INTRODUCTION

Lygodium flexuosum (L) Sw. (Schizaeaceae) is climbing or scrambling fern with glabrous, pinnately lobed leaves. The rhizomes, roots and leaves are ethnomedicinally useful in the treatment of jaundice1,2. Antheridiogens3, Lygodinolide4, O- p-coumaryldihydrocrassol, tectoquinone, kaempferol, kaempferol- 3-beta-D-glucoside, beta-sitosterol and stigmasterol were main chemical constituents studied from Lygodium flexuosum5.

Many properties of plant products are associated with the presence of phenolic compounds, which are essential for plant development and play an important role in their defense mechanisms. These compounds, present in the regular diet, might be beneficial to human health by lowering incidence of diseases. Active oxygen molecules such as (O2-, OOH), hydroxyl (OH*) and peroxyl (ROOH*) radicals play an important role in oxidative stress related to the pathogenesis of various important diseases. In healthy individuals, the production of free radicals is balanced by antioxidant defense system6.

Because of increased safety concerns about synthetic antioxidants, exploitation of cheaper and safer sources of antioxidants based on natural origin is the focus of research nowadays. Recently there has been growing interest in oxygen containing free radicals in biological systems and their implied roles as causative agents in the etiology of a variety of chronic disorders. Currently available synthetic antioxidants like butylated hydroxy anisole (BHA), butyrate hydroxy toluene (BHT), tertiary butylated hydroquinone and gallic acid esters, have been suspected to cause butyrate hydroxy toluene (BHT), tertiary butylated hydroquinone and gallic acid esters, have been suspected to cause

ANTIOXIDANT COMPOUND FROM LYgodium flexuosum (L.) SW. EXTRACTS

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ABSTRACT

In vitro antioxidant activity of different extracts of plant Lygodium flexuosum (L) Sw were evaluated using models of DPPH radical scavenging activity, total antioxidant capacity, nitric oxide scavenging activity, ion chelating activity, hydroxyl radical scavenging activity. Phenolic contents of extracts were estimated as gallic acid equivalents by Folin-Ciocalteu method and correlated with antioxidant activity. Highest phenolic content (6.24 %) was found in methanolic extract with highest antioxidant activity. The fractionation and characterization of methanolic extract showed presence of quercetin.

Keywords: Lygodium flexuosum, phenolic content, antioxidant activity, quercetin.

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Because of increased safety concerns about synthetic antioxidants, exploitation of cheaper and safer sources of antioxidants based on natural origin is the focus of research nowadays. Recently there has been growing interest in oxygen containing free radicals in biological systems and their implied roles as causative agents in the etiology of a variety of chronic disorders. Currently available synthetic antioxidants like butylated hydroxy anisole (BHA), butyrate hydroxy toluene (BHT), tertiary butylated hydroquinone and gallic acid esters, have been suspected to cause or prompt negative health effects. Hence, strong restrictions have been placed on their application and there is a trend to substitute them with naturally occurring antioxidants. Moreover, these synthetic antioxidants also show low solubility and moderate antioxidant activity7. Hence to find natural sources of antioxidants was the focus of this study.

Various antioxidant activity methods have been used to monitor and compare the antioxidant activity of foods and herbal drugs. These methods need special equipment and technical skills for the analysis. These analytical methods measure the radical-scavenging activity of antioxidants against free radicals like l-diphenyl-l-pierylbreadyl (DPPH) radical, the superoxide anion radical, hydroxyl radical (OH), or peroxyl radical. Antioxidant activity methods using free radicals are fast, easy and simple. Some methods are summarized as malondialdehyde (MDA) or thiobarbituric acid-reactive substances (TBARS) assays8, Solid-phase spectrophotometry using tetrahexenyl (h, j, n) (1,5,9,13-tetraazacyclohexadecine-Cu (II) complex immobilized9, hydroxyl radical scavenging capacity10, fluorometric analysis11, bioassay-guided rationation, high-performance liquid chromatography and spin trapping electron spin resonance (ESR) method12.

