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Total phenol, total flavonoid content and antioxidant activity of methanol extract of *Lygodium palmatum* (Bernh.) Sw. leaves.

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ABSTRACT

Lygodium palmatum (Bernh.) Sw. leaves are deliberated as worthy traditional medicine. To give a scientific basis for traditional usage of this medicinal plant, the leaf extract were appreciated for its antioxidant activity. In this study, *in vitro* antioxidant activities of the leaf extract of *L. palmatum* were determined by total antioxidant, DPPH, Reducing power capacity, total phenolic content, total flavonoid content. The extract showed a dose dependent radical scavenging effect in DPPH assay. IC_{50} for free radicals achieved by the extract is 46.94 µg/ml. The extract showed significant reducing power activity as compared to ascorbic acid and proportionally increased with the increasing concentration of the extract. Increase in absorbance of the reaction mixture indicates the increase in the reducing power of the extract. Phenol content was 96.67± 0.82 mg gallic acid/g and flavonoid content was 27.30 ± 0.30 mg quercetin/g. Our current results emerged that *L. palmatum* act as an antioxidant agent due to its free radical scavenging and cytoprotective activity. So, the plant may be further pursued to find out for its pharmacological active natural products.

Key words: *Lygodium palmatum*, phenol, flavonoid, antioxidant, DPPH.

1. INTRODUCTION

Since very old times, herbal medications have been used for remission of symptoms of disease. Despite the great advances notice in modern medicine in recent decades, plants still make an important achievement to health care. Much interest, in medicinal plants however, grows from their long use in public medicines as well as their prophylactic properties, particularly in developing countries. Large number of medicinal plants has been investigated for their antioxidant properties. Natural antioxidants either in the form of raw extracts or their chemical components are very effective to cure the blasting processes caused by oxidative stress [1, 2]. Although the toxicity profile of most medicinal plants have not been widely evaluated, it is usually accepted that medicines derived from plant products are safer than their artificial counter parts. Incarnate evidence has collected and indicated key roles for reactive oxygen species (ROS) and other oxidants in causing numerous disorders and diseases [2]. The evidence has brought the consideration of scientists to an appreciation of antioxidants for prevention and treatment of diseases, and maintenance of human health [3]. Human body has an inherent antioxidative

mechanism and many of the biological functions such as the antimutagenic, anti-carcinogenic, and anti-aging responses originate from this property [4-6]. Antioxidants stabilize or deactivate free radicals, often before they attack targets in biological cells. Recently interest in naturally occurring antioxidants has considerably increased for use in food, cosmetic and pharmaceutical products, because they possess multi-affectedness in their multitude and magnitude of activity and provide immeasurable scope in correcting imbalance.

The role of free radical reactions in disease pathology is well established and is known to be involved in many acute and chronic disorders in human beings, such as diabetes, atherosclerosis, aging, immune suppression and neuro degeneration [7]. An imbalance between ROS and the inherent antioxidant capacity of the body, directed the use of dietary and /or medicinal supplements particularly during the disease attack. Studies on herbal plants, vegetables, and fruits have indicated the presence of antioxidants such as phenolics, flavonoids, tannins, and proanthocyanidins [8]. The antioxidant contents of medicinal plants may contribute to the protection they offer from disease. The ingestion of natural antioxidants has been inversely associated with morbidity and mortality from degenerative disorders. Liver diseases remain a serious health problem. It is well known that free radicals cause cell damage through mechanisms of covalent binding and lipid peroxidation with subsequent tissue injury [9]. Antioxidant agents of natural origin have attracted special interest because of their free radical scavenging abilities. The use of medicinal plants with high level of antioxidant constituents has been proposed as effective therapeutic approach for hepatic damages [10].

Lygodium palmatum (Bernh.) Sw. is the only species of its genus native to North America. Unlike most species in the genus, this one, called the American climbing fern [11] or Hartford fern (after Hartford, Connecticut, is extremely hardy in temperate zones. This fern is on endangered or threatened species lists in several states. It requires constant moisture, high light levels, and intensely acid soil to thrive. Its range is essentially Appalachian, ranging from New England down through the Appalachians, Piedmont and Appalachian plateaus into the American south.

