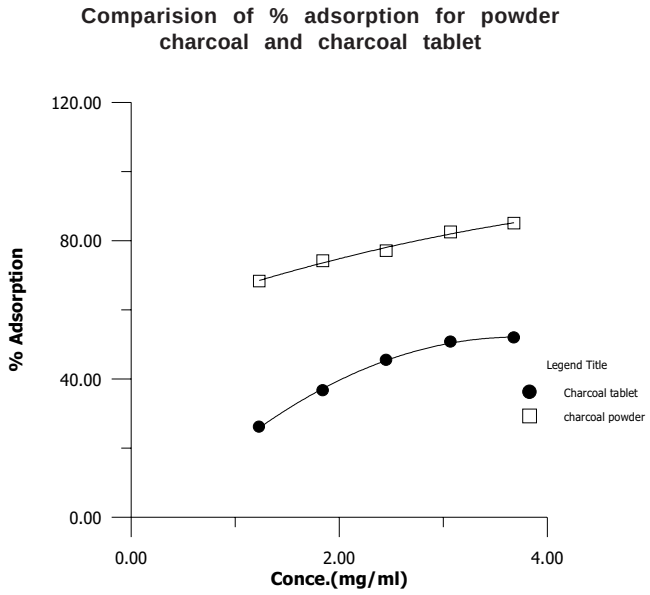

Fig.5: Comparision of % adsorption for charcoal powder and Tablet

Adsorption Isotherms : Adsorption data for wide range of Omeprazole drug concentrations are more conveniently describe by adsorption isotherms by considering amount of drug adsorbed on activated charcoal as $\mathbf{q_e}$ (mg/mg) and the residual amount of drug at equilibrium as $\mathbf{C_e}$ (mg/ml)

Freundlich adsorption isotherm represented by $q_e = K_f (C_e)^{1/n}$

Where, q_e=quantity of Omeprazole adsorbed per gram of charcoal

C_e = Equilibrium concentration of drug in solution

The constant K_f = Capacity of adsorbent (mg/mg) and 'n' is the indication of favorability adsorption. The above equaton can be kept in the linear form to find the constants as $\ln (q_e) = \ln K_f + (1/n) \ln (C_e)$

Langmuir Adsorption Isotherm represented by the linear form of equation

$$\frac{1}{q_e} = \frac{1}{Q_O} + \frac{1}{Q_O b}\frac{1}{C_e}$$ where Q_O, b are Langmuir constants signifying adsorption capacity and energy of adsorption respectively. Experimental equilibrium data for isotherms.

Table - 6

Effect of Temparature on Drug Adsorption

Initial drug (mg/ml)	37.5°C		40.0°C		43.0°C	
	q_e	C_e	q_e	C_e	q_e	C_e
1.84	0.273	0.475	0.221	0.734	0.21	0.79
2.45	0.374	0.561	0.324	0.833	0.315	0.88
3.07	0.506	0.626	0.415	0.993	0.419	0.97
3.68	0.625	0.6601	0.520	1.079	0.486	1.25

Table-7

Langmuir and Freundlich Constants

Temp °C	Langmuir		Freundlich	
	Q_O	b	K_f	1/n
37.5	0.6926	1.4237	0.1380	1.12007
40.0	0.4383	1.8493	0.2820	1.63668
43.0	0.4691	2.3027	0.3436	1.27260

The heat of adsorption was estimated from Langmuir isotherm fitness (Fig.6) and was found tobe 15.917 kcal/ gmol and is closer to the heat of condensation therefore the adsorption is due to physical adsorption phenomena.

