SCreening of lampracheniun microcephalum an endemic plant of asteraceae from Western Ghats of India for their Phenols, flavonoids and antioxidant properties

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ABSTRACTS
At the present there is a growing interest of pharmaceutical industries to replace synthetic chemicals by expected products with bioactive properties that are of plant source. The aim of present study was to evaluate the endemic medicinal plant Lamprachaenium microcephalum Dalz. Benth. of Western Ghats of India for their Phytochemicals, antioxidant properties and to determine phenolic compounds by reverse-phase high performance liquid chromatography in three different solvent systems. It is noted that solvents significantly affects the quantity of Phytochemicals and antioxidant properties. In the species total phenol content (TPC) was highest in methanol extract and lowest in acetone extract and same trend was observed for the total flavonoid content (TFC). All the extracts of the species showed 2, 2-diphenyl-1-picrylhydrazyl (DPPH), Reducing power and Hydrogen peroxide free radical scavenging activity (H\textsubscript{2}O\textsubscript{2}). The results showed that among studied antioxidant activities acetone extract of both the species showed highest Hydrogen peroxide free radical scavenging activity \textit{L. microcephalum} 78.37\%. The results provide explanation for the use of this plant in folk medicine to treat various diseases.

KEYWORDS: Phytochemicals, antioxidant properties, endemic, medicinal plants W.G.

INTRODUCTION
Herbs and botanical now materialize in more products and have more medicinal significance than ever before. India is with dissimilar agro climatic situation and is a major resource for a wide variety of medicinal plants. But the manufacture potential is principally underexploited. Pharmaceuticals, nutraceuticals, food and cosmetic industries are the major buyers of herbal products. Herbal harvests are in requiring in the form of standardized plant extracts or botanical ingredients or semi-finished products. Medicinal plants are gaining importance in health care. Majority of the world population is dependent upon traditional plant based medicine. Many medicinal herbs and spices, which find place in our daily use are used as herbal remedies. Therapeutic plants are widely expensive as they make available basic raw materials for singular industries particularly for pharmaceutical industries. Among the flowering plants, the family Asteraceae is one of the dominant families, that comprising about 1528 genera and 22750 species (Bremer K., 1984) While in India family Asteraceae possess 900 species (Hajra et al.,) The bio resources of Western Ghats are quite rich. In this exclusive region almost all groups of efficiently important plants grows that contain abundant life saving drug plants, nutraceuticals, metal intolerant plants, natural pungent plants and so many others. Still many species are await for their discoveries, the flora of this region is getting depleted in an alarming rate, therefore not just conservation of these bioresources but also their sustainable utilization for human welfare is should be the priority agenda. Western Ghats harbor about 490 medicinal plants, of which 308 are endemic and medicinally important and its exploitation ranges from cancer to rheumatism treatments. This section acquire many endemic medicinal plants, as their incidence is confidential to a slender biological range, it’s our prime duty to examine such medicinal undergrowth for their chemical potentials.

Genus Lamprachaenium is belonging to family Asteraceae. This genus is endemic and mainly distributed in the forests of Northern Western Ghats (Irwin and Narasimhan, 2011) During explorations of flowering plants from the Western Ghats it is observed that some of the endemic plants of family Asteraceae are used to cure various chronic diseases mainly by local folklore. After literature survey it is also revealed that the species have received noteworthy consideration among the researchers due to their several significant medicinal
assets. The leaves of *L. microcephalum* are reported as tonic, anti-inflammatory, diuretic and have been used for treatment of rheumatic pain and as wounds healing medicine (Gurudeva and Yoganarasimhan, 2009). The species also used for treatment of skin diseases like leucoderma and in inflammation and it is said to possess antiseptic properties (Pullaiah, 2006) Therefore to understand the important secondary metabolites of this species, the plants were analyzed for its phytochemicals including total phenol (TP), total flavonoids (TF), Saponnin, Tannin and their antioxidant potentials.

**MATERIAL AND METHODS**

**Sample collection and preparation of extracts**

The plant material of *L. microcephalum* were collected from forest of Northern Western Ghats (*L. microcephalum* - Tillari Ghats- GPS N 15° 48.795° E 074° 10’ 1978 feet MSL). The species were authentically identified with the relevant literature and deposited at Herbarium, Department of Botany, The New College, Kolhapur (*L. microcephalum* VBS-3421). Whole plant was washed with water and dried under shade at room temperature. The dried plant sample was used for further screening. One gram of dried material was pulverized in a mortar and extracted with 25 ml of solvent (aceton, ethanol and methanol). The contents were mixed on orbital shaker with constant stirring at 150 rpm for 24 hrs. and centrifuged at 10,000 rpm for 10 min. The supernatant was collected and filtered through Whatman filter paper (No.1) and was used for further analysis. All the chemicals used were analytical grades. Methanol, acetic acid and HPLC grade water purchased from Merck (Darmstadt, Germany) while gallic acid and quercitin were acquired from Sigma Chemical Co. (St. Louis, MO, USA).