The aim of the present study was to identify the antioxidant potential of methanol extract of *L. palmatum*. The extract was examined for DPPH free radical scavenging activity, reducing power capacity and for phenol and flavonoid content.

2. MATERIAL AND METHOD

2.1 Plant material

Fresh leaves of *L. palmatum* were collected from Bandarban, Chittagong, Bangladesh in the month of March 2015. It was authenticated by Dr. Shaikh Bokhtear Uddin, Professor, Department of Botany, University of Chittagong, Chittagong-4331, Bangladesh.

2.2 Preparation of Extract

The leaves were dried for a period of 10 days under shade and ground. The ground leaves (450 gm) were soaked in sufficient amount of methanol for one week at room temperature with occasional shaking and stirring then the whole mixture was filtered and the filtrate thus obtained was concentrated using a water bath to get a viscous mass. The viscous mass was kept at room temperature under a ceiling fan to get a dried extract (yield value, 5.3%). The extract prepared was for pharmacological screening.

2.3 Chemicals and equipment

All other chemicals and reagents were of analytical grade. Methanol purchased from Merck (India). Gallic acid, Folin-Ciocalteu reagent, trichloro acetic acid (TCA) was purchased from Sigma Chemicals Co. (P.O. Box 14508, St. Louis, MO 63178 USA). 1, 1-diphenyl-2-picrylhydrazyl (DPPH), aluminium chloride was purchased from Fluka (Flukachemie GmbH, CH-9471 Buchs). Ascorbic acid, Quercetin was purchased from BDH Chemicals (BDH Chemicals Ltd. Poole, England). Ferric chloride, potassium ferricyanide, sodium hydroxide and sodium nitrite were purchased from Riedel-De Haen Ag, Seelze-Hannover, Germany. Shimadzu Biospec 1601 UV visible spectrophotometer (Shimadzu, Japan) was used to measure the absorbance.

2.4 *In vitro* Antioxidant Activity

2.4.1 DPPH free radical scavenging activity

DPPH scavenging activity was carried out using the method of Braca et al. [12]. Different concentrations (400, 200, 100, 50, 25 and 12.5 µg/mL) of *L. palmatum* extract were dissolved in methanol and placed in different test tubes, and 3 mL of a 0.004% w/v methanol solution of DPPH was added to each test tube. Absorbance at 517 nm was determined after 30 min against a blank, and the percent inhibition activity was calculated from $[(A_0 – A_1) / A_0] \times 100$, where A_0 is the absorbance of the control and A_1 is the absorbance of the sample. Ascorbic acid was used as a reference standard and dissolved in methanol to make the stock solution with the same concentration. The control sample was prepared containing the same volume without any extract or reference drug.

Methanol served as a blank. The inhibition curves were prepared and the half maximal inhibitory concentration (IC_{50}) values were calculated using linear regression analysis.

2.4.2 Reducing power capacity

The reducing power of the extract was evaluated by the established method described by Oyaizu [13] with slight modification. Different concentrations of leaf extract of *L. palmatum* (125, 250, 500, and 1000 µg/mL) in 1 mL of distilled water were mixed with phosphate buffer (2.5 mL, 0.2 M, pH = 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 mL, 1% w/v). The mixture was incubated at 50 °C for 20 min. After incubation, 2.5 mL of 10% trichloroacetic acid solution was added to each tube and the mixture was centrifuged at 3000 rpm for 10 min. Subsequently, 5 mL of the upper layer solution was mixed with 5 mL of distilled water and 1 mL of ferric chloride solution (0.1% w/v), and the absorbance was measured at 700 nm. The reducing power of the extract was linearly proportional to the concentration of the sample. Ascorbic acid was taken as a reference standard. Phosphate buffer (pH 6.6) was used as a blank solution.