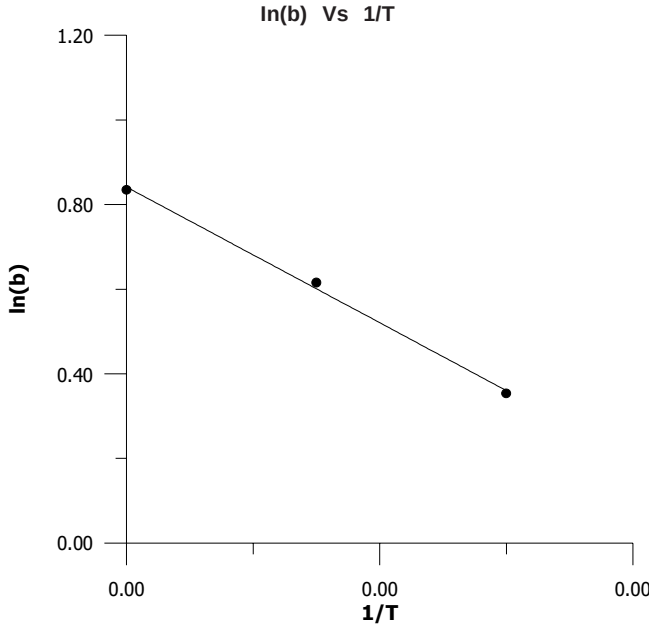

Fig.6 Langmuir isotherm fitness

CONCLUSIONS

The Omeprazole adsorption dependents on the amount of activated charcoal and increasesas with the increase of amount of charcoal up to equilibrium.

Omeprazole adsorption on to the activated charcoal increasesas with pH upto 4.0 and then decreases beyond 4.0. It was noticed that maximum adsorption attained at pH of 4.0.

A steep increase in adsorption with increase in the Omeprazole drug initial concentration was noticed.

It was also noticed that adsorption is better at lower temperatures.

Percent adsorption on to the activated charcoal powder is better than that of the activated charcoal.

The Experimental data fits well for Freundlich model rather than Langmuir model adsorption isotherms and noticed that nature of adsorption is Physical, as the heat evolved during adsorption proces is less 15.917k.cal/gr.mol while the heat of condensation of Omeprazole drug 25.983 k.cal/gr.mol.

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Synthesis and characterization of copolymer of methacrylamide with methylacrylate

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ABSTRACT

Copolymer of methacrylamide (MA) with ethyl acrylate(EA)was synthesized by free radical polymerization using1, 1' azo bis (cyclohexanecarbonitrile) (VAZO)as initiator in dimethyl formamide (DMF) at 60±1 ^{0}C. The monomer reactivity ratio was computed by both Fineman-Ross (F-R) and Kelen-Tudos (K-T) methods. The reactivity ratio values suggest the formation of random copolymers which has been supported by the azeotropic composition evaluation. The mean sequence length ($\bar{n}_1$) and probabilities *(p)* in the formation of various structural units were evaluated. The molecular weights of the polymers were determined by gel permeation chromatography, which increase with the increase of the EA content. The solubility parameters were determined with the viscometric method. The glass transition temperature (T_g) of the copolymers were determined by differential scanning calorimetry (DSC). Thermogravimetric analysis (TGA) of the copolymer was also studied.

KEYWORDS: Methacrylamide/ ethylacrylate/ copolymerization/ reactivity ratios and thermal properties.

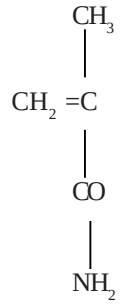
Introduction

Introduction of ethylacrylate(EA) into various copolymers appears to modify and improve the properties of a number of copolymers[1,2].The estimation of copolymer composition and determination of the reactivity ratio are important for making copolymers with required physico-chemical properties. The ^{1}H-NMR spectroscopic analysis has been used as a powerful tool for the estimation of copolymer composition[3-6].In our earlier paper[7] we discussed the copolymers of AN with isobornyl acrylate and methacrylate. In this paper we describe the synthesis, characterization, reactivity ratios, solution and thermal properties of the copolymers of MAA with EA.

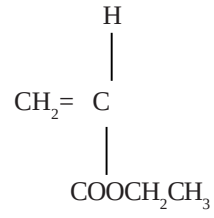
Materials and Method

MAA (Aldrich) and EA (Aldrich) were purified by washing with 5% solution of sodium hydroxide and distilled water, dried over calcium chloride before distilling under reduced pressure. The middle fraction of the distillate was collected and used for copolymerization. VAZO(Fluka) as initiator was crystallized from methanol. The solvent used in copolymerization was DMF which was a reagent grade chemical. This was dried and purified by distillation before use. All experiments were performed in glass tubes with appropriate quantities of dry monomers, solvents and initiator. The tubes were sealed in an atmosphere of nitrogen and introduced into the thermostat at 60 $\pm$ 1^{0}C and the polymerization was continued for 90 min. to get less than 10% conversion. The polymerization mixture was poured into a large amount of water to isolate the copolymer, which was filtered, washed thoroughly with water followed by ether and hexane, and finally dried under vaccum. Different samples were prepared by changing the initial monomer feed. The initiator was used at 2.5 g/dm^3 of solvent. The total monomer concentration was maintained at 1.5 M, while the feed ratio was varied. The data of composition of feed and copolymers are presented in Table1.

