

**ANTIOXIDANT ACTIVITY AND PHYTOCHEMICAL SCREENING OF TWO EDIBLE  
WETLAND PTERIDOPHYTES *DIPLAZIUM ESCULENTUM* (RETZ) SW AND  
*MARSILEA MINUTA* L.–A COMPARATIVE STUDY**Jayanta Choudhury<sup>1</sup>, Sukanta Majumdar<sup>1,2</sup>, Swarnendu Roy<sup>1,3</sup> and Prof. Usha Chakraborty\*<sup>1</sup><sup>1</sup>Plant Biochemistry Laboratory, Department of Botany, University of North Bengal, Siliguri 734013.<sup>2</sup>Microbiology and Microbial Biotechnology Laboratory, Department of Botany, University of Gour Banga, Malda, West Bengal, India.<sup>3</sup>Molecular & Analytical Biochemistry Laboratory, Department of Botany, University of Gour Banga, Malda, West Bengal, India.**\*Corresponding Author: Prof. Usha Chakraborty**

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**ABSTRACT**

Pteridophytes are recognized for their biological and medicinal properties for long time but very less work has been done on wetland pteridophytes. In our present study we evaluated the antioxidant activity of two wetland pteridophytes *Diplazium esculentum* (Retz.) Sw. and *Marsilea minuta* L. Presence of bioactive compounds like phenols, flavonoids and saponins was detected in both the plants. However, alkaloids, triterpenes and anthraquinones were exclusively detected in *Diplazium esculentum*. Methanolic extract of both the plants showed potential antioxidant activities. The IC<sub>50</sub> values of *Diplazium esculentum* for DPPH, ABTS, metal chelating and superoxide ion scavenging activity were found to be 3.8, 4.6, 1.09 and 2.24 mg/ml. Dose dependent inhibition of free radicals was also observed in case of *M. minuta*. The IC<sub>50</sub> values of *M. minuta* for DPPH, ABTS, metal chelating and superoxide ion scavenging activity were recorded to be 7.7, 6.4, 0.59 and 4.69 mg/ml respectively. The presence of compounds like total phenols, flavonoids, protein, total sugar, reducing sugar, free amino acid and chlorophyll content were also evaluated in these two plants. However, total phenol and flavonoids content was recorded higher in case of *D. esculentum*.

**KEYWORDS:** phytochemicals, phenol, flavonoid, antioxidants, DPPH, ABTS, FRAP.**INTRODUCTION**

All living organisms require oxygen for its existence, which is a highly reactive molecule that damages living organisms by producing reactive oxygen species (ROS).<sup>[1,2]</sup> Free radicals or ROS, generated from the cellular reduction and oxidation processes, are considered responsible for several diseases such as cancer, autoimmune disorders, rheumatoid arthritis, cataract, aging, cardiovascular and neurodegenerative diseases. The commonly formed reactive oxygen species are superoxide anions, hydrogen peroxide, hydroxyl radical, nitric oxide and peroxy nitrite radicals. Free radicals are extremely unstable due presence of unpaired electrons and can donate or receive single electron, which in turn accounts for their high reactivity with other biomolecules. Although, the existence of extremely developed antioxidative defence mechanism in human body balances the production of free radicals, but sometimes as a consequence of deficit in antioxidant levels, free radicals are generated which leads to oxidative stresses.<sup>[3-5]</sup> In recent years, much attention has been devoted to natural antioxidant and their associations

with health benefits.<sup>[6]</sup> Plants are potential sources of natural antioxidants.<sup>[7]</sup>

In India, from ancient past we have the rich tradition of using plants with medicinal properties as food as well as in therapeutic diseases. Even in advanced countries use of natural resources, use of herbal medicines is climbing nowadays for their fewer side effects.<sup>[8]</sup>

Due to wide range of geographic expansion India is rich in biodiversity thereby providing a rich source of plants with different therapeutic activities waiting to be explored.

Wet lands are areas with immense biodiversity and are yet not properly evaluated for therapeutic plant resources. Similarly, pteridophytes growing in wet land attained very fewer attention of scientific community. However, in recent years, many workers have explored the biological and medicinal properties of pteridophytes.<sup>[5,9-12]</sup>

*Diplazium esculentum* (Retz.) Sw. is a pantropical species belonging to the family Athyriaceae occurring commonly throughout India, China, Cambodia, Laos, Vietnam and Malaysia with no threats, hence categorized as Least Concern. This plant grows in gregarious colonies in open marshy areas, stream banks and canals from sea level to 2,300 m.<sup>[13,14]</sup> Young frond and rhizomes are used as green vegetables. The vernacular name of this plant is *dheki* in Santali *Dhekir Shaak* in Bengali, and the English name is Vegetable fern. Moreover, consuming as vegetable, *D. esculentum* has been reported to have profound use as traditional medicine in diseases like fever, dermatitis, measles dysentery, glandular swellings, indigestion, diarrhea and various skin infections.<sup>[9,15]</sup>

