PHYTOCHEMICAL CONSTITUENTS AND ANTIOXIDANT PROPERTIES OF PLEOCAULUS RITCHIEI: AN ENDEMIC PLANT FROM WESTERN GHATS OF INDIA

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ABSTRACT
The phytochemical constituents, antioxidant activities of extracts of Pleoaulus ritchiei using different solvents were evaluated. The dried powdered leaves of Pleoaulus ritchiei were extracted with acetone, ethanol and methanol. The phytochemicals were estimated according to the standard methods. 1,1-diphenyl-2-picrylhydrazyl, Hydrogen peroxide and reducing power assay were used to determine in vitro antioxidant activities and were performed using standard protocol. Phytochemical analysis of extracts showed presence of main group phytochemicals such as phenols, flavonoids, saponins and tannins. Methanolic extract was found to contain the highest phenolic and flavonoid content. In vitro antioxidant activities of all extracts were found to be good. On the basis of the results obtained, all the varied extracts of Pleoaulus ritchiei seems to be the most capable species for further exploration in order to identify the chemical ingredients responsible for their activity.

KEYWORDS: Flavonoid content, phenolic content, Antioxidant activity, Pleoaulus ritchiei.

INTRODUCTION
Plants are used as medicine since time immemorial & are a fundamental goldmine of potential drug targets. Many active molecules from plants are waiting to be discovered. An ethno pharmacological information is useful for the phytochemical research and it is generally considered an effective approach in the discovery of novel anti-infective agents from higher plants.[1] Plants have infinite potential to synthesize aromatic substances, principally secondary metabolites such as tannins, saponins, flavonoids and phenolics which play role in plants and therefore the plants defends from their invaders like fungi, bacteria, etc.

Now a day’s bioprospecting of medicinal plants has emerged as a frontier area of research. As a result, experimental studies on medicinal plants are increasingly being carried out worldwide on locally used plant species. Medicinal plant consist of some organic compounds which make available definite physiological action on the human body and these bioactive substances include phenols and flavonoids.[5,6] plants has the phenolic derivative which plays a very important role in plant defense against pathogens and herbivore predators thus these are used in the control of human pathogenic infections.[5] Flavonoids has pharmacological activities such as anti-inflammatory, antimicrobial, antioxidant and anticancer activities have been extensively reported.[5]

Less concentrations of an antioxidant compared with those of an oxidizable substrate, significantly delays or inhibits oxidation of that substrate.[6] The significance of natural antioxidants in defending from coronary heart disease, cancer and hypertension are rising day by day.[7,8]

The genus Pleoaulus Bremek. is a monotypic genus of family Acanthaceae. It is a small perennial shrub, usually grows up to half a meter tall, reaching up to 2 meters after flowering. It is one of the endemic species of Western Ghats which is mostly distributed in the Northern Western Ghats of India.[9,10]

Led by the all above considerations and our previous studies,[11] it is recognized that the phytochemical investigation and antioxidants in ethanomedicinal plant species is the need for today’s researchers because such species are valuable in both domestic and international markets due to growing recognition of its value to human health. Such information on the phytochemicals and antioxidant properties of P. ritchiei are still lacking and hence we report these parameters for first time in the present communication. The present investigation deals with the estimation of phytochemicals such as total phenolics, flavonoids, tannins, saponins and antioxidant from extracts of P. ritchiei in various solvents.

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MATERIALS AND METHODS

Chemicals

2, 2-diphenyl-1-picrylhydrazyl (DPPH), gallic acid and quercetin were purchased from Sigma Chemical Co, (St. Louis, MO, USA), while all other required chemicals and solvents used were analytical grades procured locally.

Plant collection and identification

The plant material of *P. Ritchiei* was collected from lateritic plateaus of Western Ghats (Amboli-GPS N 15° 55.950’ E 074° 00.725’ 2215 feet MSL). Species was authentically identified and deposited at Herbarium, Department of Botany, The New College, Kolhapur (Vouch. No. VBS-23478). Whole plants were washed with water to remove dust and dried under shade at room temperature. The dried plant sample was pulverized and kept in air tight bottle till further screening.

Extraction of the plant material

The shade dried plant material was pulverized and consecutively extracted with acetone (AA), ethanol (AE) and methanol (AM) by using Soxhlet apparatus for 6 hours at a temperature below the boiling points of the solvents. After which the extracts were evaporated to dryness to obtain semisolid extracts.