The monomer unit structures are represented as follows:



I) methacrylamide (MAA)



II) ethyl acrylate (EA)

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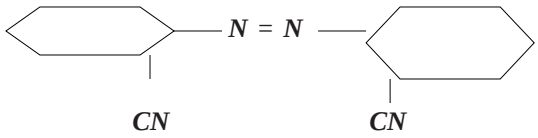
Structure of initiatior:



1, 1′ azo bis (cyclohexanecarbonitrile) (VAZO)

Results and Discussion

***IR Spectroscopy*:** Infrared spectra of the samples were recorded on a Perkin-Elmer model BX spectrophotometer in 4000 to 400 cm^{-1} range with KBr pellets. The IR spectrum of the copolymer of MAA and EA (MAA-co-EA) is shown in (Fig.1) showing the characteristic bands of both the monomer units. Appearance of strong absorption bands at 2951,1657,1164 and 3421 cm^{-1} corresponds to methylene (-CH) stretching, >C = O stretching in amide, methoxy (-OCH_2 and -NH_2 stretching vibrations respectively. The appearance of absorption bands corresponding to >C =O, -NH_2 and –OCH_2 groups and the disappearance of absorption bands corresponding to olefinic bond is the evidence for the MAA-EA copolymer.

***Determination of copolymer composition*:** ^{1}HNMR spectra of the samples were recorded using acetone-d_6 as solvent for EA copolymer on a Varian Gemini 200MHz NMR spectrometer with TMS as internal reference. The copolymer compositions were determined by ^{1}H-NMR spectra shown in (Fig.2).The characteristic peaks due to methylene (-CH_2) protons of methacrylamide unit appears at 2.3 ppm,-NH_2 protons of methacrylamide unit appears as singlet at 5.5 to 5.8ppm,-OCH_2 protons of EA appears as singlet at 3.5 to 4.0 ppm, methyl protons of MAA unit appears at 1.9 to 2.0ppm,- CH_2 protons of EA appears at 1.40ppm ,-CH (3^0)protons of EA appears at 1.93 ppm. which were considered for the composition analysis. Since the peak area corresponds to the total number of protons of a particular group, the composition of the copolymer was calculated [8] by the relation

$$\% \text{ EA in MAA} = \frac{^1\text{H-OCH}_2 \text{ group } /3}{^1\text{H-OCH}_2 \text{ group}/3 + ^1\text{H-NH}_2\text{group}/2}$$

This equations is based on the fact that the amide group of MAA unit corresponds to two protons , -OCH_2 group of EA corresponds to two protons.

Reactivity ratios: The copolymer composition data were used for the evaluation of reactivity ratios of the MAA-EA copolymer by Fineman – Ross [9] (F-R) (Fig. 3) and Kelen–Tudos[10] (K-T)(Fig.4) methods. The values of reactivity ratios were summarized in Table 2. The product of r_1r_2 values Table 2, which is less than 1 suggests that the monomers were arranged in a random sequence. The rate of polymerization depends on the value of 1/r that gives a measure of the reactivity of the ethyl acrylate towards the MAA radical. The value of $1/r_1$ for MAA-EA copolymer is 0.843.

To ascertain the normal copolymer behavior, the plots of mole fraction of monomer in the feed (m_1) vs that in the copolymer (M_1) were drawn and is given in Table 1. The shapes of the curves (Fig. 5) indicate that the azeotropic compositions of the copolymer systems and the distribution of monomeric units are random. The azeotropic composition was determined by the following equation[11-13]

$$N_1 = \frac{(1-r_2)}{(2-r_1-r_2)} \qquad (1)$$

The value of MAA-EA is 0.684 indicating that the copolymer is richer in MAA below this point than MAA and richer in EA above this point than MAA. This behaviour also suggests the random distribution of monomers in the copolymer.

