Research Article

TOTAL PHENOLIC, FLAVONOID CONTENT AND ANTIOXIDANT CAPACITY OF *MARSILEA MINUTA* L., AN INDIAN VEGETABLE

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ABSTRACT

Present study concentrated on antioxidant activity, total phenolic and flavonoid content of *Marsilea minuta* L. - an aquatic or sub-aquatic fern used as vegetable, and has wide application in traditional/folk medicine in the India and Bangladesh. Several *in vitro* and *ex vivo* model were employed in order to evaluate the antioxidant activities of methanol, ethyl acetate and petroleum ether extracts of *Marsilea minuta*. We also investigated the total phenolic and flavonoid content by Folin–Ciocalteau reagent and Aluminium nitrate - Potassium acetate reagent method respectively. Methanol extract showed highest phenolic content (72.58±0.60 mg gallic acid equivalent/g) and flavonoid content (54.87±0.11 mg quercetin equivalent/g). Results showed that the methanol extract possess significantly higher antioxidant activity among three extracts. The possible antioxidant mechanism of the methanol extract can be due to its hydrogen or electron donating and direct free radical scavenging properties. High antioxidant activity may be due high phenolic and flavonoid content. Hence, the methanol extract might be used as a readily accessible source of natural antioxidant.

Keywords: *Marsilea minuta*; vegetable; antioxidant; phenolic content; flavonoid content; methanol extract.

INTRODUCTION

Marsilea minuta L. is a fern belongs to the family Marsileaceae has been used in the treatment of respiratory disorder, psycopathy, diarrhoea, skin diseases, insomnia, mental problems, hypertension, sleeping disorders, headache, epilepsy, migraine and diabetes traditionally. The leaf and shoots of the plant is used as vegetable.[1-5] Investigation on its chemical constituent revealed the presence of a sedative and anticonvulsant photochemical named as Marceline. Consequently, different flavonoids and marsileagenin-A (a sapogenol) were also reported from the plant.[6,7] Previous researcher have reported that *Marsilea minuta* possess hypocholesterolemic,[8] anxiolytic,[9] antidepressant,[6] anti-amnestic, antistress,[10] antiaggressive,[11] and antifertility[12] activities.

The use of plants as alternative/complementary medicine has been in existence for thousand years. Consumption of different vegetables, fruits provides excellent health benefits as they are the rich source of phytochemicals that are beneficial for disease risk reduction.[13]

The role of oxidative stress in many disease conditions has been well established. Natural antioxidants through our diet play a vital role in helping endogenous antioxidants to neutralize excess free radicals. Consumption of vegetables, fruits thus considered as a major source of exogenous antioxidants which is beneficial in prevention and treatment of diseases related to oxidative stress.[14] Therefore, the present study designed to evaluate *in vitro* and *ex vivo* antioxidant activity of *Marsilea minuta*, and determined the phenolic and flavonoid content in the plant extracts.

MATERIALS AND METHODS

Chemicals

2,2-diphenyl-picrylhydrazyl (DPPH), phenazine methosulfate (PMS), and nitro blue tetrazolium (NBT) were procured from Sigma Aldrich (Bangalore, India). Trichloro acetic acid (TCA), 2-deoxy-ribose, thio barbituric acid (TBA), and quercetin were purchased from SD Fine Ltd. Mumbai, India. Folin-Ciocalteau reagent, ferrozine, butylated hydroxyanisole (BHA), ascorbic acid, and α-tocopherol acid were procured from Sisco Research Laboratories Pvt. Ltd., Mumbai, India. All other chemicals used in the study were obtained commercially and were of analytical grade.

Collection of plant materials and preparation of extracts

The whole plant of *Marsilea minuta* L. was collected in March/April 2011 from Tripura, India, and the plant was identified by Dr. BK Datta, Department of Botany, Tripura University, Tripura, India. A voucher specimen (TU/BOT/HEB/SS23072011b) was deposited at the herbarium of Plant Taxonomy & Biodiversity Laboratory, Tripura University.

