

ANTIOXIDATIVE AND FREE RADICAL SCAVENGING POTENTIALS OF *CYCLOSORUS INTERRUPTUS* (WILLD.) H. ITÔ AND *PRONEPHRIUM NUDATUM* (ROXB. EX GRIFF.) HOLTUM

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ABSTRACT

Objectives: The work aims to screen the antioxidative potentials of different crude extracts of the fronds of two medicinally important pteridophytes of Southern Assam, India, namely, *Cyclosorus interruptus* (Willd.) H. Itô and *Pronephrium nudatum* (Roxb. ex Griff.) Holttum.

Methods: Frond extracts of the pteridophytes were prepared by Soxhlet hot extraction method. Total phenolic content (TPC) and total flavonoid content (TFC) of the hexane, ethyl acetate, acetone, and methanol extracts of the fronds of the plants were done by following standard protocol. *In vitro* assessment of the antioxidative behavior of the extracts was performed using standard 2,2-diphenyl-1-picrylhydrazyl scavenging assay, reducing power assay, metal chelating assay, hydroxyl, superoxide, and 2,2'-azino-bis (3-ethylbenzothiazoline - 6 - sulfonic acid) radical scavenging methods in different *in vitro* systems.

Results: Preliminary phytochemical analysis implicated the presence of phenolic and flavonoid compounds in all the frond extracts. The methanol extract of the fronds of both the plants showed maximum phenolic and flavonoid contents in comparison to the other extracts, however, that of *C. interruptus* was found to be higher than *P. nudatum*. Antioxidative potentials of the said extracts were also found to be impressive and noteworthy. The decreasing order of the antioxidative efficacies of the extracts was found to be same as that of TPC and TFC of the extracts.

Conclusion: It is pertinent to comment that the methanol extract of the fronds of both the plants may be treated as a potential source of natural antioxidants.

Keywords: Pteridophytes, Phytochemical, Phenolic, Flavonoid, Antioxidant.

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INTRODUCTION

Prolonged persistence of free radicals followed by increasing oxidative stress in the human body triggers the advancement of many diseases including atherosclerosis, hypertension, diabetes mellitus, cancer, multiple sclerosis, liver diseases, kidney diseases, rheumatoid arthritis, aging, neurodegenerative, and cardiovascular disorders [1,2]. Antioxidant activity of plants is supposed to be due to their polyphenolic compounds especially the flavonoids [3], which are known to be potential free radical scavengers, hydrolytic as well as oxidative enzyme inhibitors and active against inflammations [4]. Many published research articles suggest that the bioactivities of these polyphenols correspond to their antioxidative nature [5-7].

Many traditionally used medicinal plants have been validated to possess natural antioxidants [8-12]. Pteridophytes are nonflowering vascular cryptogams including a large group of ignored medicinal plants which are economically significant for their medicinal as well as nutraceutical values. Out of around 12,000 identified pteridophytes from the planet, 1000 species, 70 families, and 191 genera have been documented to exist in India [13,14] and 159 species from Southern Assam, i.e., Barak Valley region of Assam, India [15]. *Thelypteridaceae* is a family of about 900 pteridophyte species belonging to the order *Polypodiales* [16] of class *Polypodiopsida* [17]. Twenty-two species of pteridophytes belonging to *Thelypteridaceae* family have been reported from this region so far [15].

Cyclosorus interruptus is pantropically distributed and is commonly found in bogs, forested wetlands, uncultivated riverine land areas, and oozing woodland areas. It is also found on floating mats of vegetation in

swamps or deep open marshes. Ethnopharmacological reports reflect the traditional use of *C. interruptus* as a remedial source to cure boils, sores, cough, liver diseases, gonorrhoea, and malaria [15,18-20]. The boiled fronds of the plant are also used to treat gastric ulcer [18,19], which is apart from being caused by the bacterium, *Helicobacter pylori* [21] is also assumed to be triggered and aggravated by oxidative stress [22].

Pronephrium nudatum (Roxb. ex Griff.) Holttum of *Thelypteridaceae* family is a bulky land-dwelling pteridophyte, forming extensive gatherings in the moist forest as vegetation, commonly near rivers. It also grows in damp places in the plane land. The fronds of *P. nudatum* are used to treat pyorrhoea and other disorders of teeth gums. A local ethnobotanical report [23] suggests the use of a cold decoction of pinnae of the plant as a mouthwash for 2-3 times/day during acute pyorrhoea [15,24,25]. Although the two plants have considerable ethnomedicinal importance, still negligible attempts have been taken to validate their medicinal potentialities. In this article, the *in vitro* antioxidant as well as free radical scavenging activities of *C. interruptus* and *P. nudatum* frond extracts in various *in vitro* models have been reported.

MATERIALS AND METHODS

Collection of plant material

Fresh fronds of *C. interruptus* (voucher specimen no. 17602) and *P. nudatum* (voucher specimen no. 46591) were collected from their natural habitats at Dorgakona village of Cachar district and Kamalpur village of Karimganj district of Southern Assam, India respectively. The herbarium sheets of both the collected specimen have been submitted to the Assam University Herbarium and identified from Botanical Survey of India, Shillong.