2.4.3 Determination of total phenolic content (TPC)

Total phenolic content of the extract was evaluated with Folin-Ciocalteu method [14]. Samples containing polyphenols are reduced by the Folin-Ciocalteu reagent there by producing blue colored complex. The phenolic concentration of extracts was evaluated from a gallic acid calibration curve. To prepare a calibration curve, 0.5mL aliquots of 12.5, 25, 50, 100, 200, and 400 µg/mL methanolicgallic acid solutions were mixed with 2.5 mL Folin-Ciocalteu reagent (diluted ten-fold) and 2.5 mL (75 g/L) sodium carbonate. After incubation at 25 °C for 30 min, the quantitative phenolic estimation was performed at 765 nm against reagent blank by UV Spectrophotometer 1650. The calibration curve was constructed by putting the value of absorbance vs. concentration. A similar procedure was adopted for the extract as above described in the preparation of calibration curve. All determinations were performed in triplicate. Total phenolic content was expressed as milligrams of gallic acid equivalent (GAE) per g of extract.

2.4.4 Determination of total flavonoid content (TFC)

Total flavonoid content of ethanol extract was evaluated with method of Jiao [15]. One ml of *L. palmatum* extract or standard of different concentrations was taken in a test tube and 3 ml of methanol was added. Then 200 µl of 10% aluminium chloride solution was added into the same test tube followed by the addition of 200 µl of 1M potassium acetate. Finally, 5.6 ml of distilled water was mixed with the reaction mixture. The reaction mixture was then incubated for 30 min at room temperature to complete the reaction. Then the absorbance of the solution was measured at 415 nm using a spectra photometer against blank. Methanol served as blank. The Total content of flavonoid compounds in *L. palmatum* extract was expressed in mg/g quercetin equivalent (QE).

2.5 Statistical analysis

All results are expressed as mean ± standard error of the mean (SEM). The results were statistically analyzed using repeated measures analysis of variance with Dunnett's multiple comparison when compared against negative control in all *in vivo* model of Sedative and Anxiolytic activities. $P<0.05$, $P<0.01$ and $P<0.001$ were considered as statistically significant. Statistical programs used were SPSS (Statistical Package for Social Science, version 22.0, IBM Corporation, NY). GRAPHPAD PRISM® (version 6.00; GraphPad Software Inc., San Diego, CA, USA) was used for graphical presentation.

3 RESULTS

3.1. *In Vitro* Antioxidant Activity

3.1.1. DPPH radical scavenging activity

Results for the free radical scavenging activity of methanol extract of *L. palmatum* are shown in **Figure 1**. The extract showed a dose dependent radical scavenging effect in DPPH assay. The half inhibition concentration (IC50) for free radicals achieved by the extract is 46.94µg/ml which is statistically significant compared to that (IC50 8 µg/ml) of reference antioxidative agent ascorbic acid.

3.1.2. Reducing power capacity

The extract showed significant reducing power activities as compared to ascorbic acid and proportionally increased with the increasing concentration of the extract, which is shown in **Figure 2**. Increase in absorbance of the reaction mixture indicates the increase in the reducing power of the sample.

3.1.3. Quantitative determination of phytochemical contents

Data for total phenolic and total flavonoid content has been summarized in **Table 1**. Data shows that moderate amount of total phenols and total flavonoids are present in the extract and the amounts are 96.67± 0.82mg Gallic acid /g dry wt and 27.30 ± 0.30mg Quercetin/g dry wt of dried extract respectively.

4. DISCUSSIONS

The antioxidant activity of a substance is usually correlated directly to its reducing capacity; the assay provides a reliable method to study the antioxidant activity of various compounds this method has been frequently used for a rapid evaluation of the total antioxidant capacity of different plant extracts containing flavonoids [16]. Antioxidant activity and this could be attributed to the presence of antioxidant phytomolecules. Antioxidant protection leads to several human diseases and conditions such as cancer, diabetes. Among the various natural antioxidants, phenolics are very important constituents because of their multiple biological effects and direct contribution to antioxidative activity. The results of our study reveal that there is a strong coincidence between antioxidant activity and phenolic content. Several studies on total phenolic content had been published over the years demonstrating its importance in the medicinal field.

DPPH radical scavenging model is widely used method to evaluate antioxidant activity of natural compound and plant extracts. The degree of discoloration indicates the scavenging potential of the antioxidant extract, which is due to the hydrogen donating ability [17]. The experimental data revealed that methanol extracts of leaves have the effects of scavenging free radicals and a dose dependent relationship in the DPPH radical scavenging activity. The involvement of free radicals, especially their increased production leads to the development of cardiovascular diseases and cancer. Thus, the consumption of *L. palmatum* leaves can be beneficial in preventing oxidative stress related numerous chronic diseases.