*Marsilea minuta* L. is a wetland Pteridophyte of the Marsileaceae family that grows in marshy places as well as on the margin of ponds ditches, paddy fields etc and is native to tropical Asia and Africa. English name of this plant is water clover and four-leaf clover. In North Bengal it is commonly known as sushni, sunsuni or sunsunia sak. It has been used in the ayurvedic system of medicine for curing several ailments. *M. quadrifolia* showed antibacterial, cytotoxic and antioxidant activities.<sup>[16]</sup>

*D. esculentum* (Retz.) Sw. and *M. minuta* L. both have been consumed frequently as leafy vegetables by different communities of North Bengal, which encompasses the Northern region of West Bengal, India. Mostly they grow in wet lands of North Bengal and constitute a remarkable part of vegetables of poor and under privileged people of this region.

Therefore, in the present study we have attempted to evaluate the comparative antioxidant activity of two wet land pteridophytes *Diplazium esculentum* (Retz.) Sw. and *Marsilea minuta* L.

## MATERIAL AND METHODS

### Collection of plant materials

Fronds of *Diplazium esculentum* (Retz.) Sw. and *Marsilea minuta* L. were collected from the wetlands of Dakshin Dinajpur (Latitude: 26°35'15" N to 25°10'55" N; Longitude: 89°00'30" E - 87 deg 48' 37" E) and Malda (Latitude: 24°40'20" N to 25°32'08" N; Longitude: 87°45'50" E to 88°28'10" E) districts of West Bengal, India. Plants were dried and submitted to Herbarium Facility at North Bengal University, Siliguri, West Bengal with the Accession Nos. 09820 and 09821 respectively.

### Phytochemical screening

Both the dried plant samples were screened for presence of compounds like alkaloids, carbohydrates, glycosides, saponins, phenols, tannins, flavonoids, proteins, amino acids and triterpenoids by following standard protocols.

### Test for phenol

The powdered plant samples were mixed with 10 ml double distilled water and stirred in magnetic stirrer for 10 min. The mixture was then filtered using Whatman filter paper No.1. To the filtrate (1ml), equal volume (1ml) of 1% FeCl<sub>3</sub> was added and observed for the appearance of blue or green color indicating the presence of phenols.<sup>[17]</sup>

### Test for flavonoid

Briefly, 2g of the sample was mixed thoroughly with 10ml of acetone which was evaporated by keeping the flask in a hot water bath for 5 min. Further, the sample was extracted using 10ml of warm double distilled water. The solution was thoroughly mixed, filtered while hot and allowed to cool at room temperature. To the filtrate (5ml), equal volume of 20% NaOH was added and change in appearance/color of the solution to yellow indicated the presence of flavonoid.<sup>[18]</sup>

### Test for tannin

The crude plant powder (200mg) was mixed with 10ml of double distilled water and allowed to stand for 10 min before filtration. Then, 1ml of 5% FeCl<sub>3</sub> was added to 2ml of the filtrate. The formation of yellow brown precipitate indicates the presence of tannin.<sup>[19]</sup>

### Test for alkaloid

The powdered sample (200mg) was mixed vigorously with 10ml of methanol for 1h at room temperature. The mixture was then filtered and to 2ml of methanolic filtrate, 2ml of 1% HCl was added. The solution was kept in boiling water bath for 5min. Then, Mayer's - Wagner's reagent (6-7 drops) was added to the filtrate. Formation of creamish/brown/red/orange precipitate indicates the presence of alkaloid.<sup>[20]</sup>

### Test for cardiac glycosides

The method described by Trease and Evans (1989) with minor modification was followed for the qualitative screening of cardiac glycosides in the samples.<sup>[20]</sup> The methanolic filtrate (2ml) was mixed with 1ml glacial acetic acid, to which further 3-4 drops of 5% FeCl<sub>3</sub> was added. Then, 1 ml of concentrated H<sub>2</sub>SO<sub>4</sub> was added carefully to the solution. Development of brown ring at the interface indicates the presence of cardiac glycosides. A violet colour may also appear below the brown ring.