***Sequence length distribution*:** The mean sequence lengths ($\bar{n}_1$) and ($\bar{n}_2$) for MAA-EA copolymer system has been calculated and listed in Table 3. The ($\bar{n}_1$) and ($\bar{n}_2$) for M_1 and M_2 units are calculated using equations of Expenyong.[14]

$$\bar{n}_1 = \frac{P_{12}}{(1-P_{11})^2} = \frac{1}{P_{12}} = \frac{r_1[M_1]+[M_2]}{[M_2]} \qquad (2)$$

$$\bar{n}_2 = \frac{P_{21}}{(1-P_{22})^2} = \frac{1}{P_{21}} = \frac{r_2[M_2]+[M_1]}{[M_1]} \qquad (3)$$

For example, at 86.7%MAA (13.3 % EA) in the monomer mixture each copolymer segment with M_1 units was approximately nine times longer than its adjoining segment with M_2 units. The sequence may be expressed as —21111111112—. This differences between $\bar{n}_1$ and $\bar{n}_2$ values are increased from 33.3-40.0% to 86.7% of MAA. Thus, we can say that MAA has higher tendencies to form more segments than that of EA. The number of MAA units in copolymer increases with increasing [MAA] in the feed, but this is not so for EA. Hence EA acts as a retarder in the copolymerization. The ratio of the mean sequence lengths distribution $\bar{n}_1/\bar{n}_2$, which theoretically[15] correspond to the ratio $[M_1]/[M_2]$, where $[M_1]$ and $[M_2]$ are the corresponding compositions of M_1 and M_2 in the copolymer for each monomer mixture of MAA-EA is given in Table 3.

Table - 1
Copolymerization data of MAA with EA

Copolymer system	Mole fraction in the feed		Intensity of amide protons (2H)(M_1)	Intensity of OCH2 protons (2H) (M_2)	Copolymer composition	
	MAA (M_1)	EA (M_2)			MAA (m_1)	EA (m_2)
MAA-EA_1	0.600	0.400	89.12	216.3	0.7082	0.2918
MAA-EA_2	0.666	0.333	55.37	166.3	0.7502	0.2498
MAA-EA_3	0.734	0.266	40.93	161.7	0.7980	0.2020
MAA-EA_4	0.800	0.200	31.64	171.2	0.8440	0.1560
MAA-EA_5	0.867	0.133	28.66	164.5	0.8516	0.1484

Table – 2
Reactivity ratios of MAA with EA by F-R and K-T methods

M_1	M_2	F-R method		K-T method	
		r_1	r_2	r_1	r_2
MAA	EA	1.185	0.21	1.24	0.20

Table - 3
Mean sequence length distribution of MAA-EA copolymers

Sl. No.	M_2 (mol%)	$\bar{n}_1$	$\bar{n}_2$	$\bar{n}_1 : \bar{n}_2$	Distribution
1	40	2.77	1.14	2:01	2112
2	33.3	3.37	1.1	3:01	21112
3	26.6	4.22	1.07	4:01	211112
4	20	5.74	1.05	6:01	2E+07
5	13.3	8.72	1.03	9:01	2E+10

Table - 6
Experimental T_g values from DSC

Copolymers	T_g°C (DSC)
MAA-MA_1	111.35
MAA-MA_3	113.89
MAA-MA_5	114.52

Table-4
Intrinsic viscosities at 25°C and molecular weights of MAA –EA copolymers

Copolymer	[η] dl/g	$\overline{M}_w$ x 10-5	$\overline{M}_n$ x 10-5
MAA – EA_1	0.4985	2.25	1.81
MAA – EA_2	0.5310	2.26	1.83
MAA – EA_3	0.5653	2.28	1.85
MAA –EA_4	0.6017	2.30	1.86
MAA – EA_5	0.6273	2.32	1.88

Table - 5
Intrinsic viscosities of MAA-EA copolymers and their dependence on solubility parameter of solvents at 30 °C

S.No	Solvent	δ(Cal/cc)$^{0.5}$	[η]dl/g MAA-EA
1	Toluene	8.9	0.325
2	Benzene	9.2	0.342
3	Chloroform	9.3	0.353
4	Acetic acid	10.1	0.37
5	Diethyl formamide	10.6	0.3
6	Dichloro acetic acid	11	0.45
7	Dimethyl formamide	12.1	0.35