The plants were collected and cleaned to remove unwanted materials, and dried under shade. The air dried samples were powdered using a mechanical grinder, and extracted with methanol, ethyl acetate, petroleum ether separately using Soxhlet apparatus. The extracts were

concentrated under reduced pressure to obtain methanol extract (MMM, 3.6% w/w), ethyl acetate extract (EMM, 3.9% w/w) and petroleum ether extract (PMM, 3.2% w/w).

Experimental animals

Wistar rats (150–200 g) were used for *ex vivo* antioxidant study. Animals were housed under standard environmental conditions (24±1°C) with 12 h light-12 h dark cycles. The animals have free access to water and food. Institutional Animal Ethical Committee (Reg. No. 1305/ac/09/CPCSEA) has approved the study.

Determination of total phenolic and flavonoid content

Folin-Ciocalteu reagent method was used to determine the total phenolic content in extracts as described by Aiyegoro and Okoh.[15] Calibration curve (ranging from 0 to 0.8 mg/ml) was prepared by taking gallic acid as standard, and results were expressed as gallic acid equivalents in milligram per gram (GAE mg/g) of dried extract.

To determine flavonoid content, 0.5 ml extract (1.0 mg/ml) was mixed with 0.1 ml of 10% aluminium nitrate, 0.1 ml potassium acetate (1 M) and 4.3 ml of 80% ethanol. The mixture was thoroughly mixed and kept a side for 40 min at room temperature; the absorbance of supernatant was measured at 415 nm.[16] Quercetin was used as standard and results were expressed as quercetin equivalents in milligram per gram (QE mg/g) of dried extract.

***In vitro* antioxidant activity of *M. mimuta* extracts**

DPPH radical scavenging assay

Methanolic solution of DPPH (1.0 ml, 0.1mM) were mixed with 3.0 ml of various concentrations extract during 30 min at room temperature in the dark and the absorbance was recorded at 517 nm.[17] The percentage of DPPH radical scavenged (I%) by the extracts was estimated using the following equation (1) :

$$I\% = (A_{control} - A_{sample})/A_{control} \times 100$$

Where, A_{sample} is the absorbance of a sample solution and $A_{control}$ is the absorbance of the control solution (containing all reagents except the test sample).

Superoxide anion radical scavenging activity

Extracts in different concentration (0.3 ml) was added into 3.0 ml mixture of Tris-HCl buffer (100 mM, pH 7.4), which contain 0.75 ml of NBT (300 µM) solution, 0.75 ml of NADH (936 µM) solution. The reaction was started by adding 0.75 ml of PMS (120 µM) into the reaction mixture and incubated at room temperature for 5 min, the absorbance was taken at 560 nm.[18] The capability of scavenging superoxide radical (I%) was calculated by equation (1).

Deoxyribose degradation assay

Briefly, 0.1 ml of sample solution mixed with 0.2 ml KH_2PO_4-KOH (100 mM), 0.2 ml of deoxyribose (15 mM), 0.2 ml of ferric chloride (500 mM), 0.1 ml EDTA (1 mM), and 0.1 ml of ascorbic acid (1 mM). Finally 0.4 ml hydrogen peroxide (2 mM) was mixed to initiate the reaction. The reaction solution incubated at 37°C for 60 min, and then 1.0 ml ice cold TCA (2.8% w/v) and 1.0 TBA (1% w/v) were added. The mixture was heated on water bath at 80°C for 30 min and cooled. The absorbance of supernatant was measured at 532 nm.[19]

Nitric oxide radical scavenging assay

Four millilitres of extract solution were added in the test tubes containing 1.0 ml of sodium nitroprusside solution (25 mM), and then the tubes incubated at 37°C for 2 h. Two millilitre of the reaction mixture was removed after incubation and mixed with 1.2 ml Griess reagent, the absorbance was measured immediately at 570 nm.[20]

Hydrogen peroxide scavenging activity

Hydrogen peroxide solution (0.6 mL, 40 mM) in phosphate buffer mixed with 3.4 ml extract solution.[21] The absorbance of the reaction mixture was measured at 230 nm. Gallic acid used as standard. The percentage of H_2O_2 scavenging (I%) of examined extracts was calculated by equation (1).