### Test for carbohydrates

The plant samples (500mg) were boiled in 30ml of double distilled water and filtered. Then, 2ml of aqueous extract was mixed with 2ml of Molish's reagent (5%- $\alpha$ -naphthol in absolute ethanol) and shaken vigorously. To it, 2ml of concentrated H<sub>2</sub>SO<sub>4</sub> was added carefully along the wall of the test tube. The presence of carbohydrate was inferred by the formation of reddish-ring at the junction of two liquids.<sup>[18]</sup>

**Test for reducing sugars**

The sample (500mg) was boiled in 30ml of double distilled water and filtered to obtain an aqueous extract/filtrate. The aqueous filtrate (1ml) was then mixed with 2ml of Fehling's solution (A: 7% CuSO<sub>4</sub> in dH<sub>2</sub>O containing 2 drops of H<sub>2</sub>SO<sub>4</sub> (dil.), B: 12% KOH and 35% Sodium potassium tartarate in dH<sub>2</sub>O. Mix A and B in equal amount) and boiled for 5mins. Formation of a brick red precipitate indicates the presence of reducing sugars.<sup>[18]</sup>

**Test for protein**

The method described by Pullaiah was followed for the detection of protein in the plant samples.<sup>[21]</sup> About 1g of the plant sample was mixed thoroughly with 10ml of double distilled water by a magnetic stirrer for 10h and filtered. 2ml of the filtrate was then mixed with 1ml of 40% NaOH in a test tube. Then, 1-2 drops of CuSO<sub>4</sub> was gently added to the solution. Change in the color of solution to violet indicates the presence of peptide linkages in a solution which in turn is an indication of the presence of proteins.

**Test for saponin**

The aqueous filtrate (0.5ml) prepared as above was mixed with 5 ml of double distilled water and shaken vigorously for about 30 seconds. The presence of saponins was indicated by the formation and persistence of the froth.<sup>[20]</sup>

**Test for terpenoid**

The methanolic filtrate (2ml) was mixed with 5ml of chloroform and 2ml of acetic anhydride. Then, to the mixture 1ml of concentrated H<sub>2</sub>SO<sub>4</sub> was added carefully along the wall of the test tube. The formation of reddish brown ring at the interface indicates the presence of terpenoid.<sup>[22]</sup>

**Test for steroid**

The methanolic filtrate (5ml) was treated with 0.5ml of anhydrous CH<sub>3</sub>COOH and cooled on an ice bath for 15mins. Then, 0.5ml of chloroform and 1ml of concentrated H<sub>2</sub>SO<sub>4</sub> was added to the cold solution. Presence of the steroid may be inferred by the formation of reddish-brown ring at the junction of two liquids.<sup>[23]</sup>

**Test for anthraquinone**

About 0.5g of powdered sample was mixed with 5ml of chloroform, shaken for 5 min and filtered using Whatman No.1 filter paper. The filtrate (3ml) was mixed with 3ml of 10% ammonia solution and shaken properly. Development of pink/red/violet color in the aqueous layer after shaking indicates the presence of free anthraquinone.<sup>[18]</sup>

**Test for amino acid**

0.5 ml methanolic plant extracts were treated with few drops of ninhydrin reagent, heated in water bath, a purple colour indicated the presence of amino acids.<sup>[24]</sup>

**Test for glycosides**

0.5 ml methanolic extracts of plant were added with 2ml of 50% hydrochloric acid. The mixtures were hydrolyzed for 2 hrs on a water bath. After that 1 ml pyridine, few drops of 1% sodium nitroprusside solution, and 5% sodium hydroxide solution were added. Pink to red colour designated the presence of glycosides.<sup>[24]</sup>

**Test for triterpenoids**

0.5 ml of methanolic plant extracts were evaporated and dissolved in 1ml chloroform. 1ml acetic anhydride was then added and chilled. After cooling, conc. H<sub>2</sub>SO<sub>4</sub> was added. If reddish violet colour appeared, the existence of triterpenoids was confirmed.<sup>[24]</sup>

**Preparation of Extracts**

The plants were collected from different wetlands and washed thoroughly initially with tap water then with double distilled water to get rid of dirt and dried using blotting paper. The shade dried plant materials were then ground to obtain fine powder that was stored at -20°C for further use. The powdered sample was then extracted using methanol (MeOH) using the method described by Okwori *et al.* with slight modification.<sup>[25]</sup> Briefly, 10 g powdered samples were soaked in 100 ml of methanol for 72 hr at room temperature in a orbital shaker and filtered 3-4 times using Whatman No.1 filter paper. The filtrates were finally concentrated using rotary evaporator at 10°C.

**DPPH free radical scavenging activity**

DPPH (2,2-diphenylpicrylhydrazyl) free radical scavenging activity was done following standard protocol with slight modifications.<sup>[26]</sup> Different concentrations of plant extracts (0.1 ml) were put in the test tube and then 2.9 ml of a methanolic solution of DPPH (0.1 mM) was added. The mixture was kept in the dark at room temperature for 30 min and absorbance was measured at 517 nm against a blank and scavenging activity was calculated using the following formula.

$$DPPH\ Scavenging\ (\%) = 100 \times (A_o - A_s) / A_o$$

Where, A<sub>o</sub> is the absorbance of the blank and A<sub>s</sub> the absorbance of the sample.