Plant phenolics and flavonoids are especially common in leaves, flowering tissues and woody parts such as the stem and bark13. Phenolics compounds are a large, heterogeneous group of secondary plant metabolites that are widespread in plant kingdom14,15. Phenolics contain different chemical classes tannins, catechins, flavonoids, steroids etc. The phenolic content is estimated by various methods like Folin Ciocalteu Method16, Chromatographic response function (HPLC)17 Reversed phase HPLC17, Protein dye binding18. On the basis availability of the facilities, we referred Folin-Ciocalteu method to estimate phenolic content of the extracts.

The aim of this study was to determine in vitro antioxidant activity of the solvents extracted material of Lygodium flexuosum and its correlation with the total phenolic content and characterization of potent extract for its main active constituents.

Experimental

Chemicals

All chemicals and solvents used in this study were of analytical grade and obtained from HiMedia Chemicals Mumbai, India.

Solvent extraction

L. flexuosum was collected from the forest of Bahuli gaon, Igatpuri District-Nashik, Maharashtra, India. The entire plant was sent to Botany Dept, University of Pune, Pune for authentication and obtained certificate of authentication. A voucher specimen was deposited in herbarium of our laboratory. The shade dried plants were pulverized and subjected to extraction. The method of extraction was successive extraction by Soxhlet apparatus. The solvents used in sequence for extractions were petroleum ether (60:80), n-hexane, benzene, ethyl acetate, chloroform, acetone, methanol and water.

In vitro antioxidant activity

DPPH radical scavenging activity

DPPH scavenging activity was measured by spectrophotometric method19. To 1 ml of various concentrations of extract, 1 ml solution of DPPH (0.1 mM) was added. An equal amount of methanol and DPPH served as control. After 20 min of incubation in dark, absorbance was recorded at 517 nm. The experiment was performed in triplicate and the percentage inhibition calculated by using the formula20:

\[
\text{Inhibition} \% = \frac{(\text{Control} - \text{Test})}{\text{Control}} \times 100
\]
Total antioxidant capacity

Total Antioxidant Capacity was measured by spectrophotometric method. 0.1 ml of the extract (10 mg/ml) dissolved in water was combined in eppendorf’s tube with 1 ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were capped and incubated in a thermal block at 95°C for 90 min. After cooling to room temperature, the absorbance of aqueous solution was measured at 700 nm against blank. Ascorbic acid was used as the standard and total antioxidant capacity is expressed as equivalents of ascorbic acid.

Scavenging of nitric oxide radical

Nitric oxide was generated from sodium nitroprusside and measured by Griess’ reaction. Sodium nitroprusside (5 mM) in standard phosphate buffer saline solution (0.025 M pH 7.4) was incubated with different concentrations (2-1000 µg/ml) of the test extract dissolved in phosphate buffer saline (0.025 M, pH 7.4) and the tubes were incubated at 25°C for 5 hr. Control experiments without the test compounds but equivalent amounts of buffer were conducted in identical manner. After 5 hr, 0.5 ml of incubation was removed and diluted with 0.5 ml of Griess’ reagent (1% sulphanilamide, 2% o-phosphoric acid and 0.1% naphthyl ethylenediamine dihydrochloride). The absorbance of the chromophore formed during diazotization of nitrite with sulphanilamide and its subsequent coupling with naphthyl ethylene diamine was read at 546 nm. The experiment was repeated in triplicate.

Ion chelating activity

Ion chelating activity was performed by colorimetric method. The reaction mixture containing 1 ml 0.05% O-phenanthroline in methanol, 2 ml ferric chloride 200 mM and 2 ml various concentrations of the test compound was incubated at ambient temperature for 10 min and the absorbance of the same was measured at 510 nm. The experiment was performed in triplicate.

Scavenging of hydroxyl radical

Extracts of different concentrations were prepared in 2% alcohol were taken in different test tubes and evaporated on a water bath. To these, 1 ml of Iron-EDTA solution (Iron was added as 2.0 mg Fe (FeSCu) mixed with Na2EDTA as an aqueous solution in a 1:1 molar ratio), 0.5 ml of 0.01% EDTA and 1 ml of DMSO (0.85% v/v in 0.1 M phosphate buffer, pH 7.4) were added and the reaction was initiated by adding 0.5 ml of 0.22% ascorbic acid to each of the test tubes. Test tubes were capped tightly and heated on water bath at 80°C-90°C for 15 min. Then the reaction was terminated by the addition of 1 ml of ice-cold trichloroacetic acid (17.5%, w/v) to all the test tubes. Kept aside for 2 min and the formaldehyde formed was determined by adding 3 ml of Nash reagent (75 g of ammonium acetate, 3 ml glacial acetic acid, 2 ml acetyl acetone in 1 L with distilled water) after 10-15 min for colour development. Intensity of yellow colour formed was measured spectrophotometrically at 412 nm against reagent blank. Percentage scavenging of hydroxyl radical was calculated by comparison of the results of the samples with that of the blank.