Preparation of extracts

Plant material collection was followed by air drying at room temperature for some days. The air-dried fronds of the ferns were powdered using a grinder. From this powder, different crude frond extracts were sequentially prepared by Soxhlet method with various solvents of differential polarity, namely, hexane, ethyl acetate, acetone, and methanol to furnish different frond extracts of less polarity, medium polarity, and high polarity.

Preliminary phytochemical analysis

Preliminary screening of phytochemicals of the frond extracts was performed following standard phytochemical methods [26].

Estimation of total phenolic content (TPC)

Total phenolic contents of the frond extracts were estimated by following the Folin-Ciocalteu (FC) method. To 0.1 mL of the sample, 0.2 mL 10% v/v FC reagent was added and was constantly shaken for 5 min, followed by addition of 0.8 mL of sodium carbonate (Na_2CO_3). This mixture was then subjected to room temperature-incubation for 2 h followed by measurement of absorbance at 765 nm of wavelength. The standard curve was prepared using different concentrations of gallic acid in methanol (10–100 $\mu\text{g}/\text{ml}$) [27]. The concentrations of phenolic compounds were calculated by employing the following equation, obtained from the standard gallic acid graph:

$$\text{Absorbance} = 0.0608 \text{ gallic acid } (\mu\text{g}) - 0.0081 \quad (R^2 = 0.9682)$$

Estimation of total flavonoid content (TFC)

TFC of the frond extracts were evaluated following Dowd's method [28]. 1 ml of 2% aluminum trichloride (AlCl_3) in methanol was added to equal volume of the methanol-dissolved frond extracts (2000 μg). Absorbances of the reaction mixtures at 415 nm were noted after 10 min. 1 mL frond extract mixed with 1 ml methanol was used as a blank solution. Calculations of flavonoid concentrations in different extracts were performed applying the following equation, which was achieved from the standard quercetin calibration curve:

$$\text{Absorbance} = 0.0355 \text{ quercetin } (\mu\text{g}) - 0.2396 \quad (R^2: 0.9886)$$

2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

Hydrogen bond donating efficiency of the frond extracts was investigated using stable DPPH radicals [29]. Different concentrations of 0.1 ml of the frond extracts in methanol were mixed with 0.004% methanolic solution of DPPH (3 ml). After ½ h, the absorbances (517 nm) of the solutions were determined. The absorbance of the DPPH solution is inversely proportional to the DPPH radical scavenging activity. The standard antioxidant quercetin (50 $\mu\text{g}/\text{ml}$) was used as a positive control. DPPH radical scavenging activity (%) was determined according to the formula as under:

$$\text{DPPH radical scavenging activity } (\%) = [(A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}}] \times 100$$

Where A_{control} corresponds to the absorbance of the control and A_{sample} denotes the absorbance of the sample solution.

Reducing power assay

The reducing power of the prepared plant extracts was evaluated following Oyaizu's method [30]. 2.5 ml of each extract of different concentrations (0.2–1.0 mg/ml) in methanol was added to 2.5 ml of sodium phosphate buffer (200 mM; pH 6.6), and 2.5 ml of potassium ferricyanide (1%). The reaction mixture was then kept for incubation at 50°C for 20 min followed by addition of 2.5 ml of 10% trichloroacetic acid to the mixture and centrifugation at 200 g for 10 min. 2.5 ml of the above layer was added to the same volume of deionized water and 0.5 ml of 0.1% ferric chloride. The spectrophotometric absorbance of the mixture was recorded at 700 nm. The standard antioxidant ascorbic acid was used as a positive control for this experiment.

Hydroxyl radical scavenging activity

Hydroxyl radical scavenging effect of the plant extracts was determined following a standard protocol [31]. 1.5 mM FeSO_4 (0.5 mL)

was mixed with 6 mM H_2O_2 (0.35 mL), 20 mM sodium salicylate (0.15 mL), and different concentrations (0.2–1.0 mg/mL) of the sample (1 ml each). Then, the mixture was kept for incubation at 37°C for 1 h. The absorbance of the formed hydroxylated salicylate complex was determined spectrophotometrically at 562 nm. Ascorbic acid was tested as a positive control. The calculation of antioxidant activity was performed as per the following formula:

$$\text{Scavenging effect } (\%) = 1 - (A_{\text{sample}} - A_{\text{blank}}) / A_{\text{control}} \times 100$$

where A_{sample} denotes the absorbance of the test sample or positive control, A_{control} corresponds to the absorbance of the solvent control, and A_{blank} is the absorbance of reagent blank without sodium salicylate.

Superoxide radical scavenging activity

Superoxide radical scavenging assay was performed by the pyrogallol acid method [32] with slight modification. To 2.5 mL of 0.1 M PBS buffer (pH 8.2), 4 mL of the sample solution and 2.5 mL of 6.0 mM pyrogallol acid were added. Then, 0.5 mL of hydrochloric acid was added to the reaction mixture to cease the reaction. The solution was then kept for incubation at room temperature and followed by taking the absorbance reading at 299 nm. Ascorbic acid was used as a standard superoxide scavenger. All experimentations were carried out in triplicate. The superoxide radical scavenging activity was determined using the following equation:

$$\text{Scavenging activity } (\%) = A_0 - (A_s - A_c) / A_0 \times 100$$

Where A_s means the absorbance of the solution containing pyrogallol acid and test extract; A_0 is that of pyrogallol acid but with no test extract; and A_c denotes the absorbance of the solution containing test extract but no pyrogallol acid.