**ABTS scavenging activity**

The ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) assay was based on a previously described method with slight modifications.<sup>[27]</sup> ABTS radical (ABTS<sup>+</sup>) was produced by the reaction of a 7 mM ABTS solution with potassium persulphate (2.45 mM). The ABTS<sup>+</sup> solution was diluted with ethanol to an absorbance of 1.5 ± 0.05 at 734 nm prior to the assay and stored in the dark at room temperature for 12 h. For assay, 100 µl of extract sample or ascorbic acid used as standard to 2 ml of diluted ABTS<sup>+</sup> solution, then absorbance was measured at 734 nm after exactly 6 min. The decrease in absorption was used for calculating scavenging effect using the following equation.

$$Scavenging\ effect\ (\%) = 100 \times (A_o - A_s) / A_o$$

Where,  $A_o$  is the absorbance of the blank and  $A_s$  the absorbance of the sample.

#### Nitric oxide scavenging activity

Nitric oxide scavenging activity was determined according to the Griess Illosvoy reaction.<sup>[28]</sup> The reaction mixture contained 2 ml of sodium nitroprusside (10 mM) in 0.5 ml phosphate buffer (0.5 M; pH 7.4). Various concentrations of the extracts (0.5 ml) were added in a final volume of 3 ml. After incubation for 60 min at 37°C, Griess reagent [N-(1-Naphthyl) ethylenediamine (0.1%) and sulphanic acid (1%) in  $H_3PO_4$  (5%)] was added. The pink chromophore generated during diazotization of nitrite ions with sulphanilamide and subsequent coupling with N-(1-Naphthyl) ethylenediamine was measured spectrophotometrically at 540 nm. Ascorbic acid was used as a positive control. The scavenging ability (%) of the nitric oxide was calculated using the formula:

$$\text{Scavenging effect (\%)} = 100 \times (A_o - A_s)/A_o$$

Where,  $A_o$  is the absorbance of the blank and  $A_s$  the absorbance of the sample.

#### Metal chelating activity

Metal chelating activity of plant extract was measured by adding 0.1 mM  $FeSO_4$  (0.2 ml) and 0.25 mM ferrozine (0.4 ml) subsequently into 0.2 ml of plant extract. After incubating at room temperature for 10 min, absorbance of the mixture was recorded at 562 nm.<sup>[29]</sup> Chelating activity was calculated using the following formula:

$$\text{Metal chelating activity} = 100 \times (A_o - A_s)/A_o$$

Where,  $A_o$  is the absorbance of the control and  $A_s$  the absorbance of the sample.

#### Superoxide scavenging activity

This assay was based on the reduction of nitro blue tetrazolium (NBT) in the presence of NADH and phenazine methosulfate under aerobic condition.<sup>[30]</sup> The 3 ml reaction mixture contained 50 ml of 1 M NBT, 150 ml of 1 M nicotinamide adenine dinucleotide with or without sample and Tris buffer (0.02 M, pH 8.0). The reaction was started by adding 15 ml of 1 M phenazine methosulfate to the mixture and the absorbance change was recorded at 560 nm after 2 minutes. Percent inhibition was calculated against a control without the extract.

#### FRAP assay

The FRAP (Ferric Reducing Antioxidant Power) of the methanolic extracts were determined by using a previously described method with slight modifications.<sup>[31]</sup> The freshly prepared FRAP reagent contained 500 ml of acetate buffer (0.3M; pH 3.6), 50 ml of 2,4,6-Tris (2-pyridyl)-s-triazine (TPTZ) (0.01M), and 50 ml of  $FeCl_3 \cdot 6H_2O$  (0.05M). 100  $\mu$ l of extracts were added to 2 ml of FRAP reagent and the absorbance was measured at 593 nm after 12 min. The results were calculated from the standard curve of  $FeSO_4$  and expressed as mM  $Fe^{2+}$  equivalent.

#### Total phenols

The total phenol content was determined by slightly modified Folin-Ciocalteu method.<sup>[32,33]</sup> The reaction mixture contained 200  $\mu$ l of extract, 800  $\mu$ l of freshly prepared diluted Folin Ciocalteu (1:1) reagent and 2 ml of sodium carbonate (7.5%), kept in the dark at ambient conditions for 2 h to complete the reaction. The absorbance was measured at 765 nm. Gallic acid was used as standard and the results were expressed as mg gallic acid/g dried extract.

#### Total flavonoids

Total flavonoid content was determined according to the standard method using quercetin as a standard.<sup>[34]</sup> A volume of 500  $\mu$ l of extract was added to 500  $\mu$ l of  $NaNO_2$  (5%). After 5 min at 25°C, 500  $\mu$ l of  $AlCl_3$  (10%) was added. After a further 5 min, the reaction mixture was mixed with 1 ml of 1 mM NaOH. Finally, the absorbance was measured at 510 nm. Quercetin was used as standard and the results were expressed as mg quercetin (QE)/g of dried extract.

