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Enzyme Inhibition studies in *Mimosa pudica*

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ABSTRACT

Inhibition of α-amylase, enzyme that plays a role in digestion of starch and glycogen, is considered a strategy for the treatment of disorders in carbohydrate uptake, such as diabetes and obesity, etc. Plants are an important source of chemical constituents with potential for inhibition of α-amylase and can be used as therapeutic or functional food sources. In this study methanolic extracts from *Mimosa pudica* plant source that have been tested for α-amylase & acid phosphatase inhibitory activity has been done. Amongst the phyto-constituents that have been investigated, flavonoids are one of them that demonstrated the highest inhibitory activities with the potential of inhibition related to number of hydroxyl groups in the molecule of the compound.The inhibition of acid phosphatase could be due to the rupture of lysosomal membrane in the presence of *Mimosa pudica*. Methanol crude extracts of the (whole plant) of *Mimosa pudica* (Mimosaceae) were screened *in vitro* for cytotoxicity studies by brine shrimp lethality bioassay and antioxidant activity using the 1,1-diphenyl-2-picrylhydrazyl-hydrate (DPPH) free radical scavenging assay. The methanol crude extracts of the whole plant showed potential cytotoxic activities (IC_{50} 82.50µg/ml). The methanol crude extract of the whole plant showed moderate antioxidant activity (IC_{50} 48.54µg/ml) compared to Rutin (IC_{50} 34.60µg/ml). The % of inhibition of alpha Amylase & Acid Phosphatase of *Mimosa pudica* was found to be *41% & 47.8%* in the concentration of 5mg/ml respectively with membrane stability *61.5%*. The overall experimental results suggested that biologically active constituents present in the methanolic extract of *Mimosa pudica* and justifies its use in folkloric remedies.

Introduction

Plants have been the traditional source of raw materials for medicine. A rich heritage of knowledge to preventive and curative medicines was available in ancient scholastic works included in the Atharva veda, Charaka, Sushruta, etc.

An estimate suggests that about 13,000 plant species worldwide are known to have use as drugs. The trend of using natural products has increased and the active plant extracts are frequently for new drug discoveries and for the presence of antimicrobials [12]. In recent years one of the areas which attracted a great deal of attention is the possible therapeutic potential of antioxidants in controlling degenerative diseases associated with marked oxidative damage.

Ayurveda, the traditional Indian herbal medicinal system practiced for over thousands of years have reports of antidiabetic plants with no apparent known side effects[8,9]. A significant inhibition was observed with extracts of *O. tenuflorum*[8].Other six Indian medicinal plants were tested for their effect on α-amylase activity. Among them, *Mangifera indica* L., *Embelia ribes* Burm., *Phyllanthus maderaspatensis* Linn. and *Punica granatum* L. showed interesting α-amylase inhibitory activity.[38]

The proteinaceous inhibitor of α-amylase (αAI), which inhibits animal salivary and pancreatic a-amylase, has been identified and isolated from various plant species[39]. Amongst this plants, seeds of *Phaseolus vulgaris* L. contain proteinaceous inhibitors of the α-amylase and the isoform inhibitor αAI-1 have been isolated and characterized[40]. The common bean αAI-1 has been reported to have relatively great potential as an extensive anti-obesity and anti-diabetes remedy[39].

Several plant extracts and different classes of phytochemicals have been found to have quite prominent antioxidant activity [62,65,64,29]. *Mimosa pudica* (Bengali name – Lajjabati Lata, Lajanti; English name – Sensitive plant; Family-Mimosaceae) is a stout strangling prostrate shrubby plant with compound leaves sensitive to touch, spinous stipules and globose pinkish flower heads, grows as a weed in almost all parts of the country [17].

Leaves and stems of the plant have been reported to contain an alkaloid, mimosine; leaves also contain mucilage and root, tannins [17] The plants also contain turgorins. *M. pudica* is regarded as diuretic, astringent and antispasmodic. Leaves and roots are used in the treatment of piles and fistula. Paste of leaves is applied to hydrocele. Cotton impregnated with juice of leaves is used for dressing sinus.