2,2'-azino-bis (3-ethylbenzothiazoline - 6 - sulfonic acid) (ABTS⁺) cation scavenging activity

The spectrophotometric determination of ABTS⁺ radical scavenging potential was performed using standard protocol [33]. 7 mM ABTS was mixed with 2.45 mM potassium persulfate and was kept for 12 h in darkness at room temperature. This solution was then diluted with phosphate buffer (0.1 M, pH 7.4) to achieve an optical density of 0.700 ± 0.025 at 734 nm. Then, ABTS⁺ solution (1 ml) was mixed with different concentrations (8–40 $\mu\text{g}/\text{ml}$) of extract solution (3 ml) in methanol. After ½ h, the inhibition (%) of ABTS⁺ cation by the plant extract was calculated for each concentration. Methanol was used as the blank solution. The ABTS⁺ radical scavenging activities of the extracts were estimated by employing the equation as under:

$$\text{ABTS}^+ \text{ scavenging effect } (\%) = [(A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}}] \times 100$$

Where A_{control} denotes the initial concentration of ABTS⁺ and A_{sample} means the absorbance of leftover ABTS⁺ after reaction with the plant extracts.

Chelating effects on ferrous ions

The chelating activity of the extracts was evaluated according to the established method [34]. Different concentrations (0.05–0.25 mg/ml) of the extracts in methanol (2 ml each) were added to 0.05 ml of 2 mM FeCl_2 , 0.02 ml of 5 mM ferrozine was then added to the reaction mixture to start the reaction. After vigorous shaking, the mixture was incubated at room temperature for 10 min, and the absorbance of the solution was taken spectrophotometrically at 562 nm. The calculation of percentage inhibition of ferrozine- Fe^{2+} complex formation was done using the following formula:

$$\text{Metal chelating effect } (\%) = [(A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}}] \times 100$$

Where A_{control} denotes the absorbance of control that contains FeCl_2 and ferrozine and A_{sample} corresponds to the absorbance of the test material. Ethylenediaminetetraacetic acid (EDTA) was used as a standard chelating ligand.

RESULTS

Preliminary phytochemical analysis

The preliminary phytochemical screening results showed the presence of phenols and flavonoids in all the prepared extracts of both the plants. Alkaloids were found to be present in ethyl acetate and methanol extract of *C. interruptus*, whereas it was found only in the ethyl acetate extract of *P. nudatum*. Hexane, ethyl acetate and acetone extracts of both the plants implicated the presence of saponins. Tannins' presence was observed in the hexane and acetone extract of *P. nudatum*, whereas, it was present in the methanol extract of *C. interruptus* only. Acetone extract of *C. interruptus* showed the presence of reducing sugar, whereas it was found in the hexane extract of *P. nudatum* only.

Estimation of TPC and TFC

The methanolic frond extract of *C. interruptus* was found to possess the highest phenolic (299.0±1.1 µg gallic acid equivalents [GAEs]/mg of extract) as well as flavonoid content (254.1±4.1 µg quercetin equivalents [QEs]/mg of extract) of all the extracts (Table 1 and Figs. 1-2). However, the methanolic extract of *P. nudatum* was found to possess the TPC and TFC

values of 223.0±6.80 µg GAEs/mg of extract and 188.4±5.74 µg QEs/mg of extract, respectively. These values were followed by the acetone, ethyl acetate, and hexane extracts of both the plants. The lowest TPC and TFC were manifested by the hexane extract of *P. nudatum* fronds (61.4±1.84 µg GAEs/mg of extract and 29.2±1.30 µg QEs/mg of extract, respectively).

DPPH radical scavenging activity

The results of the DPPH free radical scavenging ability of all the extracts are shown in Table 2 and Fig. 3. The positive control, i.e., quercetin was found to possess the highest scavenging activity in all concentrations with a half-maximal inhibitory concentration (IC₅₀) value of 0.510±0.012 mg/ml. As compared to that the methanolic frond extract of *C. interruptus* was found to possess the highest DPPH radical scavenging activities of all the extracts with an IC₅₀ value of 0.760±0.003 mg/ml followed by the other extracts of decreasing solvent polarity. However, the methanol extract of *P. nudatum* showed an IC₅₀ value of 0.980±0.030 mg/ml.

Reducing power assay

Table 3 and Fig. 4 illustrate the ferric ion reducing power of all the extracts/standard. The reducing power of all the prepared extracts

Table 1: TPC and TFC of the prepared frond extracts

Name of the plants	Extracts	TPC (µg eq. of gallic acids/mg of extract) (Mean±SD)	TFC (µg eq. of quercetin/mg of extract) (Mean±SD)
<i>C. interruptus</i>	Hexane	79.2±2.3	48.2±3.1
	Ethyl acetate	148.7±1.1	78.1±2.2
	Acetone	196.1±2.7	152.9±3.1
	Methanol	299.0±1.1	254.1±4.1
<i>P. nudatum</i>	Hexane	61.4±1.3	29.2±1.3
	Ethyl acetate	121.6±1.7	34.5±1.1
	Acetone	167.4±3.5	106.2±0.7
	Methanol	223.0±2.8	188.4±4.0