Estimation of Phenolic content

Preparation of sample

Extract solution prepared 2.5 mg/ml in ethanol, mixed, sonicated and volume was made up to 100 ml with HPLC grade water. Solution was filtered with No.1 Whatman paper. Folin-Ciocalteu Phenol reagent (0.5 ml) was added to aliquot of sample and mixed again. After 5 min, 1.5 ml sodium carbonate solution (20 %w/v) was added. The volume was adjusted to 10 ml with HPLC grade water, mixed and filtered after 2 hours. The absorbance at 760 nm was recorded. The same solution without the extract solution was used as blank solution. This procedure was repeated for each extract.

Preparation of standard

Gallic acid 0.5mg/ml solution prepared in ethanol and volume was made up to 100 ml with HPLC grade water. Again 1ml resultant solution diluted to 10 ml with water. Remaining procedure was done as mentioned in previous section.

Calculation of phenolic content

Calculation of percent Total Phenols content was based on GAE gallic acid equivalents

\[ \text{A sample} \times \frac{W \text{ std}}{X} = \text{Total Phenolic content} \times \frac{W \text{ sample}}{A \text{ std}} \]

Characterization of methanolic extract

Isolation of compounds

The dried samples were separately Soxhlet extracted in 80% methanol (100 ml/gm dry weight) on a water bath for 24 hrs. Each of the extracts was defatted in petroleum ether (40°C-60°C) (fraction-I), ethyl ether (fraction-II) and ethyl acetate (fraction-III) in succession. Each of the steps was repeated three times to ensure complete extraction in each case. Each reaction was analyzed for the free flavonoids in each of the samples. Fraction III was hydrolyzed by refluxing with 7% H2SO4 (10 ml/gm residue) for 5 hours. The mixture was filtered; the filtrate was extracted with ethyl acetate in a separating funnel. The ethyl acetate layer was washed with distilled water till neutrality and dried in vacuum. The residues were taken up in small volumes of ethanol separately and then subjected to preliminary phytochemical screening. Fraction I, II and III were analyzed for antioxidant activity.

Preparative thin layer chromatography

Glass plates (20 x 20 cm) thickly coated (0.4-0.5 mm) with silica gel ‘G’ (45 gm/80 ml water) and activated at 100°C for 1 hour and cooled at room temperature were used for preparative thin layer chromatography. The fraction III was applied on separate plates and developed in n-butanol, acetic acid and water (4: 1: 5, upper layer v/v). Developed plates were air dried and visualized under UV light. Each of the fluorescent spots coinciding with those of standard reference compound of quercetin was marked. The spotted marks were scrapped and collected separately along with the silica gel G and eluted with ethanol. Each of eluate was then crystallized with chloroform. The purified material was subjected to spectral studies.

Fraction III Compound was obtained as a yellow powder of melting point 300°C and EIMS m/z: 302 [M]+. IR (KBr, nmax in cm-1): 1674, 827, 942, 1220, 1001, 1210, 1382, 1400, 1466, 1662, 3407. IR absorption band at 3407, 1660, 1662 and 3407. IR absorption band at 3407, 1660, 1408 and 1210 cm-1 were consistent with the presence of hydroxyl, carbonyl and aromatic ring and ether groups respectively.

RESULTS

The anti-oxidant activity of petroleum ether (60:80), n-hecane, benzene, ethyl acetate, chloroform, acetone, methanol and aqueous extracts of L. flexuosum were measured by different methods like DPPH scavenging activity, Total Antioxidant Capacity, Scavenging of nitric oxide radical, Ion chelating activity, Scavenging of hydroxyl radical. The aim was to evaluate the activity and isolate possible activity responsible compound. The results obtained by these studies are mentioned in Table 1. According to DPPH assay, methanol extract showed maximum activity with IC50 = 5.1 µg/ml and the activity was comparable to that observed with standard ascorbic acid having IC50 = 2.76 µg/ml. DPPH is relatively stable free radical. The assay based on measurement of scavenging ability of antioxidants towards stable DPPH radical. From the present result it may be postulated that some hydrogen donors in antioxidant principles of L. flexuosum reduces the radical when it react with hydrogen donors in antioxidant principles. DPPH radicals react with suitable reducing agents, the electrons become paired off and solution looses colour stochiometrically depending on number of electrons taken up.