Plant is also useful in the treatment of sore gum and is used as a blood purifier. It is also used for treating convulsions of children [17]. Few chemical and biological studies have been carried out on this plant [16,63,7,13,10,24]. In this paper, the cytotoxicity, enzyme inhibitions (amylase and acid Phosphatase), membrane stability and the preliminary antioxidant activities of the organic extractives are being reported.

Materials And Methods

Chemicals

All of the chemicals used in this work were purchased are the linoleic acid and β- carotene were purchased from across organic (Greel, Belgium), 1,1 –diphenyl-2-picryl-hydrazyl (DPPH) S. D. Fine Chem. Ltd - (Mumbai - India), and solvents

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and other reagents of analytical grade were from E.Merk (Mumbai , Maharashtra, India).

Plant material

Plant materials collected from different natural habitats in south Karnataka .India, in March 2012. Voucher specimens were deposited in the Herbarium of R&D, C.D. Sagar Centre Dept of P.G. Studies Biotechnology Department. Dayananda Sagar College. India, chopped, dried powdered (40- mesh) and stored in polythene bags at 4°c, i.e. *Mimosa pudica* (Fabaceae) (whole plant*)*.

Preparation of extracts and solutions

Plant materials were air dried at room temperature and finely grounded. Each sample (100 g) was macerated with methanol 90% (500 ml) three times. Solvent was evaporated under reduced pressure at approximately 40 °C. The dried extracts were dissolved in methanol 90% to a final concentration of 1000 µg ml-1 (sample stock solution) was prepared.

Preliminary Phytochemical Screening:

Methanol extract was then treated with various reagents which revealed the presence of various phytoconstituents. The total flavanoid content of the extract was determined by employing HPLC technique which was carried out in Pavan Labs (P) Ltd, Bangalore.

Biological screening Cytotoxicity study

Brine shrimp lethality bioassay [49] technique was applied for the determination of cytotoxic property of methanol extracts of whole plant of *M. pudica*.

Preparation of positive control group

Vincristine sulphate was used as the positive control. Measured amount of the vincristine sulphate was dissolved in DMSO to get an initial concentration of 20 mg/ml from which serial dilutions are made using DMSO to get 10 mg/ml, 5 mg/ml, 2.5mg/ml, 1.25 mg/ml, 0.625 mg/ml, 0.3125 mg/ml, 0.15625 mg/ml, 0.078125 mg/ml and 0.0390 mg/ml. Then the positive control solutions are added to the pre-marked vials containing ten living brine shrimp nauplii in 5 ml simulated sea water to get the positive control groups.

Preparation of negative control group

30 ml of DMSO was added to each of three pre-marked glass vials containing 5 ml of simulatedseawater and 10 shrimp nauplii to use as control groups. If the brine shrimps in these vials show a rapid mortality rate, then the test is considered as invalid as the nauplii died due to some reason other than the cytotoxicity of the compounds.

Counting of nauplii

After 24 hours, the vials were inspected using a magnifying glass and the number of survived nauplii in each vial were counted. From this data, the percent (%) of lethality of the brine shrimp nauplii was calculated for each concentration.

Screening for antioxidant activity

Antioxidant activities of the aerial part of methanol extract was determined on the basis of their scavenging potential of the stable DPPH free radical in both qualitative and quantitative assay.

i) Qualitative assay: A suitably diluted stock solutions were spotted on pre-coated silica gel TLC plates and the plates were developed in solvent systems of different polarities (polar, medium polar and non-polar) to resolve polar and non-polar components of the extracts. The plates were dried at room temperature and were sprayed with 0.02% DPPH in ethanol. Bleaching of DPPH by the resolved band was observed for 10 minutes and the color changes (yellow on purple background) were noted [55]

ii) Quantitative assay: Quantitative assay was performed on the basis of the modified method[18]. Stock solution (10 mg/ml) of the plant extracts were prepared in methanol from which serial dilutions were carried out to obtain concentrations of 25, 50, 75, 100µg/ml. Diluted solutions (2 ml) were added to 2 ml of a 0.004% methanol solutions of DPPH (Himedia), mixed and allowed to stand at 25^0 C for 30 min for reaction to occur. The absorbance was determined as 570 nm and from these values corresponding percentage of inhibitions were calculated. Then % inhibitions were plotted against log concentration and from the graph IC_{50} was calculated. The experiment was performed in triplicate and average absorption was noted for each concentration. Rutin (Loba, India) was used as positive control.