TFC: Total Flavonoid Content, *C. interruptus*=*Cyclosorus interruptus*, *P. nudatum*: *Pronephrium nudatum*, TPC=Total Phenolic Content, SD=Standard deviation

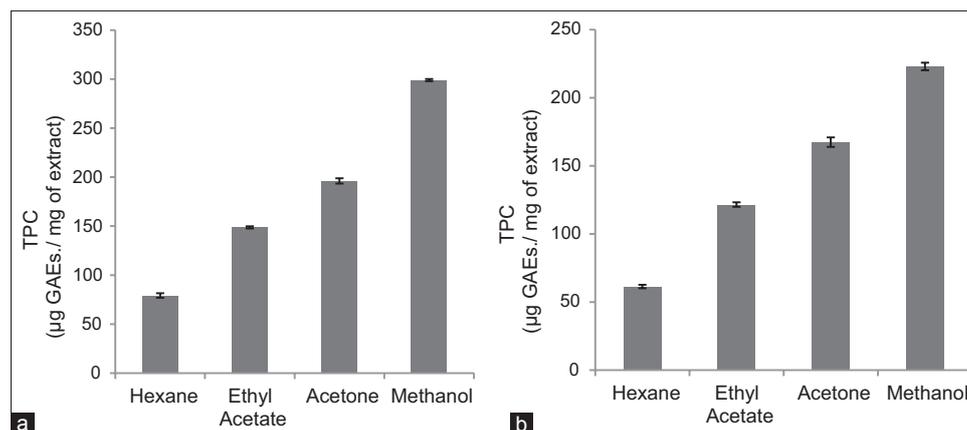
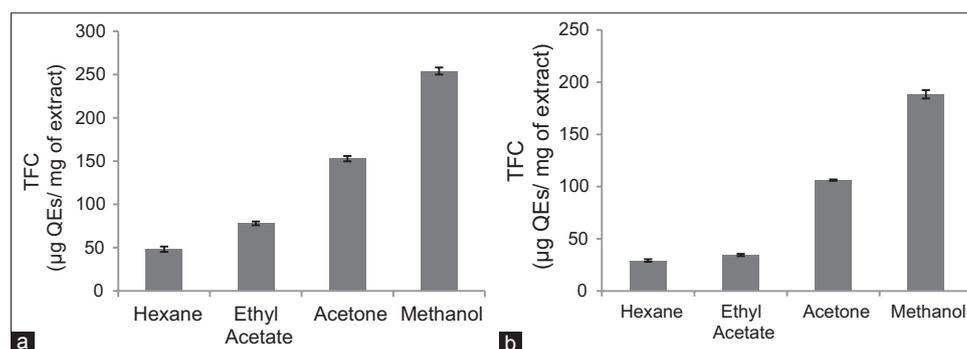
Fig. 1: (a and b) Total phenolic content of different frond extracts of (a) *Cyclosorus interruptus* and (b) *Pronephrium nudatum*Fig. 2: (a and b) Total flavonoid content of different frond extracts of (a) *Cyclosorus interruptus* and (b) *Pronephrium nudatum*

Table 2: DPPH free radical scavenging activities of the plant extracts

Extracts/standard	IC ₅₀ values of <i>C. interruptus</i> (mg/ml) (Mean±SD)	IC ₅₀ values of <i>P. nudatum</i> (mg/ml) (Mean±SD)
Hexane	1.460±0.010	1.910±0.007
Ethyl acetate	1.110±0.005	1.840±0.011
Acetone	0.860±0.021	1.360±0.004
Methanol	0.760±0.003	0.980±0.030
Quercetin	0.510±0.012	0.510±0.012

DPPH=2,2-diphenyl-1-picrylhydrazyl, SD=Standard deviation, *C. interruptus*=*Cyclosorus interruptus*, *P. nudatum*: *Pronephrium nudatum*, IC₅₀: Half-maximal inhibitory concentration

Table 3: Reducing power assay of the frond extract

Name of the plants	Extracts/standard	Absorbance at 700 nm (mg/ml) (Mean±SD)				
		0.2	0.4	0.6	0.8	1.0
<i>C. interruptus</i>	Hexane	0.098±0.007	0.142±0.009	0.178±0.010	0.252±0.004	0.398±0.005
	Ethyl acetate	0.189±0.006	0.244±0.008	0.312±0.009	0.395±0.022	0.482±0.008
	Acetone	0.636±0.008	0.686±0.002	0.779±0.009	0.825±0.004	0.888±0.003
	Methanol	0.701±0.012	0.771±0.009	0.790±0.008	0.799±0.002	0.921±0.007
<i>P. nudatum</i>	Hexane	0.042±0.001	0.137±0.004	0.152±0.002	0.199±0.011	0.234±0.009
	Ethyl acetate	0.089±0.002	0.152±0.005	0.203±0.010	0.256±0.012	0.316±0.003
	Acetone	0.182±0.007	0.287±0.004	0.355±0.013	0.381±0.006	0.495±0.021
	Methanol	0.333±0.007	0.392±0.003	0.477±0.019	0.599±0.022	0.678±0.003
	Ascorbic acid	0.215±0.004	0.298±0.008	0.387±0.002	0.452±0.015	0.561±0.003