**Total phenolic content (TPC)**

The total phenolic content was estimated by the method of Bray and Thorpe with Folin–Ciocalteu reagent (FCR), 1 gm of dried plant sample ground with pestle and mortar in 80% solvents (ethanol, methanol and acetone), centrifuged at 10,000 rpm for 20 min. and supernatant was evaporated to dryness, the residue was dissolved in 5 ml water then different aliquots (0.2- 2 ml) were kept in test tubes and diluted it up to 3 ml with d/w, 0.5 ml of Folin–Ciocalteu reagent was added, after 3 min. 2 ml of 20% Na₂CO₃ was mixed, then tubes were placed in boiling water bath for 1 min. and cooled. The absorbance was measured at 650 nm against reagent blank. A calibration curve was done for standard phenol (gallic acid) using different concentration (R²=0.958) (fig. 1A). The total phenolic content was expressed in mg of gallic acid equivalents (GAE)/g DW of extract (Bray and Thorpe, 1954).

**Total flavonoid content (TFC)**

Estimation of total flavonoid content was carried out by the AlCl₃ 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% AlCl₃ was added and then 0.1 ml of 1M potassium acetate and 2.8 ml of distilled water was added and kept for 30 min. at room temperature. The absorbance was measured at 415 nm. A calibration graph was performed for quercetin which was used as standard flavonoid (R² =0.979) (fig.1B). The total flavonoid content was expressed in mg of quercetin equivalents (QE)/g DW of extract (Chang et al., 2002).

**Determination of saponnin**

About 5gm of plant sample was placed into a conical flask. And 50 ml 20% ethanol was added and then sample was heated for 4h. With continuous stirring at 55°C The mixture was filtrate and extracted. The concentration transfer into separating funnel and 10 ml diethyl was added and shaken vigorously. The aqueous of ether layer was discarded. The decontamination method was frequent and 20 ml of n-butanol was added. The combined n-butanol extract were washed twice with 10 ml of 5% aqueous sodium chloride .The remaining solution was heated in water bath for 40 min. After the evaporation the samples were dried and calculate the percentage of saponnin (Obadoni and Ochuko, 2001).

**Total tannin determination**

About 5gm of sample was weighed into a conical flask and 50 ml of D/W was added and then shaken thoroughly for 30 min in a mechanical shaker. Solution was centrifuge and filtered into a 50 ml volumetric flask. Take the supernatant and dilute to 100 ml D/W. This diluted supernatant was used for tannin estimation. 5 ml of the diluted extract was pipette out into a test tube and mixed with 1ml Folin-Ciocalteu reagent. Then addition of 2.5 ml saturated Sodium carbonate. Make up the volume of 50 ml incubate at room temperature (RT) for 90 min then read the absorbance at 760 nm by using Spectrophotometer (Chemito UV 2100) at 760 nm.

**Identification and quantification of the phenolic compounds by RP-HPLC**

The analysis of phenolic compounds was carried out by using High performance liquid chromatography (HPLC-DAD). The HPLC system (Shimadzu, Kyoto, Japan), equipped with Shimadzu LC20AD reciprocating pumps connected to the degasser DGU 20A and UV-VIS detector DAD (Diode array detector) SPD-M20A and software LC solution.

Reverse phase chromatographic analysis were carried out under gradient conditions using C18 column, the mobile phase was water containing 0.1% acetic acid (A) and methanol (B) and the composition gradient was 5% (B) for 1 min.; 10% (B) for 5 min.; 15% (B) for 10 min.; 40% (B) for 25 min.; 95% (B) for 45 min.; 5% (B) until 65 min. Stock solutions of standards references were prepared in the HPLC mobile phase at a concentration of 20 ppm. Quantification was carried out by integration of the peaks using the external standard method, at 272 nm for gallic acid and 370 nm for quercetin. The flow rate was 1ml/min and the injection volume was 20μl. The
Antioxidant activity
DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical scavenging assay