Antioxidant assay:

DPPH method: 0.3mM of 1,1 Diphenyl 2 picryl hydrazyl (DPPH) was dissolved in 90% ethanol. Stock solution of the plant was prepared. 3ml of 90% ethanol with 3ml of DPPH was served as blank while different concentrations of the plant extract with 1ml of DPPH was added. The test tubes were incubated for 30minutes at rom temperature in the dark. The absorbance was recorded at 570 nm.

Membrane stability Assay:

Preparation of Drugs: Standard drug (Ibuprofen 2.5mg/ml) and various extracts were prepared in iso-saline (0.85% w/v NaCl) to final concentrations of 1.0-5.0 mg/ml.

Preparation of Sheep Red Blood Cell : Fresh Sheep blood samples were collected into an anticoagulant. Blood samples were centrifuged at 3000rpm on a Bench centrifuge for 10 min at room temperature. The supernatants were carefully removed while the packed RBC was washed in fresh normal saline (0.85% w/v NaCl).The process of washing and centrifugation were repeated five times until the supernatants were clear.

Assay of membrane stabilizing activity: The membrane stabilizing activity assay was carried out using 2%(v/v) sheep erythrocyte suspension while Ibuprofen was used as standard drug. The assay mixture consisted of 2ml hypo saline (0.25% w/v) sodium chloride, 1.0 ml of 0.15 M sodium phosphate buffer, pH 7.4, 0.5ml of 2% (v/v) sheep erythrocyte suspension, 0.0-1.0 ml of drugs(standard extracts/fractions) and final reaction mixtures were made up to 4.5 ml with iso saline. Drugs were omitted in the blood control while the drug control did not contain the erythrocyte suspension. The reaction mixtures were incubated at 56^0 C for 30 minutes on a water bath, followed by centrifugation at 5000 rpm for 10 minutes at room temperature. The absorbance of the released haemoglobin was read at 560 nm. The percentage membrane stability was estimated using the expression

$$100 - \frac{\text{(Abs of test drug - Abs of drug control)}}{\text{Abs of blood control}} \times 100$$

Enzyme Inhibition Assay:

Alpha amylase (Saliva)

Preparation of Enzyme:

a) Salivary amylase: 10ml of the saliva was collected and diluted to 100ml with cold phosphate buffer pH 7.1. The solution was centrifuged at 8000 rpm for 20mts and the clear supernatant was used.

b) Extraction of crude inhibitor: 100mgs of dried plant powder was extracted with 2.5% of cold TCA with magnetic stirrer for 45mts. The solution was centrifuged to get a clear supernatant, which was neutralized to pH 7 with dilute sodium carbonate and used for the assay of enzyme inhibition. This is referred as the crude inhibitor.

Amylase assay:

1ml of the enzyme solution was added to 2ml of phosphate buffer pH6.9 containing 2MNacl and the reaction started with the addition of 2ml 1% soluble starch solution. The tubes were incubated at 37°C fir 20mintues. The reaction was arrested by the addition of 1ml for dinitrosalicylic acid (DNS) colour reagent. The tubes were kept in a boiling water bath for 10minutes, cooled and diluted to a final volume of 10ml with distilled water. The absorbance was measured at 530nm. The standard curve was constructed using pure anhydrous maltose. The assays were run along suitable blank (without enzyme).

$$\% \text{ inhibition} = \frac{\text{Abs of control (no inhibitor)} - \text{Abs of sample}}{\text{Abs of control (no inhibitor)}} \times 100$$