Determination of total phenolic content (TPC)

The total phenolic content was estimated using Folin–Ciocalteu reagent. The extracts were oxidized with 10% Folin–Ciocalteu reagent and were neutralized with 100mM sodium carbonate solution. The absorbance was measured at 765 nm after 60 minutes against reagent blank using UV-Vis spectrophotometer.

A calibration curve was plotted for standard phenol (gallic acid) using 20-100 μg/mL concentration (Fig.1). The total phenolic content was expressed as mg/g of gallic acid equivalents (GAE)/g DW of dry weight of extract (DW).

Determination of total flavonoid content (TFC)

Total flavonoid estimation was carried out by the aluminium chloride colorimetric method. 0.5 mL of extract of different solvents (ethanol, methanol and acetone) was mixed with 1.5 mL of methanol, to this mixture 0.1 mL 10% aluminium chloride was added and then 0.1 mL of 1M potassium acetate and 2.8 mL of distilled water was added and tubes were kept for 30 min at room temperature for incubation. The absorbance was measured at 415 nm on UV-Vis spectrophotometer against blank. The total flavonoid content was estimated using linear regression equation obtained from standard calibration curve of quercetin shown in fig.1. The total flavonoid content was calculated as mean ± SD (n=3) and expressed as mg/g of quercetin equivalents (QE) DW of dry weight extract (DW).

Determination of saponins contents

The determination of saponins was estimated by the method of Obadoni and Ochuko. 5g of fine powder of plant sample was mixed with 50mL of 20% v/v aqueous ethanol. The sample was heated over hot water bath at 55°C for 4h bath with continuous stirring. The mixture was filtered and re-extracted the residue with another 50mL 20% ethanol and reduced to 20mL over hot water bath at about 90°C. The concentrate was transferred into separating funnel and 10mL diethyl ether was added and shaken vigorously. The aqueous layer was collected while ether layer was discarded. The collected layer was purified and repeated the process. The 20mL of n-butanol was added and then washed with 10 mL of 5% w/v aqueous sodium chloride. The whole mixture was heated to evaporation on hot water bath and then oven dried at 40°C to a constant weight. The saponins percentage was calculated using the formula.

\[ \% \text{Saponins} = \frac{\text{Weight of final filtrate}}{\text{weight of sample}} \times 100 \]

Determination of tannins

0.5g of plant sample was mixed with 75mL distilled water and transferred to a 250mL conical flask. The flask was heated and boiled for 30 min. The extract was centrifuged at 2000 rpm for 20 min and supernant was collected in 100 mL volumetric flask and the volume was made to 100mL. 1mL of the sample extract was then transferred to 100 mL volumetric flask having 75 mL water. 5 mL of folin denis reagent and 10 mL of sodium carbonate was added and diluted upto 100 mL with water and after incubation of 30 min at room temperature absorbance was measured at 760nm. The standard calibration curve was obtained by tannic acid.

Antioxidant activity

**DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical scavenging assay**

The antioxidant activity of different concentration of the plant extracts in various solvents were estimated using the DPPH radical scavenging protocol as given by Sutharsingh. DPPH solution (0.004% w/v) was prepared in 95% ethanol. The stock solutions of various extracts and standard ascorbic acid were prepared in the concentration of 100μg/mL. From the stock 2,4,6,8 and 10μL were taken and diluted upto 10μL with the same solvent to make final concentration as 20, 40, 60, 80 and 100μg/mL to this solution 2mL of DPPH solution was added in each test tube. The mixture was incubated in the dark for 15 min. then the absorbance was measured of each sample against the blank at 523 nm. Control sample was prepared by adding 2mL of DPPH in 10 mL of ethanol and absorbance was recorded after 30 min. The ability of scavenging DPPH radical was calculated using the following formula:

\[ \text{DPPH scavenged (\%)} = \left( \frac{A_{\text{control}} - A_{\text{test}}}{A_{\text{control}}} \right) \times 100 \]

Where, \( A_{\text{control}} \) = absorbance of the control, \( A_{\text{test}} \) = absorbance of the sample

**Hydrogen peroxide scavenging activity**

The hydrogen peroxide scavenging activity of extracts was performed as per reported method.
buffer saline (pH 7.4) was used to prepare 20 mM hydrogen peroxide solution. Different concentrations 20, 40, 60, 80 and 100µg/mL of plant extracts and standard ascorbic acid 1mL were added to 2 mL of H₂O₂ solution in Phosphate buffer saline. The absorbance was measured after 10 min at 230 nm against a blank solution containing Phosphate buffer saline without H₂O₂. The hydrogen peroxide scavenging percentage was calculated using the following formula:

% scavenged [Hydrogen peroxide] = A₀ - A₁ / A₀ x 100

Where, A₀ was the absorbance of the control, A₁ was the absorbance of the sample.