#### Chlorophylls

From the samples Chlorophyll was extracted following the method of Harborne with minor changes.<sup>[35]</sup> 1g of the dried powder was crushed with 80% acetone in a mortar pestle and filtered through Whatman No.1 filter paper in a dark room. The residue was re-extracted with 80% acetone until it became completely colorless attaining final volume upto 10 ml. OD was measured at 663nm and 645nm in a UV-VIS spectrophotometer against a blank of 80% acetone. Using the following formula the chlorophyll content was calculated.

$$\text{Total chlorophyll} = (20.2 A_{645} + 8.02 A_{663}) \text{ mg g}^{-1} \text{ dry weight.}$$

$$\text{Chlorophyll a} = (12.7 A_{663} - A_{645}) \text{ mg g}^{-1} \text{ dry weight}$$

$$\text{Chlorophyll b} = (22.9 A_{645} - 4.68 A_{663}) \text{ mg g}^{-1} \text{ dry weight.}$$

#### Carotenoid content

Carotenoid content was estimated by the procedure of Lichtenthaler.<sup>[36]</sup> The desired amount of crude or diluted filtrate was taken directly into the cuvette and the absorbance was taken at 480nm, 645nm and 663nm wavelength against a blank in UV-VIS spectrophotometer 118 systronics. The amount of carotenoid was calculated using the standard formula as follows:

$$A_{480} - (0.114 \times A_{663}) - 0.638 (A_{645}) \mu\text{g g}^{-1} \text{ dry weight}$$

#### Protein content

1 g of fresh plant tissue was homogenized in a prechilled mortar and pestle using 5 ml of 50 mM sodium phosphate buffer (pH 7.2) and PVP (polyvinylpyrrolidone) under ice cold condition and centrifuged at 10,000 rpm at -4°C for 15 mins.<sup>[37]</sup> The supernatant obtained was used as the crude extract for estimation. For estimation, 1ml of extract and 5ml alkaline reagent was mixed thoroughly and allowed to



stand for 15mins.<sup>[38]</sup> Then, FolinCiocalteu's phenol reagent was added and incubated for 20 minutes and the absorbance was read at 690 nm.

#### Free amino acid

Free amino acids were estimated with standardized protocol with slight modifications.<sup>[39]</sup> 0.5 g leaf tissue was extracted in ethanol and filtered using Whatman No. 1 filter paper. To 1 ml of the filtrate, 1 ml of ninhydrin reagent was added and boiled in a water bath for 20 min. The absorbance was read at 570 nm and the free amino acid content was estimated using a standard curve of L-proline.

#### Total sugars and reducing sugars

Total and reducing sugar was extracted following the method of Harborne.<sup>[22]</sup> 1 g fresh leaf tissue was extracted in 10 ml of 95% ethanol and the alcoholic fraction was evaporated on a boiling water bath. Then the residue was dissolved in dH<sub>2</sub>O and the final volume was made up to 5 ml which was then centrifuged at 5000 rpm for 10 min.

Estimation of total sugar was done by Anthrone reagent following the method of Plummer.<sup>[40]</sup> To 1 ml of test solution, 4 ml of Anthrone reagent (0.2% Anthrone in conc. H<sub>2</sub>SO<sub>4</sub>) was added. The reaction mixture was mixed thoroughly and was incubated in boiling water bath for 10 mins. Then the reaction mixture was cooled under running tap water and absorbance was measured at 620 nm and sugar content was quantified using a standard curve of D-glucose.

The amount of reducing sugar present in the plant samples was determined by Nelson-Somogyi method as described by Plummer.<sup>[40]</sup> In brief, 1 ml of the test solution was mixed with 1 ml of alkaline copper tartarate solution and heated over a boiling water bath for 20 mins. The reaction mixture was then cooled under running tap water and 1 ml Nelson's Arsenomolybdate reagent was added along with 2 ml of dH<sub>2</sub>O and mixed vigorously. A blue colour was developed, the absorbance of which was the measured in a colorimeter at 515 nm

**Table 2: Nutritional analyses in terms of total proteins, free amino acid, total sugars and reducing sugars in the extracts.**

Sample	Protein (mg/g dwt)	Free amino acids (mg/g dwt)	Total Sugars (mg/g dwt)	Reducing sugars (mg/g dwt)
DE	26.50±01.41	0.15±0.010	35.00±0.32	0.130±0.01
MM	25.50±02.04	0.42±0.210	11.00±0.13	0.435±0.03

DE =*Diplazium esculentum*; MM=*Marsilea minuta*; dwt= Dry Wt.