Acid Phosphatase (Green gram):

i). 1.0 mL of citrate buffer, of pH 5.0 was added to each of four test tubes, and label them 0, 5, 10 and F (the numbers represent the number of drops of enzyme added to each, F represents the presence of fluoride inhibitor). Do not add any enzyme to the tube labeled "0", add 5 drops of enzyme solution to the tube labeled "5" and the tube labeled "F", add 10 drops of enzyme solution to the tube labeled "10". Add 5 drops of sodium fluoride solution to the tube labeled "F". Add de-ionized water to tubes "0" and "5" to give each tube the same volume of liquid, that will be 10 drops of water in tube "0", 5 drops of water in tube "5", and no water in tubes "10" and "F".

ii). Place each tube in the 37°C water bath for 5 min before adding substrate. Be careful not to mix them up with other sets of tubes.

iii). After the temperature has equilibrated, add 5 drops of p-nitrophenylphosphate substrate solution to each test tube and allow them to incubate in the 37°C water bath for 10 min.

iv). While they are incubating, prepare 4 test tubes with 3.0 mL of 0.1 M NaOH solution and label them 0B, 5B, 10B and FB. Alternatively, you can use the tubes labeled 1B thru 5B from part A, but be sure to keep track of which solution is added to each of these tubes.

v). After each buffer solution has been in the 37°C water bath for exactly 10 min, pour the contents of the tube into the respective tubes containing 0.1 M NaOH solution.

vi). Measure the absorbance of the solution in each tube and report it in the second table on the Report Sheet. These solutions can be discarded in the hazardous waste bottle.

$$\% \text{ inhibition} = \frac{\text{Abs of control (no inhibitor)} - \text{Abs of sample}}{\text{Abs of control (no inhibitor)}} \times 100$$

Results:

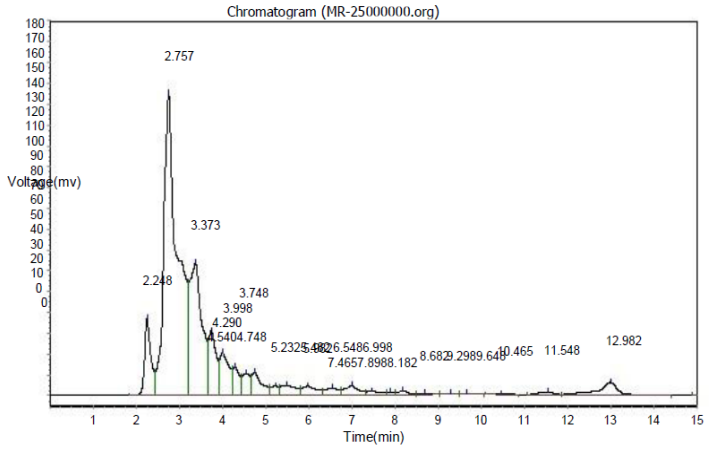
Phytochemical Screening: The phytochemical studies when brought to little existence, revealed the presence of phytoconstituents like alkaloids, carbohydrates, steroids, flavonoids and glycosides.

Table 1 : Phyto-chemical screening of methanol extract of *Mimosa pudica*. "+" Indicates the present, "-" Indicates the Absent.

Sl no.	Tests	Methanolic Extract of *Mimosa pudica*		
1	Flavanoids	+		
2	Alkaloids	+		
3	Glycosides	+		
4	Proteins	+		
5	Tannins	-		

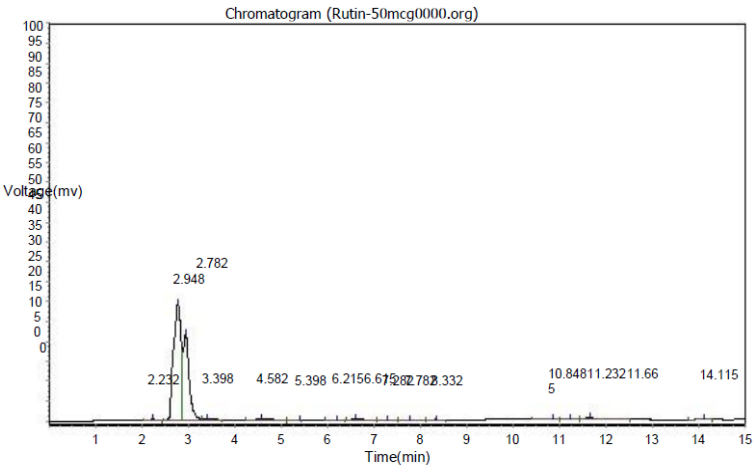
Estimation of flavonoid content in Mimosa Pudica Plant extract by HPLC