Reducing power Assay

The determination of reducing power of extracts was carried out using method described by Oyaizu (1986). Different concentrations of plant extracts in various solvents were mixed with phosphate buffer of pH 6.6 (2.5 mL) and 1% potassium ferricyanide (2.5 mL). The mixture was incubated at 40ºC for 20 min., after cooling 2.5 mL trichloroacetic acid (10%) was added and centrifuged for 5 min. From this reaction mixture 2.5 mL of solution mixed with 2.5 mL distilled water and 0.1% ferric chloride (0.5 mL). Ascorbic acid was used as standard and control was prepared in the same manner lacking of sample. The absorbance was measured at 700nm. Higher is absorbance greater is the reducing power of the plant extract.

Statistical analysis

The statistical analysis was carried as mean± S.D. Dunnett comparison test and One way ANOVA applicable and used to analyze level of significance between groups. P value <0.01 were considered statistically significant.

RESULTS AND DISCUSSION

Total phenolic and flavonoid content

The phenolic compounds are one of the major and most common groups of plant metabolites. They have various biological activities including antiapoptosis, antiaging, antiinflammation and many other.

The P. ritchiei was studied for phenolic contents and observations on total phenolic content are tabulated in Table 1. The TPC in the different solvent extracts of P. ritchiei ranged from 1.6 ± 0.5 mg GAE/g DW to 3.27 ± 0.6 mg GAE/g DW. In the present study the greatest TPC was found in methanolic extract and lowest in acetone.

The results obtained in the quantitative investigation of flavonoids are presented in the Table 1. The amount of total flavonoid in different extracts ranged from 0.56 ± 0.05 mg QE/g DW to 2.1 ± 0.4 mg QE/g DW. During this investigation the highest total flavonoid content was found in methanolic extract and lowest in ethanol. Similarly solvent dependent TFC variation was previously reported for Limonium delicatulum.

In the present investigation we observed the presence of phenols and flavonoids in all plant extracts.

![Standard curve for gallic acid](image1.png)

![Standard curve for quercetin](image2.png)

**Fig. 1: Standard curves of phytochemicals.**

**Table 1: Total phenolic and flavonoid content in plant extracts of P. ritchiei (n=3).**

<table>
<thead>
<tr>
<th>Plant extract</th>
<th>Acetone</th>
<th>Ethanol</th>
<th>Methanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total phenols</td>
<td>1.6 ± 0.5</td>
<td>2.60 ± 0.16</td>
<td>3.27 ± 0.6</td>
</tr>
<tr>
<td>Total flavonoids</td>
<td>1.2 ± 0.8</td>
<td>0.56 ± 0.05</td>
<td>2.1 ± 0.4</td>
</tr>
</tbody>
</table>

**Tannins and saponins content**

Tannins exhibits general antimicrobial and antioxidant activities. The tannin concentration of the plant extract was evaluated by the linear regression equation obtained from the standard graph of tannic acid. It was found to be shown in Table 2. 3.61 ± 0.34 mg/gm of plant sample.

Other phytochemicals such as saponins are mild detergent used in intracellular histochemistry staining to
allow antibody access to intracellular protein. In medicine, it is utilized in hypercholesterolemia, antioxidant, anticancer, anti-inflammatory and weight loss, etc. It is also shown to have anti-fungal properties. The percentage of saponins was obtained in P. ritchiei plant powder and it was 11.50 ± 0.03 mg/gm of plant sample shown in Table 2.

Table 2: Saponins and tannins compositions of P. ritchiei plant (Mean ± SD).

<table>
<thead>
<tr>
<th>Phytoconstituent</th>
<th>Amount of compound (%)</th>
<th>Extracts equivalent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saponins</td>
<td>11.50 ± 0.03</td>
<td>ND</td>
</tr>
<tr>
<td>Tannins (TE/g)</td>
<td>3.61 ± 0.09</td>
<td>Tannic acid</td>
</tr>
</tbody>
</table>

ND: Not detected; TE: Tannic acid equivalent.