1H-NMR (Fig. 2) (400MHz, CDCl3): δ 6.17 (1H, s, H-6, H-8) [C-6, C-8],
6.37 (1H, s, H-8), 6.86 (1H, d, H-5’)[C-5’], 7.62 (1H, d, H-6’)[C-6’],
7.72 (1H, s, H-2’)[C-2’]. The 13C-NMR (Fig. 3) (100MHz, CD3OD): δ
94.58 (C-8), 99.41 (C-6), 104.69 (C-10), 116.39 (C-2’), 116.85 (C-6’), 124.32 (C-1’), 137.37 (C-3), 146.38 (C-3’), 148.19 (C-4’), 148.93 (C-2), 158.41 (C-9), 162.67 (C-5), 165.72 (C-7), 177.50 (C-4). From these results, fraction III compound may consider as quercetin.
The antioxidant activity of *L. flexuosum* extracts were evaluated by five different *in vitro* free radicals. The results showed that methanolic extract was rich in polyphenol and flavonoid content and had significant antioxidant activity which is in correlation with phenolic content. The fractionation of methanolic extract gave biologically active fraction which on characterization by spectral studies found to be quercetin.

Thus this study gives support for expanding future investigations of pharmacological activities associated with free radicals. 

**ACKNOWLEDGEMENT**

The authors are thankful to Dr. H. N. More, Principal, Bharati Vidyapeeth College of Pharmacy, Kolhapur, Maharashtra, India for providing facilities to carry out this work.

### Table 1: Phenolic content of extracts and effect of *L. flexuosum* extracts on various *in vitro* free radicals

<table>
<thead>
<tr>
<th>Extracts</th>
<th>Phenolic content</th>
<th>DPPH activity</th>
<th>Total antioxidant capacity</th>
<th>Nitric oxide activity</th>
<th>Ion chelating activity</th>
<th>Hydroxyl radical activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Petroleum Ether</td>
<td>2.30</td>
<td>0.62</td>
<td>11.43±0.05*</td>
<td>34.7±0.98*</td>
<td>13.6±0.85*</td>
<td>11.31±0.51*</td>
</tr>
<tr>
<td>n-hexane</td>
<td>3.14</td>
<td>0.67</td>
<td>11.34±0.24*</td>
<td>23.29±0.84*</td>
<td>20.07±0.94*</td>
<td>14.08±0.34*</td>
</tr>
<tr>
<td>Benzene</td>
<td>2.84</td>
<td>1.54</td>
<td>19.63±0.78*</td>
<td>19.5±0.64*</td>
<td>21.94±0.87*</td>
<td>19.24±0.45*</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>2.01</td>
<td>1.50</td>
<td>20.31±0.65*</td>
<td>22.59±0.54*</td>
<td>24.21±0.64*</td>
<td>26.48±0.86*</td>
</tr>
<tr>
<td>Chloroform</td>
<td>3.65</td>
<td>1.14</td>
<td>15.92±0.46*</td>
<td>18.14±0.67*</td>
<td>21.83±0.35*</td>
<td>18.32±0.67*</td>
</tr>
<tr>
<td>Acetone</td>
<td>4.53</td>
<td>0.66</td>
<td>19.7±0.78*</td>
<td>18.43±0.87*</td>
<td>28.8±0.82*</td>
<td>20.15±0.74*</td>
</tr>
<tr>
<td>Methanol</td>
<td>7.86</td>
<td>6.24</td>
<td>5.1±0.25*</td>
<td>6.14±0.54*</td>
<td>4.5±0.21*</td>
<td>8.7±0.28*</td>
</tr>
<tr>
<td>Aqueous</td>
<td>9.68</td>
<td>4.38</td>
<td>6.92±0.36*</td>
<td>6.71±0.21*</td>
<td>6.18±0.34*</td>
<td>5.72±0.26*</td>
</tr>
<tr>
<td>Standard (Ascobic acid)</td>
<td>2.76±0.12</td>
<td>--</td>
<td>--</td>
<td>3.1±0.20</td>
<td>2.87±0.21</td>
<td>4.5±0.34</td>
</tr>
</tbody>
</table>

