PHARMACOGNOSTICAL STUDIES AND EVALUATION OF ANTIOXIDATIVE PROPERTIES OF DRYNARIA QUERCIFOLIA (L.)

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ABSTRACT
Free radicals are responsible for many oxidative stress related diseases. Antioxidants are those substances capable of scavenging free radicals and in recent years, antioxidants of plant origin are gaining interest globally. Ferns are an interesting group of plants, many of which still remain unexplored in the area of phytochemical properties. Thus, the present study was carried out to quantify the phytochemicals and evaluate the in vitro antioxidant activity of fertile fronds of Drynaria quercifolia, a common fern growing in this region. Methanolic (MeOH), ethanolic (EtOH) and hot water (HW) extracts were used for determination of antioxidative activity. Results revealed the presence of phenol, flavonoid, vitamin C and E in the sample. MeOH extract showed higher antioxidative activity in comparison to the EtOH and HW extract in dose-dependent manner. However, DPPH was seen to be scavenged slightly better by HW extract. From the overall data it can be concluded that methanolic extract (MeOH) of Drynaria quercifolia could be one of the potential source of natural antioxidant for the treatment of free-radical and age-related diseases.

Keywords: Drynaria quercifolia, phytochemicals, antioxidants, phenol, flavonoid.

INTRODUCTION
Free radicals or ROS (reactive oxygen species) are highly unstable molecules or molecular fragments that contain one or more unpaired electrons in their outer orbit which contributes to its high reactivity, as the unpaired electrons can receive/donate electrons from/to the...
neighbouring substance. The production of free radical is regulated strictly but sometimes excessive free radicals are generated at lower antioxidant levels leading to oxidative stresses causing diabetes, arthritis, cardiovascular, cancer, neurologic and other disorders. Nevertheless, the advantageous roles of ROS at low or moderate concentrations have been reported. Antioxidants are the substances that counteract free radicals and their harmful activity. The synthetic antioxidants are sometimes associated with adverse effect which has encouraged many to exploit the biologicals as they are natural, cost effective and has lesser side effects.

Historically, medicinal plants which produce numerous secondary metabolites such as phenolic acids, flavonoid and tannins have provided a source of inspiration for novel drug compounds owing to its huge contributions for human health and well being. The role of secondary metabolites as potent ROS scavengers thereby protecting cells against various oxidative stresses are well established and reported by many authors.

As folk medicine, pteridophytes have been in use for over 2000 years and are well documented in ancient literature. Drynaria quercifolia (L.) J. Smith, (Polypodiaceae) an epiphytic fern commonly known as “Oak Leaf Fern”, is used in traditional medicine to treat various life threatening diseases. Extensive pharmacognostical studies have been carried out on the rhizome of Drynaria quercifolia by many authors. Although the fronds are used as poultice on swelling and as antibacterial, pectoral and expectorant agents, the detailed study with respect to their antioxidative activity is still obscure. Thus, the present study was carried out to investigate in-vitro antioxidative activities along with the quantification of various bioactive constituents.

MATERIALS AND METHODS
Chemicals
2,2-Diphenyl-1-picyrylhydrazyl (H), acetic acid (M), chloroform (M), H₂SO₄ (M), methanol (M), aluminium chloride, NaNO₂ (M), catechin, BSA, L-ascorbate acid, ferulic acid (H), acetone (M), NaOH (M), ethanol, Nelson’s arseno molybdate, 2,2’– Bipyridyl (H), hexane (M), 2,4-dintrophyldrazine (H), thiourea (H), Folin-Ciocalteau reagent (M), Na₂CO₃ (H), disodium hydrogen phosphate (H), sodium dihydrogen phosphate di-hydrate (M), NaOH, trichloroacetic acid (TCA), gallic acid monohydrate (H), sodium nitroprusside (H), sulphanilic acid (M), glacial acetic acid, N-(1- naphyl) ethylenediamine dihydrochloride (H), nicotinamide adenine dinucleotide (NADH) (H), nitroblue tetrazolium chloride (NBT)(H), phenazine
methosulphate (PMS)(H), mercury (II) chloride (M), potassium iodide. All chemicals used including solvents were of analytical grade obtained from Merck (M) and Himedia (H) India Ltd, Mumbai.