Reducing power ability

Different concentration of 1.0 ml of sample solutions were mixed with 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of 1% (w/v) $K_3Fe(CN)_6$. After incubation for 20 min at 50°C, 2.5 ml of 10% TCA was added. The mixture was centrifuged and 2.5 ml of supernatant solution taken to mix with 2.5 ml of distilled water, 0.5 ml of 0.1% (w/v) $FeCl_3$. The absorbance of the solution was recorded at 700 nm.[22]

Ferrous chelating ability

Briefly, 0.4 ml of sample solution was mixed with 0.05 ml of $FeCl_2$ (2 mM) and 0.2 ml of ferrozine (5 mM). Total volume of the mixture was adjusted to 4.0 ml with ethanol. The mixture was shaken vigorously and kept for 10 min, absorbance was measured at 562 nm.[23] The percentage of inhibition of ferrozine-Fe^{2+} complex formation (I%) was calculated by using equation (1).

Ferric thiocyanate (FTC) method

A reaction solution containing extract sample (200 µg) in ethanol (4.0 ml), 2.5% linoleic acid (4.0 ml), phosphate buffer (8.0 ml, 0.05 M, pH 7.0) and distilled water (4.0 ml) was placed in screw cap tube, mixed properly and incubated in the dark at 40°C. After every 24 h, 0.1 ml incubated solution was withdrawn and mixed with 75% ethanol (9.7 ml) and 30% ammonium thiocyanate (0.1 ml). Exactly 3 min after the addition of 0.1 ml 20 mM ferrous chloride in 3.5% hydrochloric acid to the reaction mixture, the absorbance was measured at 500 nm until the absorbance of the control reached a maximum.[24]

Ex vivo antioxidant activity of crude extracts

Inhibition of lipid peroxidation

Aliquot of 1.0 ml of rat liver homogenate (5.0% w/v) and 100 µl of sample solution was mixed and incubated for 2 h at 37°C, followed addition of 1.0 ml of TCA (15% w/v) and 1.0 ml TBA (0.67% w/v). The mixture was warmed in boiling water bath for 15 min and cooled in room temperature. The volume of the mixture was made up to 5.0 ml with deionized water and centrifuged at 2800 rpm for 10 min. The absorbance of the supernatant solution was measured at 532 nm.[25] The inhibition of lipid peroxidation in percent (%) was calculated according to equation (1).

Oxidative haemolysis inhibition assay

Briefly, 0.5 ml of 5% erythrocyte suspension was mixed with 0.5 ml extract solution (0.02-0.16 mg/ml) and 0.05 ml of H_2O_2 (100 mM). Mixture was incubated at 37ºC for 60 min, and after incubation 4.2 ml of distilled water was added. The solution was centrifuged at 1000 rpm for 10 min, and supernatant absorbance was read at 415 nm.[26].

Statistical analysis

Experimental results were expressed as mean±SEM. P values < 0.05 were regarded as statistically significant. A one-way analysis of variance followed by Turky tests was employed for the data analysis, using SPSS (Statistical Package for Social Sciences) version 10.0 software.

RESULTS AND DISCUSSION

Antioxidants are closely associated to their biofunctionalities, such as the decrease of DNA damage, acute and chronic diseases, and inhibition of pathogenic microorganism growth. Therefore, antioxidant activity and free radical scavenging capacity is widely used as a parameter for medicinal bioactive components.[14, 23]

The quantity of total phenolic content was determined from gallic acid calibration curve, with the regression equation Y = 0.0044x + 0.031 (R^2 = 0.9995), while calibration curve of quercetin (Y = 0.0288x + 0.0058, R^2 = 0.9991) was used to estimate total flavonoid content. Methanol extract possessed the highest concentration of phenolic (72.6 GAE mg/g of dry material) and flavonoid compounds (54.9 QE mg/g of dry material). The total phenolic and total flavonoid content in ethyl acetate and petroleum ether extract were 47.0, 28.3 GAE mg/g, and 23.0, 9.1 QE mg/g respectively.