Antioxidant properties
The different solvents for extracts of plant material of P. ritchiei had good DPPH free radical scavenging, hydrogen peroxide scavenging and reducing antioxidant power capabilities (Table 3). Among three extracts of P. ritchiei, ethanolic extract had the highest DPPH free radical scavenging activity (60.85 ± 71%) followed by methanolic plant extract (50.84 ± 46%) while it was lowest in acetone plant extract (55.30 ± 3.95%).

Antioxidant ability of various extract of P. ritchiei is determined by hydrogen peroxide scavenging activity presented in the Table 3. The better results were showed by methanolic extracts as (84.03 ± 2.73%) followed by ethanol and acetone 75.69 ± 3.22% and 78.09 ± 2.35% respectively.

In the present study antioxidant ability of various extract has been affected by solvent system. Similar observations on fluctuations in the total phenolics, flavonoids and antioxidant properties after using different solvents also made by Patil et al., (2012) in the Carrisa carandus and Eleagnus conferta.

Table 3: Percentage inhibition of (A) DPPH free radical and (B) hydrogen peroxide scavenging assay by extracts.

<table>
<thead>
<tr>
<th></th>
<th>20ppm</th>
<th>40ppm</th>
<th>60ppm</th>
<th>80ppm</th>
<th>100ppm</th>
</tr>
</thead>
<tbody>
<tr>
<td>A) I % inhibition AM</td>
<td>16.99 ±1.60**</td>
<td>24.05 ±2.74**</td>
<td>36.42 ±2.12**</td>
<td>45.99 ±1.69**</td>
<td>50.84 ±2.46**</td>
</tr>
<tr>
<td>A) II % inhibition AE</td>
<td>32.34 ±3.65**</td>
<td>42.96 ±1.97**</td>
<td>47.67 ±2.60**</td>
<td>55.11 ±1.67**</td>
<td>60.85 ±1.71**</td>
</tr>
<tr>
<td>A) III % inhibition AA</td>
<td>32.55 ±5.69**</td>
<td>42.09 ±4.31**</td>
<td>44.75 ±4.42**</td>
<td>48.30 ±2.98**</td>
<td>55.30 ±3.95**</td>
</tr>
<tr>
<td>B) I % inhibition AM</td>
<td>68.19 ±2.28**</td>
<td>72.02 ±2.67**</td>
<td>76.77 ±2.37**</td>
<td>79.59 ±3.49**</td>
<td>84.03 ±2.73**</td>
</tr>
<tr>
<td>B) II % inhibition AE</td>
<td>36.93 ±2.67**</td>
<td>42.68 ±2.54**</td>
<td>51.25 ±2.43**</td>
<td>63.57 ±2.48**</td>
<td>75.69 ±3.22**</td>
</tr>
<tr>
<td>B) III % inhibition AA</td>
<td>57.92 ±2.63**</td>
<td>62.84 ±1.99**</td>
<td>66.02 ±3.15**</td>
<td>75.76 ±2.62**</td>
<td>78.09 ±2.35**</td>
</tr>
</tbody>
</table>

Values in table indicate mean of three readings ±sd; P value <0.01 considered significant, calculated using Dunnett comparison test.

As presented in the Fig. 2, the reducing power values ranged from 0.106±0.03 to 0.341±0.05 O.D. (optical density) for methanolic extract which was highest. The ethanolic extract showed 0.108±0.03 to 0.320±0.07 O.D. and followed by acetone extract 0.047±0.04 to 0.106±0.05 O.D. at 700 nm. In the present study antioxidant ability of various extract has been affected by solvents. Reducing power assay estimates the electron-donating ability of an antioxidant. Presence of reducers causes the alteration of the ferrous form which acts as a significant indicator of its antioxidant capacity.

Figure 2: Antioxidant activities of various extracts of P. ritchiei by Reducing power assay.
CONCLUSION
The various solvent extracts obtained from the P. richieii showed the phenolic, flavonoid, tannin and saponin content and antioxidant ability. From the present work, the result showed that P. richieii is a good source of phytochemicals including phenolic compounds and gives opportunities for development of value added products, properties that may suggest applications in pharmaceutical and food industry. It is also noted that selective extraction of bioactive molecules from P. richieii can be isolated by using specific solvents. In overall the selected plant species had relatively high antioxidant capacities and total phenolic contents. Because of their strong antioxidant capacities, the selected species may be used also used as potential source in anti-inflammatory abilities.

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