**Plant collection and extract preparation**

The fertile fronds of *Drynaria quercifolia* (L.) J. Smith was collected from the campus of University of North Bengal, identified and deposited in the North Bengal University Herbarium, Department of Botany, University of North Bengal, India.

The fine powder obtained from clean, air and shade dried plant material was extracted using ethanol (EtOH), methanol (MeOH) and hot water (HW) in 1:10 ratio, filtered and concentrated using rotary evaporator at 40°C and were lyophilized for complete solvent removal.\(^{[19,20]}\)

**Qualitative analysis of phytochemicals**

Various standard methods were used for the phytochemical detection of crude powder.\(^{[21,22]}\)

**Quantitative estimation**

**Total flavonoids estimation**

Total flavonoids were quantified using Aluminium Chloride colorimetric method from the standard graph of (+) catechin.\(^{[23]}\) Briefly, 500µl aliquot of solution was mixed with 4ml of distilled water and 300µl of NaNO₂ (5%). After incubation for 5 min at room temperature 300µl of 10% AlCl₃.6H₂O was added. At 6th min 2ml of NaOH, followed by 2.4ml of distilled water was added. The absorbance of the mixture was read at 510 nm.

**Total phenol determination**

Total phenolic content estimation was done using the method given by Mahadevan & Sridhar(1982).\(^{[24]}\) In brief, 1ml of extract, 1ml of 1N Folin ciocalteau’s phenol reagent and 2ml of 20% Na₂CO₃ solution was mixed thoroughly and boiled in water bath for 1min. The reaction mixture was cooled under running tap water and then diluted with distilled water to make the final volume up to 25ml. The absorbance was recorded at 650nm in a colorimeter and evaluated from ferulic acid standard graph.

**Quantification of Ascorbate** (Vitamin C)

The method described by Mukherjee *et al.*, 1983\(^{[25]}\) was used to estimate the vitamin C content in the sample. Plant sample (1g) was homogenised using 6% Trichloroacetic acid.
under chilled condition and filtered. The filtrate (4ml), 2ml (2% Dinitrophenylhydrazine) and 1 drop of 10% thiourea was mixed properly and kept in boiling water bath for 15mins. After cooling, 5ml of 80% (v/v) sulphuric acid (H2SO4) was added at 0°C and the absorbance was observed at 530nm against blank solution and quantified from the standard curve of ascorbic acid.

**α-Tocopherol (Vitamin E) content**

Vitamin E (α-Tocopherol) was estimated following the method of Jayaraman (1996). In brief, 1g of the sample was homogenized in 5ml of hexane, shaken vigorously and filtered. The filtrate (2ml) was mixed with 2ml of absolute ethanol followed by the addition 0.2ml of 2,2’- Bipyridyl solution (0.5% in ethanol) and 0.2ml of ferric chloride solution (0.2% in ethanol). The mixture was shaken properly and then incubated in dark for 15mins. After the development of red colour 4ml of distilled water was added and mixed well. The red coloured aqueous layer was collected and absorbance was measured against a blank at 520nm and quantified using a standard curve of α-tocopherol.

**In vitro antioxidant assay**

**DPPH radical scavenging activity**

The radical scavenging activity of the plant extract and reference substance was determined using the stable DPPH radical. Briefly, different concentration of EtOH, MeOH and HW extract and reference sample (ascorbic acid) was mixed with 1ml of DPPH methanolic solution. Absorbance was recorded at 517nm after 30minutes. The control was prepared taking all the reagents except the extract. The percentage inhibition was calculated according to the formula

\[
\%\text{DPPH inhibition}= \frac{(A_0- A_1)}{A_0}\times 100
\]

Where, A0 was the absorbance of the control and A1 was the absorbance of the extract/standard

**Superoxide scavenging activity**

The superoxide anions generated in a non-enzymatic system through the reaction of PMS, NADH and oxygen was detected by the reduction of nitro blue tetrazolium (NBT). The reaction, in the reaction mixture containing 1ml sample, 1ml of NBT (phosphate buffer pH 7.4) and 1ml of NADH (936 µM in phosphate buffer pH 7.4) was accelerated by adding 0.2ml of PMS (120 µM) which was incubated for 5mins at 25°C. The absorbance was read at
560 nm against blank samples taking vitamin-C as a positive control. Percentage of superoxide anion radical scavenged was measured using the equation as follows:

\[
\% \text{superoxide scavenging activity} = \frac{(A_0 - A_1)}{A_0} \times 100
\]

Where, \(A_0\) was the absorbance of the control and \(A_1\) was the absorbance of the extract/standard.