All the extracts process concentration dependent antioxidant activity. IC_{50} values of extracts in different assay model were tabulated in table 1. The MMM had highest DPPH radical scavenging effect compare to EMM and PMM, with an IC_{50} value of 39.2±0.29 µg/ml. $DPPH^{\bullet}$ scavenging assay is based on the reduction of the stable $DPPH^{\bullet}$ to diphenyl-picrylhydrazine by antioxidants.[17,24] The strong $DPPH^{\bullet}$ scavenging capacity extracts was possibly due to the hydrogen donating ability of polyphenolic compounds present in extracts.

Table 1: Antioxidant activities of the extracts of *Marsilea minuta* by using different *in vitro* models

Extracts/ Standard	*In vitro* models (IC_{50} µg/ml) (Mean ± SEM)					
	DPPH radical	Superoxide radical	Hydroxyl radical	Nitric oxide radical	Hydrogen peroxide	Fe^{2+} chelating ability
Methanol extract	39.2 ± 0.29	48.8 ± 1.01	26.9 ± 0.98	33.5 ± 0.52	140.0 ± 1.99	136.8 ± 3.21
Ethyl acetate extract	53.9 ± 1.21	70.0 ± 1.31	48.0 ± 1.09	54.0 ± 0.40	265.0 ± 3.01	177.5 ± 2.89
Petroleum ether extract	66.1 ± 1.30	86.9 ± 1.09	60.9 ± 2.11	141.5 ± 0.98	349.9 ± 3.65	203.6 ± 4.01
Ascorbic acid	3.3 ± 0.01			23.0 ± 0.08		
BHA		23.8 ± 0.89				
α- tocopherol						107.0± 1.23
Quercetin			20.8 ± 0.78			
Gallic acid					65.0 ± 1.32	

Values are expressed as the mean ± SEM, n =3 with P < 0.01 compared to positive control.

All the extracts effectively scavenged the superoxide anion generated by the system at concentration of 20-160 µg/ml. Among the levels used in the experiment, 160 µg/ml of methanol extract was the strongest one with a scavenging rate of 79.46%, while BHA as standard produced 82.53% scavenging effect at the concentration of 120 µg/ml. In hydroxyl radical scavenging assay, the degradation of deoxyribose by Fe^{+3}-ascorbic acid-EDTA-H_2O_2 system was markedly decreased by all extracts of *M. mimuta*. The IC_{50} was higher for methanol extract (26.9±0.98 µg/ml) than the other two extracts, but at a lower rate constant then the standard drug (IC_{50}=20.8±0.78 µg/ml). Superoxide and hydroxyl radical are the reactive oxygen species involved in lipid peroxidation and generation of other reactive species, which can damage almost every molecule in living tissue.[23,27,28] Superoxide and hydroxyl radical scavenging effects by extracts possibly render them as promising antioxidants.

Nitric oxide is as an important pleiotropic mediator of physiological process and regulation of cell mediated toxicity. Nitric oxide also involved in host defence but over production of $NO^{\bullet}$ contributes in the pathogenesis of certain inflammatory diseases.[18,28] Extracts also effectively reduced the generation of NO radical. MMM exhibited superior $NO^{\bullet}$ scavenging activity with an IC_{50} of

33.5±0.52 µg/ml than the EMM (IC_{50}=54.0±0.40 µg/ml) and PMM (IC_{50}=141.5±0.98 µg/ml).