#### In vitro Antioxidant activity

##### DPPH scavenging activity

DPPH is a stable free radical having characteristic purple colour having absorption maxima at 517 nm. It loses its colour when react with antioxidant. In case of both the plants dose-dependent scavenging activity was observed. Methanolic extract of *D. esculentum* showed much more scavenging activity than *M. minuta*. The IC<sub>50</sub> value for

and reducing sugar content was quantified using a standard curve of D-glucose.

## RESULTS

#### Phytochemical screening

The dried plant samples were screened for the presence of different phytochemicals. The result revealed that both plants contain carbohydrate, saponins, phenols, tannins, flavonoids, proteins, triterpenes and alkaloids. Phytosteroid, fat and fixed oil, glycosides and anthroquinone were found to be absent in both the plant samples (Table 1).

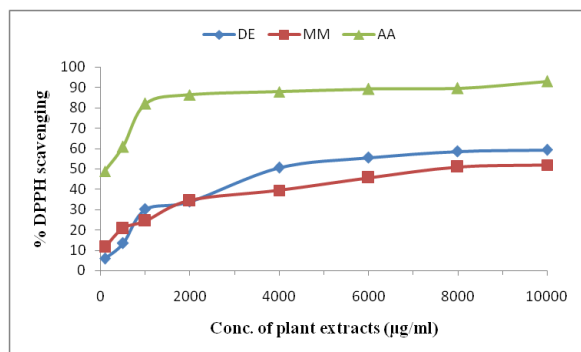
#### Nutritional factors

The amount of different nutritional factors such as protein, carbohydrate and free amino acid in both the dried plant samples was quantified. Results revealed that the amount of total sugar is much higher in *D. esculentum* in comparison to *M. minuta* but in contrary the reducing sugar content is much higher in *M. minuta*. The protein content of both plant samples were quite similar but free amino acid content was found to be higher (0.42 mg/gm tissue) in MM (Table 2).

**Table 1: Phytochemical compounds detected in the two plant extracts.**

Phytochemicals	<i>D. esculentum</i>	<i>M. minuta</i>
Alkaloid	+	-
Carbohydrate	+	+
Glycosides	-	-
Saponins	+	+
Phytosteroid	-	-
Phenols	+	+
Tannins	-	+
Flavonoids	+	+
Protein	+	+
Amino acid	-	+
Triterpens	+	-
Anthroquinones	+	-
Cardiac glycosides	-	-

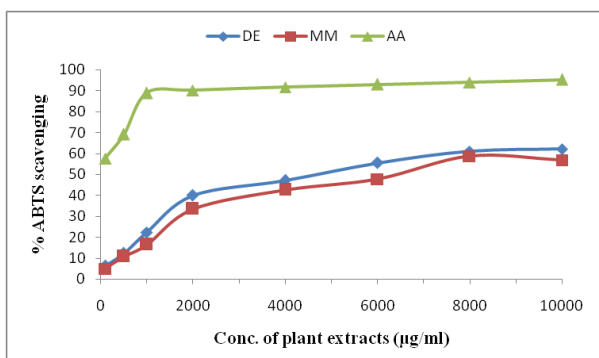
DE was found to be 3.8 mg/ml and 7.7 mg/ml for MM (Fig. 1).



**Figure 1: DPPH scavenging activity of different concentrations of plant extracts (µg/ml); DE-*Diplazium esculentum*, MM-*Marsilea minuta*, AA-Ascorbic acid (standard).**

#### ABTS scavenging activity

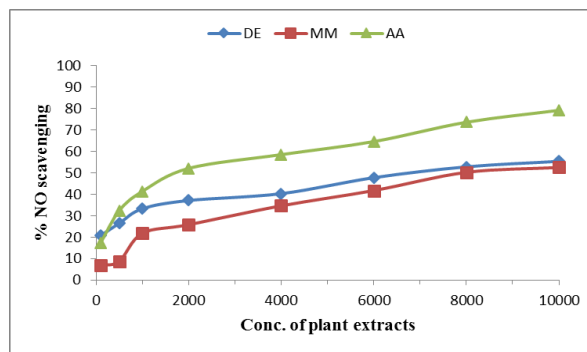
Proton radical scavenging is an important feature of antioxidants. ABTS is a protonated radical that has characteristic absorption maxima at 734 nm, which decreases in presence of antioxidants. In both the plant samples, scavenging effect of ABTS radical increased with concentration. The scavenging activity of DE was relatively higher than MM. The  $IC_{50}$  values were recorded 4.6 mg/ml and 6.4 mg/ml for DE and MM respectively (Fig. 2).



**Figure 2: ABTS radical scavenging activity of different concentrations of plant extracts (µg/ml), DE-*Diplazium esculentum*, MM-*Marsilea minuta*, AA-Ascorbic acid (standard).**

#### Nitric oxide scavenging activity

Nitric oxide scavenging activity of DE and MM was determined with varied concentration ranging from 100 to 10000 µg/ml. At low concentration the scavenging activity of DE was found to be greater than MM, however with increase in concentration both the plant samples showed almost similar type of scavenging efficiency (Fig. 3).