**Nitric oxide scavenging activity assay**

Sodium nitroprusside in an aqueous solution at physiological pH produce nitric oxide which interacts with oxygen to generate nitrite ions that can be estimated by the Griess reagent. Nitric oxide scavengers compete with oxygen reducing the production of nitric oxide. Briefly, the reaction mixture containing 2 ml of sodium nitroprusside (10mM), 0.5 ml of phosphate buffer saline (pH: 7.4, 0.1M) and 0.5 ml of the extract was incubated at 25°C for 150 mins. The incubated solution (0.5 ml) was mixed with 1 ml of sulphanilic acid (0.33% in 20% glacial acetic acid) and allowed to stand for 5 min. After 5 min, 1 ml of naphthylethylenediamine dihydrochloride (NED) was added, mixed thoroughly and incubated for another 30 mins at 25°C. The absorbance was taken at 540 nm. L-ascorbic acid was taken as the reference standard. The nitric oxide scavenging percentage was calculated according to the formula:

\[
\% \text{nitric oxide scavenged} = \frac{(A_0 - A_1)}{A_0} \times 100
\]

Where, \(A_0\) was the absorbance of the control and \(A_1\) was the absorbance of the extract/standard.

**Ferric Reducing Antioxidant Power (FRAP) assay**

The ferric reducing power of ethanol, methanol and water extract was evaluated using an assay described by Oyaizu (1986) with slight modification. 1 ml (20-100 µg/ml) of extracts was mixed with 2.5 ml (0.2 M, pH 6.6) of phosphate buffer and 2.5 ml of 1% potassium ferricyanide. The mixture was incubated for 20 minutes at 50°C. The solution was allowed to cool at room temperature after which 2.5 ml of Tri-carboxylic acid (10%) was added and centrifuged at 3000 rpm for 10 minutes. Upper layer of the centrifuged solution was taken and mixed with equal volume of double distilled water. To this, 0.5 ml of 0.1% ferric chloride was added and incubated for 10 min. Absorbance was taken against appropriate blank solution at 700 nm after allowing the solution to stand for 10 minutes at room temperature. Vitamin C was taken as a positive control. Higher the absorbance, greater is the reducing power of the extract.
RESULTS & DISCUSSION
Qualitative analysis showed the presence of diverse compounds such as flavonoid, phenol, tannin, cardiac glycosides, saponin, terpene, steroid and cholesterol which are known to play an important role in human therapy\cite{32} and was in accordance to the findings of Anuja et al. (2014).\cite{33} However, anthraquinine and alkaloid was not detected in the sample (Table. 1).

Table 1: Qualitative analysis of phytochemicals

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Present/Absent</th>
</tr>
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<tbody>
<tr>
<td>Flavonoids</td>
<td>+</td>
</tr>
<tr>
<td>Phenols</td>
<td>+</td>
</tr>
<tr>
<td>Tannins</td>
<td>+</td>
</tr>
<tr>
<td>Cardiac glycosides</td>
<td>+</td>
</tr>
<tr>
<td>Saponins</td>
<td>+</td>
</tr>
<tr>
<td>Terpenes</td>
<td>+</td>
</tr>
<tr>
<td>Steroid</td>
<td>-</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>+</td>
</tr>
<tr>
<td>Anthraquinine</td>
<td>-</td>
</tr>
<tr>
<td>Alkaloid</td>
<td>-</td>
</tr>
</tbody>
</table>

+ = present, - = absent

Plant sample showed appreciable amount of total phenol, flavonoid, vitamin E and C. Flavonoid was found to be higher than other phytochemicals tested (Table. 2). Various environmental stresses (abiotic and biotic) lead to the production of phenolic compounds in the plants which acts as an antioxidant systems for protecting themselves against adverse conditions \cite{34-36} and these antioxidative properties of plants are beneficial to humans. Antioxidants such as vitamin C, vitamin E and polyphenols (flavonoid and phenolic acid) are known to play pivotal role in combating various oxidative damages.\cite{37}