Detector: UV
Wavelength(nm): 220
Sample Description:
SAMPLE CODEST- 1000mcg/ml
MOBILE PHASE: Methanol: Phosphate buffer 80:20
pH: 3.5
Flow Rate: 1ml/min
Injection Volume: 20 µL
Column Dimension ID : 250X4.6 mm, 5µ
Diluent Water



Estimation of flavonoid Std Rutin content by HPLC

Detector:UV
Wavelength(nm):220
Sample Description:
SAMPLE CODERUTIN
MOBILE PHASE = Methanol: Phosphate buffer :: 80:20
pH3.5
Flow Rate = 1ml/min
Injection Volume = 20 µL
Column Dimension ID : 250X4.6 mm, 5µ
Diluent Water



Cytotoxicity Study: The result of the brine shrimp lethality test (BST) showed good activity in all the fractions of the methanolic extract of *M pudica* with highest toxicity observed IC_{50} value 82.50µg/ml.

Table 2: LC_{50} data of the test samples of *M. pudica* in brine shrimp lethality bioassay

Mimosa pudica Whole plant Crude extracts Methanol	IC_{50} (µg/ml)	82.50
Standard: Vincristine Sulphate	LC_{50} (µg /ml)	0.34

Antioxidant Assay:

Table 3: Enzyme inhibition activity of *M pudica over acid phosphatase*

Plant	AOA%	TPC mg/g gallic acid equivalent (5 - 500 mg/g gallic acid)	IC_{50} by DPPH method (µg/ml)	TFC(µg/mg) ± SEM
Mimosa pudica (Fabaceae) whole plant	92.40± 0.028	68.32 ± 0.142	48.54#	26.426 ± 0.204*
Standard Rutin,	35.66±0.05	76.4 ± 0.052	34.32 (32.20-36.78)	48 – 12.0

***Enzyme Inhibition*:**

α-Amylase: In the inhibition process of alpha amylase enzyme the absorbance of control and the absorbance of sample was found to be ***1.24*** and ***0.72***. Therefore by using the formula which is mentioned in the above the % of inhibition of alpha amylase over *Mimosa pudica* was found to be ***41%*** when the concentration was taken to be 5mg/ml**.**

Acid Phosphatase: In the inhibition process of acid phosphatase over *Mimosa pudica*, the absorbance of control was found to be 0.19. By using the formula which is mentioned in the above, the inhibition of acid phosphatase over various concentrations of *Mimosa pudica* in the labeled test tubes such as 0, 5, 10 and F as follows*:*

Table 4: Enzyme inhibition activity of *Mimosa pudica on Acid phosphatase*

Sl No.	Drops of enzyme stock	Concentration/percentage(%) of inhibition				
		1mg/ml	2mg/ml	3mg/ml	4mg/ml	5mg/ml
1.	0	21.05	15.79	5.26	31.58	47.37
2.	5	52.63	36.84	26.31	5.26	10.51
3.	10	15.79	15.79	5.26	26.31	47.37
4.	F	42.10	52.63	68.42	63.13	15.79

Membrane Stability Assay:

When the sheep erythrocyte suspensions were incubated with standard drug (Ibuprofen) and with the extract of *Mimosa pudica* the membrane stability was found to be as follows.