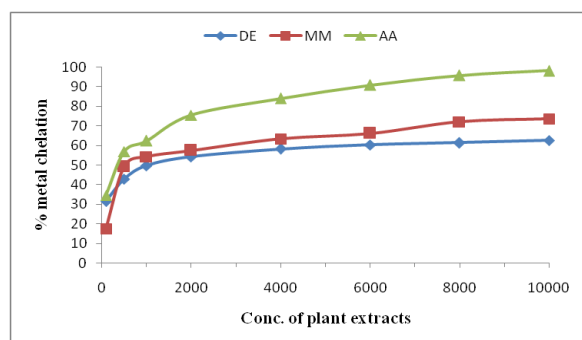
**Figure 3: NO scavenging activity of different concentrations of plant extracts (µg/ml).**

#### Metal chelating activity

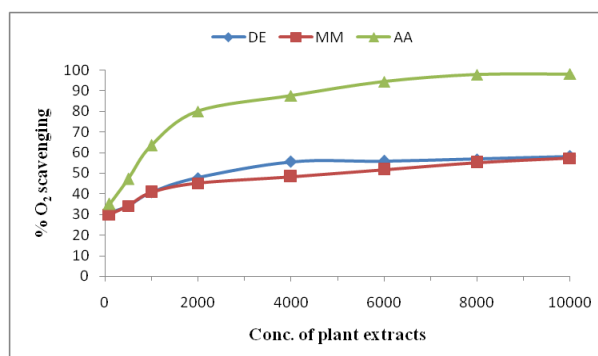
The methanolic extract of both the samples evaluated for their chelating capacity. With increasing concentration of plant extract decrease in absorbance of  $Fe^{+2}$ -ferrozine complex was observed in both the cases. Efficacy of metal chelating was found to be greater in MM in comparison to DE. The  $IC_{50}$  values for metal chelating activity were recorded 0.59 and 1.09 mg/ml for MM and DE respectively (Fig.4).

#### Superoxide scavenging activity

Dose dependent superoxide radical scavenging activity was demonstrated by methanol extract of DE and MM. Though at higher concentration both the samples showed almost similar degree of scavenging, but  $IC_{50}$  value for superoxide anion scavenging activity were recorded 2.24 and 4.69 mg/ml for DE and MM (Fig. 5).



**Figure 4: Metal chelating activity of plant extracts at different concentrations (µg/ml).**



**Figure 5: Superoxide anion scavenging activity of different concentrations of plant extracts (µg/ml).**

### Hydroxyl ion scavenging activity

The potential of methanolic extracts of DE and MM to inhibit hydroxyl-radical-mediated deoxyribose damage was assessed at a concentration of 100 to 10000  $\mu\text{g/ml}$ . A concentration dependent inhibition of hydroxyl ion was observed in both plant extracts (Fig. 6).

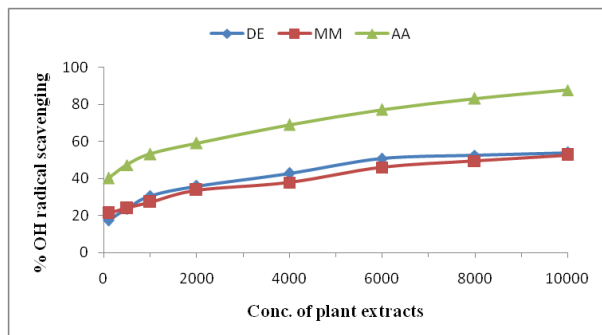


Figure 6: Hydroxyl ion scavenging activity of plant extracts at different concentrations ( $\mu\text{g/ml}$ ).

Table 3: *In vitro* antioxidant potential of the extracts quantified in terms of FRAP, total phenols, total flavanoids, ascorbic acid, carotenoids and total chlorophyll.

Sample	FRAP assay (mM Fe <sup>2+</sup> eq.)	Total Phenols (mg/g dwt)	Total Flavonoids (mg/g dwt)	Ascorbic acid (mg/g dwt)	Carotenoids (mg/g dwt)	Total chlorophyll (mg/g dwt)
DE	0.095 $\pm$ 0.002	0.054 $\pm$ 0.04	0.238 $\pm$ 0.021	0.459 $\pm$ 0.11	0.279 $\pm$ 0.005	0.648 $\pm$ 0.004
MM	0.121 $\pm$ 0.005	0.018 $\pm$ 0.01	0.127 $\pm$ 0.090	0.478 $\pm$ 0.09	0.517 $\pm$ 0.003	1.424 $\pm$ 0.100

DE = *Diplazium esculentum*; MM = *Marsilea minuta*; dwt = Dry Wt.