Hydrogen peroxide, a non radical species capable to cross cell membranes quickly and can inactivate different enzymes, usually by oxidation of essential thiol groups.[18,21,28] All extracts moderately neutralise H_2O_2 (IC_{50} was 140.0±1.99 µg/ml for MMM, 265.0±3.01 µg/ml for EMM and 349.9±3.65 µg/ml for PMM). Hence, H_2O_2 scavenging effect is significant for human health and protection of pharmaceutical and food system. In metal chelating activity, all extracts interfered with the formation of ferrous and ferrozine complex suggesting the significant ($P < 0.05$) chelating activity of extracts. The ferrous ion breaks down of hydrogen and lipid peroxides to reactive free radicals via Fenton reaction which increases lipid oxidation.[18,28] Therefore, minimizing ferrous ions by extracts may afford protection against oxidative damage.

The antioxidant action of compound depends on the breaking of free radical chain reaction by donating a hydrogen atom, or reacting with certain precursors of peroxide to prevent peroxide formation.[18,24,27] Figure 1 shows the concentration-response curves for the reducing powers of the extracts. The sequence for reducing power was ascorbic acid > MMM > EMM > PMM. The reducing power of MMM, EMM, and PMM increased from 1.264±0.002, 0.991±0.004 and 0.783±0.003 at 50 µg/ml to 2.417±0.002, 2.000±0.002 and 1.818±0.005 at 800 µg/ml respectively.

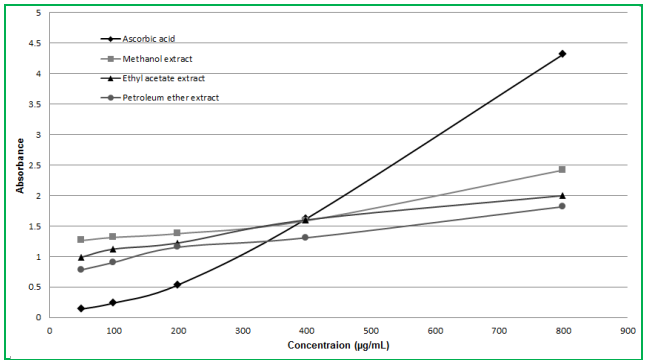

Figure 1: Reducing power ability of different extracts from the *Marsilea minuta* at different concentrations. Each value represents a mean ± SD (n = 3).

The amount of peroxides produced at initial stage of lipid oxidation can be measured by FTC method.[24,27] The effects of various solvent extracts in preventing the peroxidation of linoleic acid were shown in Figure 2. After 8 day of incubation, the formation of peroxides was stopped because of non-availability of linoleic acid. The intermediate products had been also converted to stable end-products, which results in the stoppage of oxidation of Fe^{2+}. The oxidations of linoleic acid slow down in the presence of antioxidants. The results clearly showed that all extracts exhibited significant ($P<0.05$) antioxidant activity.

Methanol extract possess a higher inhibition of MDA formation in liver tissue homogenates than other extracts and may comparable to rutin (Figure 3). IC_{50} value of rutin, methanol, ethyl acetate and petroleum ether extract was 75.9±0.92, 90.0±2.02, 103.9±2.33, and 143.4±3.07 µg/ml respectively. Figure 4 showed erythrocyte haemolysis was effectively inhibited by *M. minuta* extracts at concentrations of 20-160 µg/ml. Methanol extract showed highest activity, and at concentrations of 20, 80 and 160 µg/ml, the inhibition rate was 24.77%, 62.66% and 75.94%, respectively.

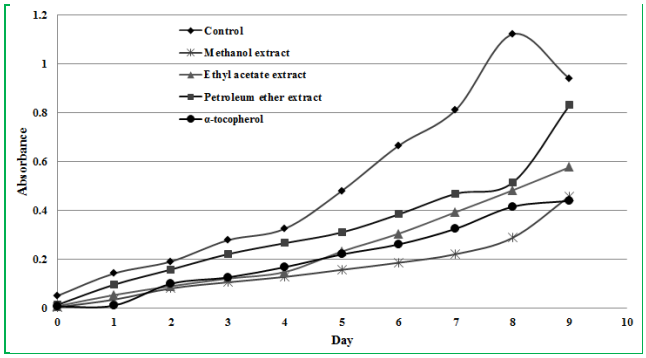

Figure 2: Antioxidant activity of *Marsilea minuta* extracts by the FTC method. Results are of triplicate measurements.