The reducing power of MELP was determined by direct electron donation in the reduction of ferri cyanide $[Fe(CN)^6]^{3-}$ to ferro cyanide $[Fe(CN)^6]^{4-}$. The product was visualized by addition of free Fe^{3+} ions after the reduction reaction, by forming the intense Prussian blue colour complex, $(Fe^{3+})_4[Fe^{2+}(CN^-)^6]_3$, and quantified by absorbance measurement at 700 nm [18]. The presence of reductants (i.e. antioxidants) in *L. palmatum* leaves cause the reduction of the Fe^{3+} /ferricyanide complex to the ferrous form which was monitored by measuring the formation of Perl's Prussian blue at 700 nm. Figure 2 shows the reductive capabilities of MELP compared to ascorbic acid. Therefore, like the DPPH radical scavenging activity, the observed reducing power of leaves was in agreement with the chemical constituents in the extracts of *L. palmatum* leaves.

Phenolic compounds possess the ideal chemistry for antioxidant activity because they have high reactivity as hydrogen or electron donors and are also capable of chelating metal ions [19]. These antioxidants also possess diverse biological activities, such as anti-inflammatory, anti-atherosclerotic, and anti-carcinogenic activities. These activities may be related to their antioxidant activity [20]. Thus, TPC and TFC of MELP were also evaluated. Polyphenolic flavonoids occurr ubiquitously in food and medicinal plants; they occur as glycosides and contain several phenolic hydroxyl groups. They are known for their efficient radical scavenging activity owing to their hydroxyl group at various positions and an ortho-dihydroxy structure in their B ring [21]. Recent investigation has shown that many flavonoids and related polyphenols contribute significantly to the antioxidant activity of many fruits, vegetables, and medicinal plants [22].

The results obtained from correlation between polyphenols (phenol and flavonoid) and DPPH scavenging suggested that phenolic compounds are dominant contributors to the antioxidant activity of the extract.

5. CONCLUSIONS

The present study indicated that *L. palmatum* contains considerable amount of total polyphenols and flavonoids and exhibited good antioxidant activity by effectively scavenging various free radicals. The antioxidant and biological activities might be due to the synergistic actions of bioactive compounds present in them. However, it is still unclear which components are playing vital roles for this activity. Therefore, further studies are still needed to elucidate mechanistic way how the plant contributes to this property.

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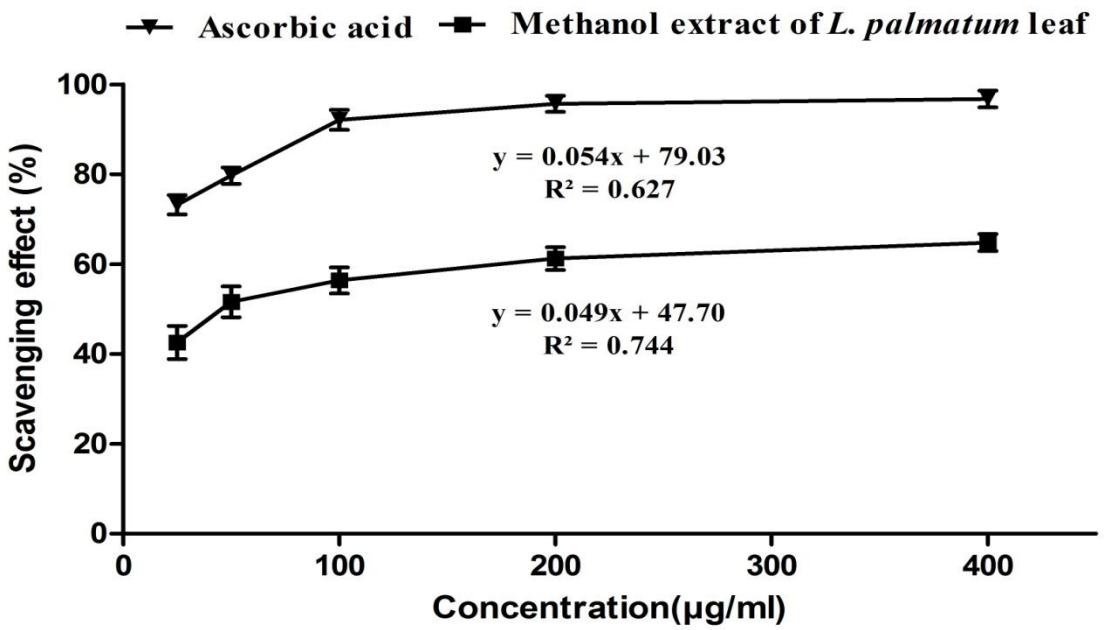