Hydroxyl radical scavenging activity of extracts was assayed by generating hydroxyl radicals using ascorbic acid-iron-EDTA.$^{31}$ The hydroxyl radical formed by the oxidation reaction with formaldehyde. The formaldehyde production from DMSO provides a convenient method to detect hydroxyl radicals formed during oxidation of DMSO by Fe+3/ascorbic acid system which was used to detect hydroxyl radical.

The total phenolic content was obtained and was mentioned in Table 1. It was revealed that methanol extract had the maximum phenolic content 6.24 % The correlation coefficients for phenolic content and antioxidant activity of different extracts were studied and it was found that methanol extract showed good correlation coefficient (r2) of 0.87 to 0.97 for all antioxidant methods. Among all the extracts analyzed, a significant phenolic content and antioxidant activity were found for methanolic extract so it can be predicted that the antioxidant activity may be due to the total phenolic content in the plant. Previously it was revealed that the antioxidant activity of phenolics is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers.$^{32}$ We estimated antioxidant activity of extracts of *L. flexuosum*; the activity maybe due to redox properties of the phenolic content. In characterization of methanolic extract three fractions were collected and screened for preliminary phytochemical screening with phenolic content. Fraction III showed maximum phenolic content with presence of flavonoids. IC50 values for Fraction I, II and III are mentioned in Table 2.

### Table 2: Effect of *L. flexuosum* methanolic extract fractions on various *in vitro* free radicals

<table>
<thead>
<tr>
<th>Fractions of methanolic extract</th>
<th>DPPH activity</th>
<th>Total antioxidant capacity</th>
<th>Nitric oxide activity</th>
<th>Ion chelating activity</th>
<th>Hydroxyl radical activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>47.8±0.85*</td>
<td>57.40±0.87*</td>
<td>60.33±0.76*</td>
<td>52.54±0.86*</td>
<td>65.2±0.78*</td>
</tr>
<tr>
<td>II</td>
<td>69.2±0.94*</td>
<td>69.25±0.78*</td>
<td>66.65±0.68*</td>
<td>68.05±0.67*</td>
<td>68.86±0.26*</td>
</tr>
<tr>
<td>III</td>
<td>3.53±0.78*</td>
<td>5.65±0.64*</td>
<td>3.01±0.56*</td>
<td>3.96±0.68*</td>
<td>3.29±0.67*</td>
</tr>
<tr>
<td>Standard (Ascobic acid)</td>
<td>2.76±0.12</td>
<td>3.1±0.20</td>
<td>2.87±0.21</td>
<td>4.5±0.34</td>
<td>2.76±0.30</td>
</tr>
</tbody>
</table>

Values are in the Mean ± S.D., n = 3 for each experiment, data were analyzed by one way ANOVA followed by Turkey test using Graph pad Instat software, *P > 0.001* compared with standard ascorbic acid.

Fracion III showed maximum antioxidant activity with IC50 values 3.53, 5.65, 3.01, 3.96, 3.29 µg/ml for DPPH, total antioxidant capacity, nitric oxide activity, ion chelating activity, hydroxyl radical activity assays respectively. Further Fraction III was characterized by spectral studies. It resembled presence of quercetin in Fraction III. Phenolic components phytochemically associated with quercetin like anthocyanins, anthocyaninides catechins and myricetins may be responsible for the observed activity.

### CONCLUSION

The antioxidant activity of *L. flexuosum* extracts were evaluated by five different *in vitro* testing systems. The results showed that methanolic extract was rich in polyphenol and flavonoid content and had significant antioxidant activity which is in correlation with phenolic content. The fractionation of methanolic extract gave biologically active fraction which on characterization by spectral studies found to be quercetin.

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*a% Phenolic Content represents Gallic Acid Equivalents*

*Values are in the Mean ± S.D., n = 3 for each experiment, data were analyzed by one way ANOVA followed by Turkey test using Graph pad Instat software, *P > 0.001* compared with standard ascorbic acid.*
REFERENCES