Table 5: Membrane stability assay of sheep erythrocyte when incubated with different concentrations of methanolic extract of *M pudica*

Sl No.	Concentration	Membrane stability
1.	200mg/ml	6%
2.	400mg/ml	21.5%
3.	600mg/ml	33.5%
4.	800mg/ml	47.55%
5.	1000mg/ml	61.5%

Table 6: Membrane stability assay of sheep erythrocyte when incubated with different concentrations synthetic drug (Ibuprofen)

Sl No.	Concentration	Membrane stability
1.	200mg/ml	4%
2.	400mg/ml	20.5%
3.	600mg/ml	31%
4.	800mg/ml	45%
5.	1000mg/ml	56.5%

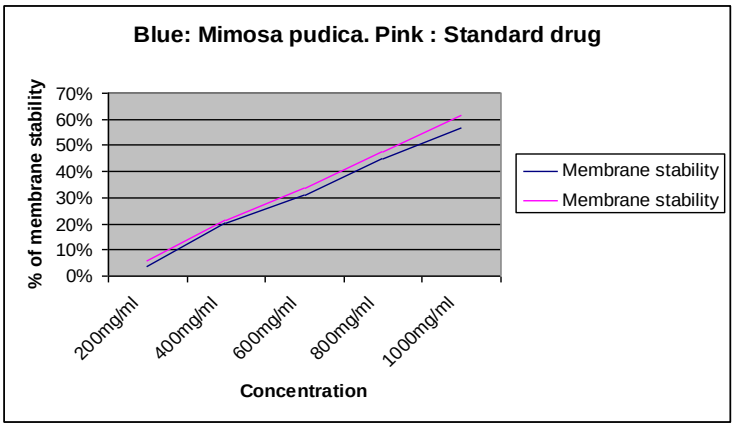


Fig I: Comparative representation of membrane stability assay at different concentrations of methanolic extract of *M pudica* and synthetic drug (Ibuprofen)

Discussion:

The phytochemical studies when brought to existence, revealed the presence of phytoconstituents like alkaloids, proteins, flavonoids and glycosides. Their total flavonoid (TPC) content was analyzed using the technique of HPLC taking Rutin as the standard. The presence of potent bioactive compounds in the cytotoxic study revealed the crude extract of *M pudica* which might be very useful as antiproliferative, antitumor, pesticidal and other bioactive agents (Meyer et al., 1982).In our study IC_{50} value of plant extract revealed a dose of 82.50µl/ml which showed cytotoxic activity over the brine shrimp larvae. The antioxidant assay was carried out for the methanolic extract of *Mimosa pudica* which showed an antioxidant activity of **92.40± 0.028 %** over Rutin which showed an antioxidant activity of **35.66±0.05%.** These findings can be proved to be useful in the treatment of kapha and pitta , wound healing, blood coagulatin and treatment of sexual weakness.

Alpha amylase showed an inhibition of 41% when the concentration was taken to be 5mg/ml while that of acid phosphatase showed the highest inhibition of 52.63%, 36.84%, 26.31%, 31.58% and 47.38% when the concentrations were taken to be 1mg/ml, 2mg/ml, 3mg/ml, 4mg/ml and 5mg/ml respectively. It was noted that methanolic extract which gave positive test for Flavonoids exhibited highest membrane stabilities of **45% and 56.5%** at the concentration of 800mg/ml ans 1000mg/ml respectively. Moreover the standard anti-inflammatory drugs (Ibuprofen) at 800mg/ml and 1000 mg/ml exerted maximum membrane stabilities **of 47.55% and 61.5%.** The comparative study showed a slight decrease in the membrane stabilizing activity of the extract. It may be concluded that the membrane stabilizing activities of these fractions were aided by the presence of flavonoid.

Conclusion:

Hence in this present study active constituents are subjected to HPLC standardized bu Rutin and Catechin.Form the phytochemical investigations and the results, it is observed that the methanol extract of flowers and leaves of *Mimosa pudica* posses active constituents which acts on microorganisms and inhibits the activity of alpha amylase and acid phosphatase enzyme.

On the basis of the results of membrane stability, it can be inferred that extracts of *M. pudica* contained principles that were capable stabilizing sheep red blood cells membranes against heat and hypotonic-induced lyses. The plant therefore could be regarded as a natural source of membrane stabilizes and was capable of providing an alternative remedy for the management and treatment of inflammatory related disorders.

Moreover cytotoxic study (BSLT) proves it is devoid of cytotoxicity and hence safe for therapeutic consumption. The results obtained may prove to be helpful in treatment of diabetes and liver disorders.

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