Table –7
Thermal behaviour of MAA-EA copolymer

Copolymer	IDT (°C)	IPDT (°C)	Temperature (°C) at wt. loss		
			10%	20%	50%
MAA-EA_1	110	130	130	130	175
MAA-EA_2	111	131	132	131	182
MAA-EA_3	113	133	132	133	199
MAA-EA_4	114	134	151	220	229
MAA-EA_5	117	136	152	224	238

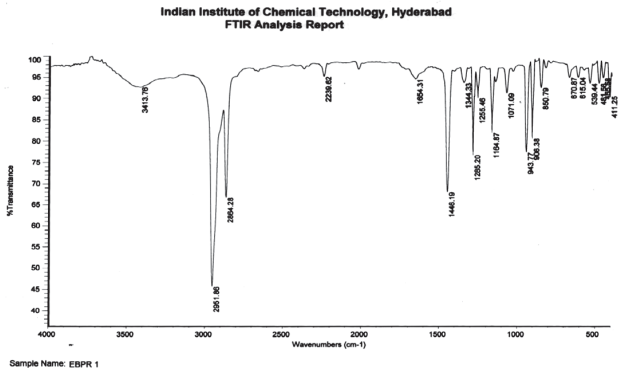


Fig. 1IR Spectrum of MAA-EA$_1$

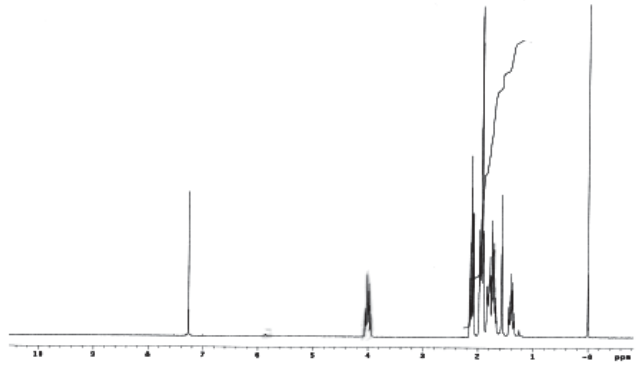


Fig. 2: NMR Spectrum of MAA-EA$_1$

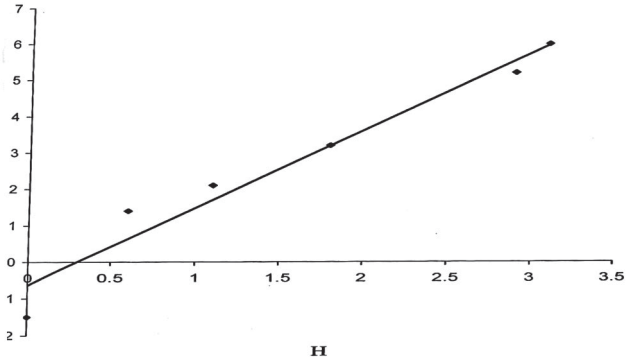


Fig.3 : F-R Plot of MAA-MA

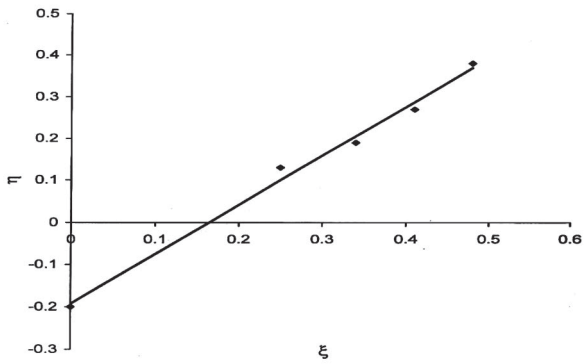


Fig.4 : K-T Plot of MAA-MA

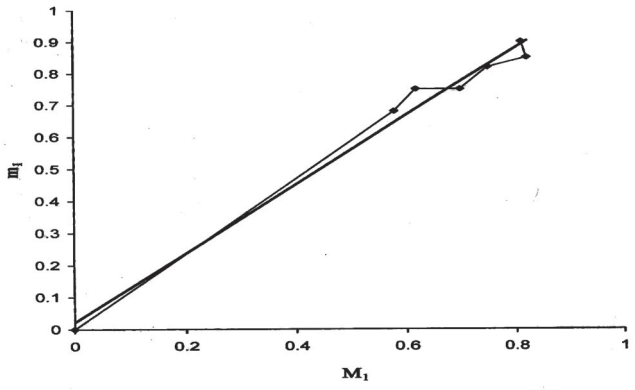


Fig. 5: Plot of m_1 Vs M_1 of MAA-MA

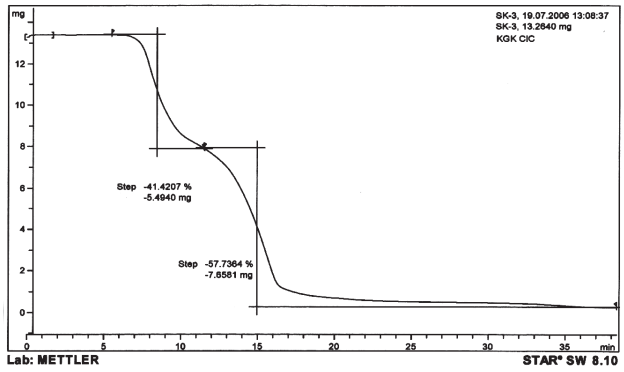


Fig. 6: Thermogram of MAA-EA$_1$

Solution properties: The molecular weights of the copolymers were determined with a KNAUER (WG) GPC with THF as eluent. The intrinsic viscosity of the copolymers was measured with an Ubbelhode viscometer in toluene at 30 ± 0.1°C. The weight and number average molecular weights ($\overline{M}_w$ and $\overline{M}_n$) values of copolymer for different compositions are given in Table 4. These values increase with the increase in the MAA content of the copolymer. This trend is in consonance with variation in intrinsic viscosity.The solubility parameter helps in explaining the viscosity behavior of solution, polymer-polymer compatibility, dispersion and tolerance for dilution with non-solvents. Proper solvents can be selected to control the viscosity of polymer solution by using these values. The evaporation of solvent can also be adjusted by selecting proper solvents using this solubility parameter concept to get good film with no defects.

Plots of η_{sp}/c against concentration are found to be linear and the intrinsic viscosity values are obtained by extrapolating it to zero concentration. In all the copolymers