Figure 1. DPPH radical scavenging activity of *L. palmatum* leaves.

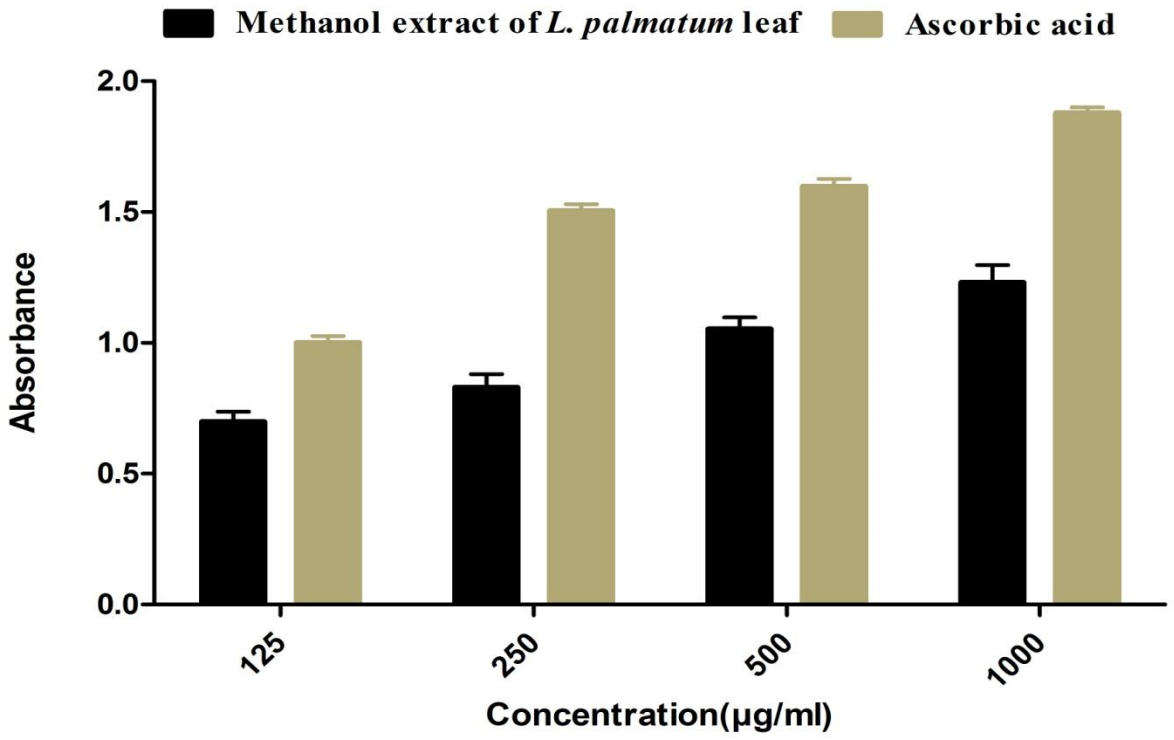

Figure 2. Reducing capacity of the methanol extract of *L. palmatum* leaf.

Table 1. Phytochemicals of methanol extract of *L. palmatum*.

Phytochemicals (mg/gm)	*L. palmatum* (methanol)
Total Phenol (mg Gallic acid /g)	96.67± 0.82
Total Flavonoid (mg Quercetin/g)	27.30 ± 0.30

Values are the mean of triplicate experiments and represented as mean ± SEM (n=3).

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