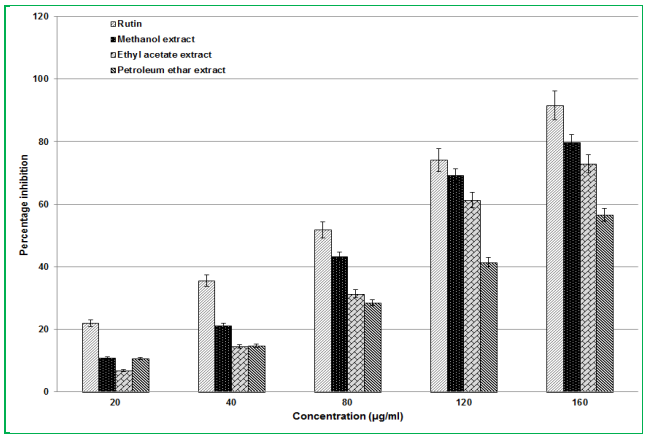

Figure 3: Inhibition of lipid peroxidation in liver tissues of mice by the extracts of *M. minuta* and rutin. Each value is expressed as mean ± SD (n = 3).

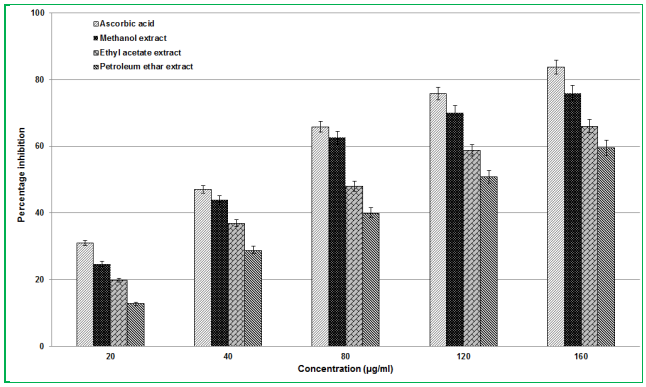

Figure 4: The inhibition of *M. minuta* extract for erythrocyte haemolysis in rat blood.

Genomic and mtDNA can be damaged by lipid peroxidation, an oxidative deterioration process of polyunsaturated fatty acids. Alteration in stability of membrane ligand-binding domains, membrane transport protein disruption and deactivation of membrane-

associated enzymes can be caused by peroxidation of membrane lipids.[24,26] Therefore inhibition of lipid peroxidation by the *Marsilea minuta* extracts is an elementary property of antioxidants. The cells like erythrocyte are highly susceptible to oxidation; consequently erythrocytes are one of the suitable cellular models to investigate oxidative damage in biomembranes.[16,26] Hence protective effect of *Marsilea mimuta* extracts can be helpful prevent cell films from lipid oxidation and protect erythrocyte.

The antioxidative effectiveness of natural compound has been reported mostly due to presence of phenolic and flavonoid compounds. Phenolic compounds contains hydroxyl group which is important for their scavenging ability.[29,30] The present study found a correlation between the polyphenolic content and the antioxidant activity, as methanol extract content highest amount of phenolic and flavonoid content and exhibit higher antioxidant activity.

CONCLUSION

Present work suggested high level of phenolics, flavonoids and excellent antioxidant activity of *Marsilea minuta* leaf extract. Presence of highest polyphenolic content may be responsible for highest antioxidant activity of methanolic extract. This plant can be considered as a good natural source of phytochemicals to be utilising as vegetable and the plant can provide precious functional ingredients and can be used for the prevention of diseases related to oxidative stress by repairing the oxidant/antioxidant balance in human body. Present study also indicates that the possible antioxidant mechanism of the extract can be due to its hydrogen or electron donating and direct free radical scavenging properties, but extract the antioxidant mechanisms and identification of antioxidant constituent the methanol extract should be further studied to gain more application for use as natural antioxidants.

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