C. interruptus=*Cyclosorus interruptus*, *P. nudatum*: *Pronephrium nudatum*, SD=Standard deviation

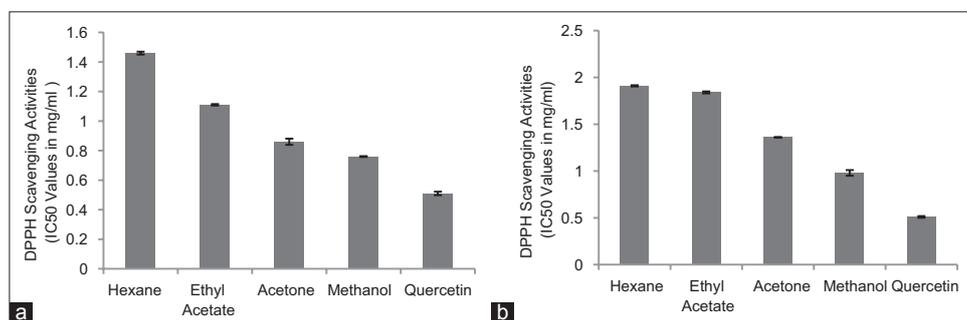


Fig. 3: (a and b) 2,2-diphenyl-1-picrylhydrazyl radical scavenging activities of different extracts of (a) *Cyclosorus interruptus* and (b) *Pronephrium nudatum*

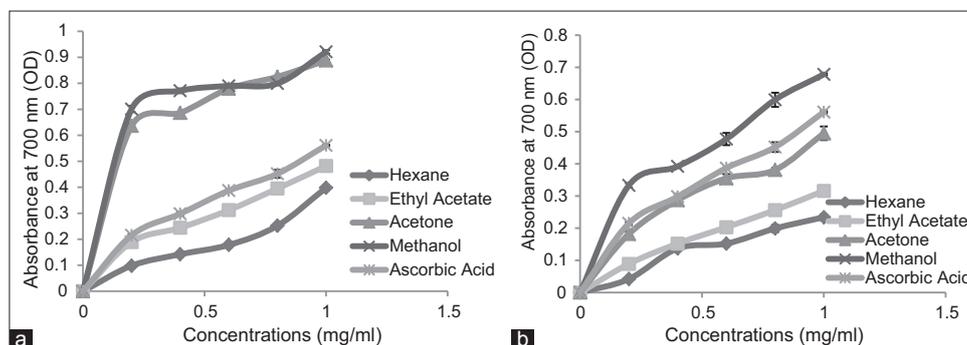


Fig. 4: Reducing power of the different extracts of (a) *Cyclosorus interruptus* and (b) *Pronephrium nudatum*

increased with increase in concentration. At 1.0 mg/ml concentration, the reducing potential of acetone (0.888±0.003) and methanol extract (0.921±0.007) of *C. interruptus* was found to be higher than the ascorbic acid control (0.561±0.003). However, the methanol extract of *P. nudatum* fronds was also found to possess higher reducing power with an absorbance value of 0.678±0.003 than the standard compound.

Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity of all the extracts is shown in Table 4 and Fig. 5. The ascorbic acid standard was found to exhibit

the highest hydroxyl radical scavenging activity with an IC₅₀ value of 0.290±0.007 mg/ml followed by the methanol extract of *C. interruptus* having an IC₅₀ value of 0.330±0.005 mg/ml and methanol extract of *P. nudatum* having an IC₅₀ value of 0.490±0.005 mg/ml. It was found to be the lowest in the hexane extract of *P. nudatum*.

Superoxide radical scavenging activity

The superoxide radical scavenging activity of the plant extracts/standard is shown in Table 5 and Fig. 6. The methanolic frond extract of *C. interruptus* revealed the highest superoxide radical scavenging

activity with an IC₅₀ value of 0.260±0.006 mg/ml which was slightly less than that of the ascorbic acid standard having an IC₅₀ value of 0.230±0.006 mg/ml. The methanolic frond extract of *P. nudatum* was found to possess good activity against superoxide radicals with an IC₅₀ value of 0.690±0.009 mg/ml. However, it was much less than that of *C. interruptus*.

ABTS⁺ cation scavenging activity

Table 6 and Fig. 7 illustrate the ABTS⁺ cation scavenging activity of the plant extracts. As a result of this assay, the highest activity was observed in the methanolic extract of *C. interruptus* (67.42±2.09%) at the concentration of 40 µg/ml followed by the other extracts of decreasing solvent polarity. The percentage scavenging effect of the methanolic frond extract of *P. nudatum* was found to be 51.18±2.16% at the same concentration of the extract.

Chelating effect on ferrous ions

The percentage metal-chelating effect (Table 7 and Fig. 8) of the standard chelating ligand, i.e., EDTA was found to be the highest (97.24±5.6%) at the concentration of 0.250 mg/ml followed by the methanol extract of *C. interruptus* fronds (94.27±2.7%) which was much higher than that of methanolic frond extract of *P. nudatum* (69.14±4.1%). The metal-chelating effect of the extracts was also found to be decreasing concerning the decreasing solvent polarity.

Statistical analysis

All the data have been reported as the mean±standard deviation of triplicate measurements. Calculation of mean, SD, and preparation of graphs was made in Microsoft Excel-2013. ANOVA was performed by using SPSS software for Windows, Version 16.0. p<0.05 was regarded as significant.