intrinsic viscosity and hence molecular weight increases with increase in the EA content Table 4. This may be attributed to the greater reactivity of amide content, which facilitates propagation in preference to termination. Solubility parameter values of the copolymer determined in different solvents are presented in Table 5. Solvents which cover a range of solubility parameter from 8.9 to 12.1 (cal/cc) $^{0.5}$ have been selected. The solubility parameter value of MAA-EA is 11.0 (cal/cc).$^{0.5}$

Thermal studies: The thermogravimetric analysis of the polymers was performed on a V51A Dupont 2000 thermalanalyzer at a heating rate of 15^0C/min. Glass transition temperature (T_g) of the copolymers was determined using a Q 2000 U23.9 Build 78 analyzer at a heating rate of 15^0C/min.T_g values of the copolymers obtained from the DSC curves are summarized in Table 6. T_g of the copolymer increases with increase in the MAA content.. When the MAA content increases intramolecular interaction increase and the polymer segments become less mobile and T_g occurs at higher temperature[16-21]. The relative thermal stabilities are evaluated by the comparison of the initial decomposition temperature (IDT), the integral procedural decomposition temperature (IPDT) and decomposition temperature (DT) at 50% weight loss shown in (Fig.6).To obtain a comparative picture of relative thermal stability of the copolymer their IDT, IPDT and DT values are given in Table 7.

Conclusions

The copolymer of MAA with EA has been synthesized using a VAZO as initiator in DMF. The copolymer is characterized by I-R and ^{1}H NMR. Reactivity ratio of the copolymer was determined using the F-R and K-T methods which suggest that random copolymers were formed. Intrinsic viscosities and solubility parameter was determined to establish interactions in polymer solutions. Thermal properties like T_g, IDT and IPDT have been evaluated to find the thermal stability of the polymer.

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