Table 2: Quantitative analysis of phytochemicals in D.quercifolia

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Content (mg/g dwt)</th>
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</thead>
<tbody>
<tr>
<td>Flavonoids</td>
<td>63.85± 0.008</td>
</tr>
<tr>
<td>Total phenol</td>
<td>13.17± 0.040</td>
</tr>
<tr>
<td>Ascorbate/Vit C</td>
<td>1.06± 0.0003</td>
</tr>
<tr>
<td>α -Tocopherol/Vit E</td>
<td>2.04± 0.003</td>
</tr>
</tbody>
</table>

Dwt=dry weight tissue, ± = SD
Various *in vitro* antioxidative assay such as DPPH assay, Superoxide scavenging activity, Nitric oxide (NO) scavenging activity assay and Ferric Reducing Antioxidant Power (FRAP) assay were carried out to evaluate the antioxidative potential of *Drynaria* fertile fronds. DPPH is a stable free radical showing purple color at 517nm which gets reduced to yellow colored diphenyl picrylhydrazine in the presence of hydrogen donating antioxidant. All the extracts were seen to reduce the DPPH radical in a dose dependent manner. The HW extract showed higher DPPH inhibition activity compared to MeOH and EtOH. However, ascorbic acid which was used as positive control showed highest inhibition at the same concentration (Fig. 1). Like-wise, superoxide anion was seen to be scavenged effectively by all the extracts in the dose-dependent manner. MeOH extract exhibited the highest activity in comparison to the EtOH and HW extract (Fig. 2). Superoxide anion is one of the most reactive free radicals and causes various cellular damages. Superoxide anions are seen to be scavenged effectively even at a very low sample concentration which may be attributed to its flavonoid content and is in accordance to the findings of Robak, 1988.

![Figure 1: DPPH scavenging activity of three extracts of *Drynaria quercefolia*](image1.png)

![Figure 2: Superoxide scavenging activity of *Drynaria quercefolia*](image2.png)
Similarly, dose-dependent inhibition was observed in case of nitric oxide scavenging activity, MeOH extract exhibited slightly higher activity than EtOH and HW though it was lesser than the reference standard (Fig. 3). The ability of extracts to scavenge nitric oxide may be considered beneficial for health as it can evade the harmful effects of excessive NO generation. NO is considered to be detrimental as they are related to diverse pathological conditions.\(^{[40]}\) The reducing ability of extracts was measured using FRAP method. In the assay, ability of an antioxidant to donate electron to Fe (III) resulting in the reducing of Fe\(^{3+}\)/ferricyanide complex to Fe\(^{2+}\) complex could be monitored at 700 nm. The higher the FRAP value, the greater is the reducing power, thus the greater the antioxidant activity. The reducing ability was found to be appreciable, which increased gradually with the rise in concentration. MeOH extract showed highest reducing activity among the three extract, however it was lesser than the positive control (Fig. 4).

![Figure 3: Nitric oxide scavenging activity of HW, MeOH and EtOH extract of Drynaria quercifolia](image)

![Figure 4: FRAP assay of the extract (Ascorbic acid MeOH, HW and EtOH extract of Drynaria quercifolia)](image)
Overall, the antioxidative property may be attributed to the hydroxyl groups of phenolic compounds that are prone to donate a hydrogen atom or an electron to a free radical, thereby scavenging them. Flavonoids may also aid in the scavenging activity as they are known to possess the best electron donating property.\cite{41-43} Owing to its better antioxidative capabilities, the DQ extract particularly MeOH extract could be considered as one of the possible source of natural antioxidant for treating various oxidative stress related diseases.

CONCLUSION
From the present study it can be concluded that the presence of various phytochemicals, mainly the flavonoids and phenols may be responsible for the free radical scavenging activity of the fertile fronds of *Drynaria quercifolia* extract making them one of the potential source of natural antioxidant for the treatment of free-radical and age-related diseases. However, further investigations are essential to evaluate their bio-efficacies, active constituents, and molecular and biological mechanisms *in vivo* to characterize them as biological antioxidants.

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REFERENCES