Table 4: IC₅₀ values of the plant extracts for eliminating hydroxyl radicals

Extracts/standard	IC ₅₀ values of <i>C. interruptus</i> (mg/ml) (Mean±SD)	IC ₅₀ values of <i>P. nudatum</i> (mg/ml) (Mean±SD)
Hexane	0.980±0.018	1.410±0.021
Ethyl acetate	0.470±0.004	0.980±0.005
Acetone	0.380±0.009	0.740±0.007
Methanol	0.330±0.005	0.490±0.001
Ascorbic acid	0.290±0.007	0.290±0.007

C. interruptus=*Cyclosorus interruptus*, *P. nudatum*: *Pronephrium nudatum*, SD=Standard deviation, IC₅₀: Half-maximal inhibitory concentration

Table 5: IC₅₀ values of the plant extracts for eliminating superoxide radicals

Extracts/standard	IC ₅₀ values of <i>C. interruptus</i> (mg/ml) (Mean±SD)	IC ₅₀ values of <i>P. nudatum</i> (mg/ml) (Mean±SD)
Hexane	0.860±0.006	1.500±0.003
Ethyl acetate	0.590±0.011	1.110±0.001
Acetone	0.350±0.004	0.920±0.002
Methanol	0.260±0.005	0.690±0.006
Ascorbic acid	0.230±0.006	0.230±0.006

C. interruptus=*Cyclosorus interruptus*, *P. nudatum*: *Pronephrium nudatum*, SD=Standard deviation, IC₅₀: Half-maximal inhibitory concentration

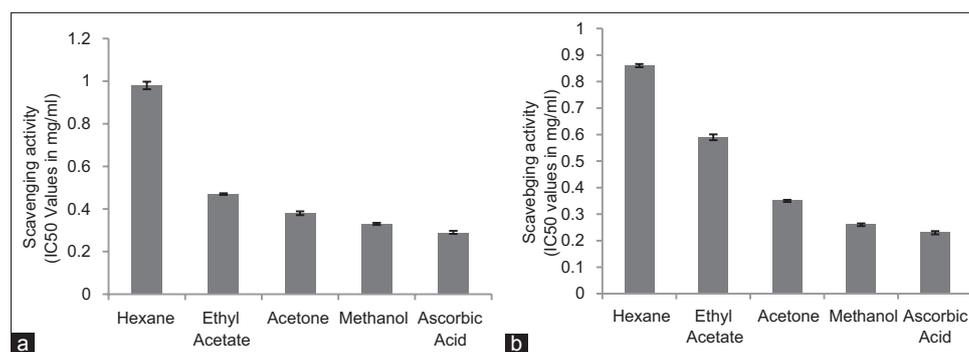


Fig. 5: (a and b) Hydroxyl radical scavenging activities of different extracts of (a) *Cyclosorus interruptus* and (b) *Pronephrium nudatum*

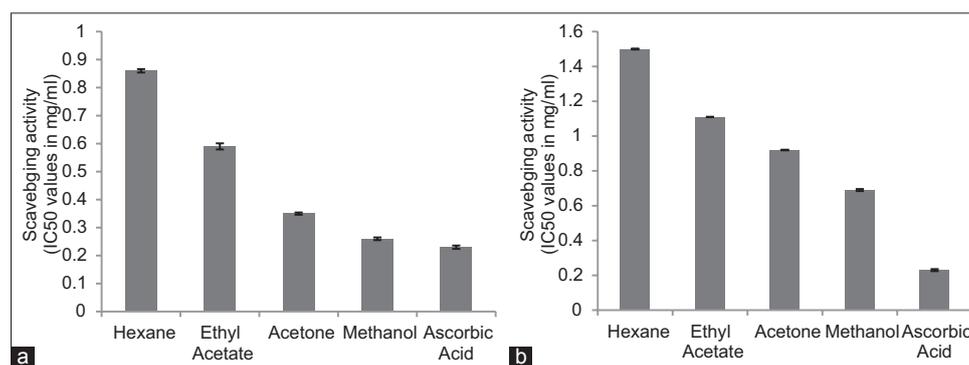


Fig. 6: (a and b) Superoxide radical scavenging activities of different extracts of (a) *Cyclosorus interruptus* and (b) *Pronephrium nudatum*

Table 6: Scavenging (%) effect of the extracts on the stable ABTS⁺ radical

Name of the plants	Extracts/standard	% Scavenging effect (Mean±SD) (µg/ml)		
		8	20	40
<i>C. interruptus</i>	Hexane	19.58±2.26	28.63±1.52	35.69±1.18
	Ethyl acetate	31.89±0.079	41.95±2.16	52.63±1.32
	Acetone	36.91±0.08	49.38±1.56	57.26±2.76
	Methanol	43.21±1.14	56.91±2.12	67.42±2.09
<i>P. nudatum</i>	Hexane	4.81±0.076	10.09±1.01	22.21±1.77
	Ethyl acetate	12.67±0.12	26.09±1.19	31.87±2.10
	Acetone	19.44±1.01	29.43±2.01	37.86±1.22
	Methanol	26.51±1.09	38.77±1.91	51.18±2.16