### DISCUSSION

Pteridophytes are known to have different bioactive compounds which have therapeutic activity. In present study we have evaluated the antioxidant activity of two wet land pteridophytes *D. esculentum* and *M. minuta*, which are generally consumed by poor and underprivileged peoples. The phytochemical screening revealed that both the plant samples contain carbohydrate, saponins, phenols, tannins, flavonoids, proteins, triterpenes. These compounds are known to contribute for antioxidant activities of plants.<sup>[41]</sup> Presence of hydroxyl functional group in flavonoids empower them to act as antioxidant.<sup>[42]</sup>

DPPH scavenging activity was exhibited by methanolic extract of both plant samples. DPPH scavenging activity of DE was much greater than that of MM. This may be due to the presence of much more phenolic compounds in DE in comparison to MM. Similar type of observation was also recorded previously.<sup>[43]</sup> The ABTS radical cation decolorization assay can measure the relative antioxidant ability to scavenge the radical ABTS.<sup>[44]</sup> The ABTS scavenging activity is one of the most important parameters for determination of antioxidant activity. Methanolic extract of both plant samples showed considerable amount of scavenging activity. Nitric oxide is a free radical produced in mammalian cells involved in regulation of various physiological processes. However, excess production of nitric oxide is associated with several inflammatory diseases.<sup>[45,46]</sup> So, development of

### FRAP Assay

Ferric Reducing Antioxidant Power capacity of the plant extracts was also determined. Results revealed that FRAP of DE is higher than that of MM. Total phenol and flavonoids content also showed similar type of observation. The phenolic and flavonoids content of DE was found to be higher in comparison to MM. However, chlorophyll, carotenoids and ascorbic acid content was recorded higher in *M. minuta* methanol extract (Table 3).

antioxidant mechanism to combat nitric oxide has become a recent area of research. In present study, the methanolic extract of *Diplazium esculentum* and *Marsilea minuta* was evaluated for its inhibitory effect on nitric oxide production. It was found that both the isolates could inhibit the production of nitric acid from sodium nitroprusside at physiological pH.

In present study, methanolic extracts of *Diplazium esculentum* and *Marsilea minuta* inhibited the formation of chelating agent complex, which reduces the red color of the complex. This measurement of color reduction therefore allows estimation of the chelating ability of the coexisting chelator.<sup>[44]</sup> Superoxides are produced from molecular oxygen by both enzymatic and nonenzymatic pathway.<sup>[47]</sup> These superoxide anions are responsible for the formation of other reactive oxygen species such as hydrogen peroxide, hydroxyl radical and singlet oxygen, which induce oxidative damage in lipids, protein and DNA.<sup>[48,49]</sup> A dose dependent superoxide radical scavenging activity was demonstrated by methanol extract of both the plants.

Hydroxyl ion scavenging activity was also demonstrated by methanolic extract *Diplazium esculentum* and *Marsilea minuta*. Hydroxyl ion, which can react with biomolecules in living cells cause severe damage, scavenging activity is considered as prime criterion to be an antioxidant.<sup>[50]</sup> In presence of antioxidant, ferric-ferric cyanide complex is reduced to the ferrous form, thus providing the basis of FRAP assay. Higher absorbance

indicates a higher ferric reducing power.<sup>[51,52]</sup> In our study we showed that in presence of both the plant extracts ferric-ferric cyanide complex was reduced to the ferrous form.

Quantification of bioactive compounds revealed that methanolic extract of *Diplazium esculentum* and *Marsilea minuta* contain significant amount of phenols, flavonoids, chlorophyll and carotenoids. Phenolics are important plant secondary metabolites and are known to be distributed throughout plant kingdom. Phenols are one of the most commonly occurring groups of phytochemicals, with significant morphological and physiological importance in plants. Phenolic compounds have been reported as major group of compounds that contribute to the antioxidant activity of plant extracts and has been correlated with DPPH scavenging assay.<sup>[53]</sup> Along with chlorophyll presence of carotenoids was observed in both the plant samples in present study. Carotenoids are one of the key non-enzymatic antioxidants that have capacity to suppress lipid peroxidation in all photosynthetic organisms.<sup>[54]</sup>

## CONCLUSION

In present study we have evaluated the antioxidant activity of two wet land pteridophytes *Diplazium esculentum* and *Marsilea minuta*. Both the plants are used as vegetables by local peoples. In our study we found that both the plants have potential antioxidant activity as well as nutritional values. However, the overall result indicated that, *Diplazium esculentum* had more nutritional and antioxidative properties in comparison to *Marsilea minuta*. Finally, it could be concluded that the consumption of these underutilized vegetables should be popularised as they could be natural sources of antioxidative phytochemicals and nutrients.

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