C. interruptus=*Cyclosorus interruptus*, *P. nudatum*: *Pronephrium nudatum*, SD=Standard deviation, IC₅₀: Half-maximal inhibitory concentration

Table 7: Metal chelating effect of the extracts on Fe²⁺ ions

Name of the plants	Extracts/standard	% chelating effect (Mean±SD)		
		0.050 mg/ml	0.150 mg/ml	0.250 mg/ml
<i>C. interruptus</i>	Hexane	19.26±2.6	26.84±1.3	33.48±2.1
	Ethyl acetate	34.08±1.9	46.07±4.6	52.23±3.1
	Acetone	71.17±5.2	79.39±2.9	84.27±2.0
	Methanol	81.42±2.1	90.00±1.4	94.27±2.7
<i>P. nudatum</i>	Hexane	06.41±0.7	16.65±2.0	19.89±1.4
	Ethyl acetate	11.91±0.9	29.88±1.2	39.67±2.2
	Acetone	39.11±3.7	47.10±4.2	61.77±2.9
	Methanol	51.99±1.3	64.43±3.9	69.14±4.1
	EDTA	89.29±3.6	92.83±2.40	97.24±5.6

C. interruptus=*Cyclosorus interruptus*, *P. nudatum*: *Pronephrium nudatum*, SD=Standard deviation

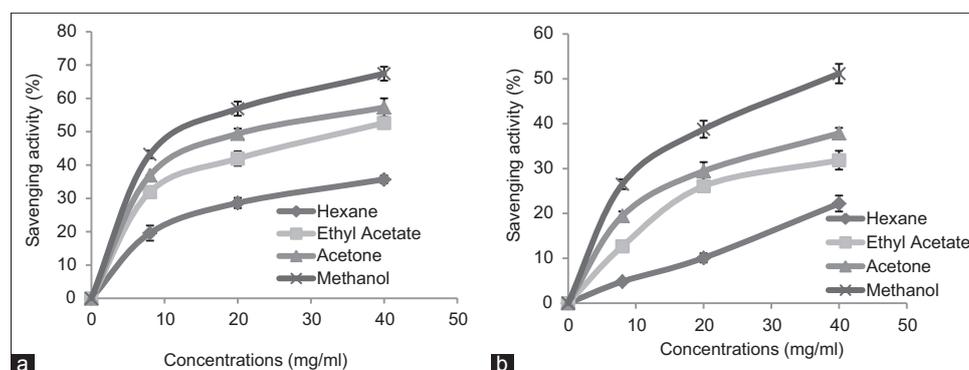


Fig. 7: (a and b) 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) cation scavenging activity (%) of various frond extracts of (a) *Cyclosorus interruptus* and (b) *Pronephrium nudatum*

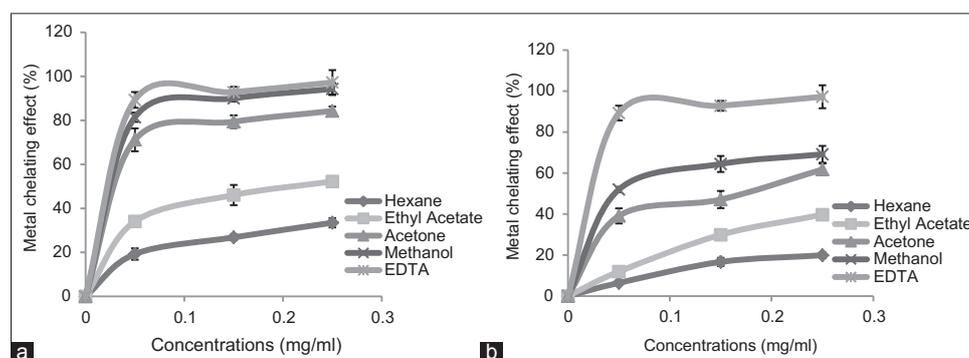


Fig. 8: (a and b) Metal chelating effect of different frond extracts of (a) *Cyclosorus interruptus* and (b) *Pronephrium nudatum*

DISCUSSION

A detailed study on the earlier published literature indicates the traditional medicinal uses of *C. interruptus* and *P. nudatum* (*Thelypteridaceae*) by different tribes of people for the

treatment of various oxidative stress associated diseases and disorders [15,18,19,22-24] and hence it can be inferred that the selected ferns should possess potent antioxidant activities. The validated antimicrobial potentials of pteridophytes also correspond to the traditional utilization of ferns as folk medicine against various

microbial infections [35-38]. The presence of phenolics, flavonoids, and the alkaloids is attributed to exhibit the antioxidant and hepatoprotective activity.

Since the preliminary phytochemical screening results suggest the presence of phenolic and flavonoid compounds in all the prepared frond extracts of both the selected plants; hence, the TPC and TFC were quantified for all the extracts of both plants. Literature suggests the important role of polyphenolic compounds in stabilizing lipid peroxidation as well as possessing diverse antioxidative properties [39,40]. The phenolic compounds are known to possess direct antioxidative action against the free radicals [8]. Polyphenolic compounds are known to inhibit the progress of mutagenesis and carcinogenesis in humans [41]. As the TPC and TFC of the plant extracts are supposed to be directly proportional to the antioxidative potentials of the extracts [3,4]; hence, all the extracts showed more or less antioxidant activities with respect to their TPC and TFC (Table 1 and Figs. 1-2).

The DPPH radical scavenging activity assay is one of the most convenient methods to investigate the ability of the phenolic components in extracts to perform the activity as hydrogen atom or electron donors [29]. Based on the hydrogen donating potential of the stable DPPH radical, the free radical scavenging capability of the plant extracts was tested [29]. As the methanolic frond extracts of both the plants revealed the highest DPPH scavenging activities (Table 2 and Fig. 3) of all other low polar solvent extracts; hence, it can be inferred that the most potent DPPH radical scavenging compounds in both the two plants are of high polarity. This activity has been found to be relevant with the TPC and TFC of both the plant extracts. Similar findings have been reported in some published research articles [42-44].

The reducing potential of the plant extracts was analyzed by reduction of Fe^{3+} ions to Fe^{2+} form in the presence of reducing antioxidants in the plant extracts. The Fe^{2+} ions were then explored spectrophotometrically by measuring the optical density of developed Perle's Prussian Blue color at 700 nm of wavelength [30] (Table 3 and Fig. 4). The standard antioxidant, i.e., ascorbic acid was used as a positive control for this experiment. The methanolic frond extract of *C. interruptus* was found to possess the maximum reducing potential in comparison to the other extracts as well as ascorbic acid. The reducing activities of all the extracts have also been found to increase with the increase in the extract concentrations. Similar results have been described in some published literature [45,46].

Hydroxyl is a very reactive free radical which can react with almost all biological macromolecules and is also supposed to be a highly potent contributor of oxidative stress-mediated tissue injuries [39,47,48]. Hydroxyl radical scavenging assay was performed as per standard protocol [31]. As shown in Table 4 and Fig. 5, the methanolic extract of *C. interruptus* fronds possesses the highest scavenging activity against the hydroxyl free radical ($IC_{50}=0.330\pm 0.005$ mg/ml) which was slightly less than the ascorbic acid standard (0.290 \pm 0.007 mg/ml). In this case, also the methanol extract of *P. nudatum* fronds ($IC_{50}=0.490\pm 0.005$ mg/ml) was found to possess less scavenging activity than that of *C. interruptus*. However, the scavenging activity of both the plants decreased along with the polarity of other solvent extracts.

Superoxide is a highly harmful free radical and can be produced by various photochemical reactions. These radicals may undergo decomposition reactions to generate singlet oxygen and hydroxyl radicals as a result of which oxidative stress associated cellular damage, lipid peroxidation, and diseases such as arthritis and Alzheimer's disease may get triggered [49]. The pyrogallol acid method was used to create superoxide radicals [32]. The superoxide scavenging activity of ascorbic acid standard was found to be the highest ($IC_{50}=0.230\pm 0.006$ mg/ml) followed by the methanolic extract of *C. interruptus* ($IC_{50}=0.260\pm 0.006$ mg/ml) (Table 5 and Fig. 6). The methanolic frond extract of *P. nudatum* possessed a good but less scavenging activity than *C. interruptus*. ($IC_{50}=0.690\pm 0.009$ mg/ml).

The method to study the ABTS⁺ radical scavenging activities of the plant extracts by decolorization assay [33] is a consistent and prompt method to determine the total antioxidative potential for hydrophilic as well as lipophilic antioxidants/systems [50]. The spectrophotometric analysis of ABTS⁺ radical (Table 6 and Fig. 7) reveals that at the concentration of 40 μ g/ml the methanolic extract of *C. interruptus* possesses the most potent ABTS⁺ radical scavenging activity (67.42 \pm 2.09%) of all other extracts. The result shown by that of *P. nudatum* at the same concentration was also noteworthy (51.18 \pm 2.16%).

The metal chelating effect (Table 7 and Fig. 8) was determined according to the method of Dinis *et al.* [34]. Lipid peroxidation can be triggered by iron by Fenton reaction, thereby boosting up peroxidation by the degradation of lipid hydroperoxides and formation of peroxy and alkoxy radicals that can continue lipid peroxidation reaction by abstracting hydrogen [1]. The percentage metal (Fe^{2+}) chelating effect was also found to be the highest in case of methanolic frond extract of *C. interruptus* (94.27 \pm 2.7%) as compared to the other extracts as well as that of *P. nudatum*. However, the highest activity of *C. interruptus* was a bit less than that of EDTA standard (97.24 \pm 5.6%) at the concentration of 0.250 mg/ml. Some published articles also describe similar findings [6,7,45,51].

CONCLUSION

It can be assumed that the methanol extract of the fronds of both the plants, i.e., *C. interruptus* and *P. nudatum* possess potent free radical scavenging as well as antioxidative capabilities in comparison to the standards and other extracts. However, the overall antioxidant activities of *C. interruptus* frond extracts were found to be much higher than that of *P. nudatum*.

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AUTHORS' CONTRIBUTIONS

Abhijit Mitra performed the experiments and prepared the research article. Prakash Roy Choudhury and Subrata Das helped in the calculation and statistical analysis of the data. Deepa Nath assisted in the collection of medicinal plants and their identification. Manabendra Dutta Choudhury and Anupam Das Talukdar directed the whole work and helped in the preparation of the manuscript.

CONFLICTS OF INTEREST

There are no conflicts of interest.

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