The antioxidant activity of the plant extracts in various solvent such as ethanol, methanol and acetone was estimated by using the DPPH radical scavenging protocol (Sutharsingh et al., 2011). The ability of scavenging DPPH radical was calculated using the following formula:

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

Hydrogen peroxide scavenging activity

The hydrogen peroxide scavenging activity of extracts was estimated by the method. Phosphate buffer saline (PBS) was used to prepare 20 mM hydrogen peroxide solution. Different concentrations of plant extracts and ascorbic acid as a standard (1ml) in ethanol, methanol and acetone solvents were added to 2 ml of H2O2 solution in PBS. The absorbance was measured after 10 min. at 230 nm against a blank. The hydrogen peroxide scavenging percentage was calculated using the following formula:

\[
% \text{ scavenged [Hydrogen peroxide]} = \left( \frac{A_0 - A_1}{A_0} \right) \times 100
\]

Where, \( A_0 \) was the absorbance of the control, \( A_1 \) was the absorbance in the presence of the sample in different solvents and standard as ascorbic acid (Chand et al., 2012).

Reducing power Assay

The determination of reducing power of extracts was completed as method. 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 40°C for 20 min., after cooling 10% trichloroacetic acid (2.5 ml) 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 but lacking of sample. The absorbance was measured at 700 nm. Higher is absorbance greater is the reducing power (Oyaizu, 1986).

Statistical analysis

The statistical analysis was carried out by using Microsoft Excel 2010 software. The determination was repeated at least three times for each sample.

RESULTS AND DISCUSSION

Total phenol and flavonoid content

It is reported that the antioxidant activity of plant origin components can be ascribed mainly due to the presence of phenolic compounds (Cai et al., 2004). The results obtained in the present study for quantitative investigation for phenols and flavonoids are presented in Table 1. The observations showed that in both the species total phenolic content was highest in methanol solvent extract followed by ethanol and acetone extract (Table-1). In \( L. \) microcephalum total phenolic content was ranged from 2.28±0.12 mg GAE/g DW to 3.35±0.8 mg GAE/g DW (Table 1). In present study the result exposed a considerable diversity in the total phenol after change in the solvent.

It is proved that flavonoids are plant nutrients that when consumed in the form of fruits and vegetables are non-toxic as well as potentially beneficial to the human body (Taiz et al., 2006). It is also noted that most of the flavonoidic compounds exhibit antipyretic, analgesic, anti-inflammatory, anti-arthritic, antioxidant and immune-modulatory properties. (Gill et al., 2011 and Wang et al., 2012). The amount of total flavonoid content of \( L. \) microcephalum in different solvent of plant extract assorted broadly ranging from 2.35±0.1 to 5.4±0.9 mg QE/g DW. The result exposed that higher level of flavonoid was found in ethanolic extract (5.4±0.9 mg QE/g DW) and lowest level of total flavonoids was estimated from acetone extracts (2.35±0.1 mg QE/g DW). Before the \( L. \) microcephalum was analyzed for its various phytochemicals including flavonoidic content and similar observations were also made by ( Mohansundaram et al., 2011). High level of flavonoids justifies the use of species as a source of vegetable since flavonoids are biological antioxidants and good for management of cardiovascular diseases. Total saponin content was maximum 03±0.12 (% W/W) and total tannin content was found maximum 03.45±0.18 (%W/W) in \( L. \) microcephalum plant extracts.
Table 1: Total phenol and flavonoid content in different solvent extract of *L. microcephalum*.

<table>
<thead>
<tr>
<th>SN.</th>
<th>Solvent</th>
<th>Total phenol content (mg/gm)</th>
<th>Total flavonoid content (mg/gm)</th>
<th>Total Tannin content (% w/w)</th>
<th>Total Saponnin content (% w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Methanol</td>
<td>3.35±0.8</td>
<td>3.04±0.21</td>
<td>0.33±0.34</td>
<td>0.31±0.34</td>
</tr>
<tr>
<td>2.</td>
<td>Ethanol</td>
<td>2.88±0.6</td>
<td>5.04±0.9</td>
<td>0.3±0.12</td>
<td>0.34±0.18</td>
</tr>
<tr>
<td>3.</td>
<td>Acetone</td>
<td>2.28±0.12</td>
<td>2.35±0.1</td>
<td>0.29±0.26</td>
<td>0.289±0.26</td>
</tr>
</tbody>
</table>

*Measurement are mean ± SE of three parallel determination and expressed as Gallic acid equivalent per gram dry weight.*

*Measurement are mean ± SE of three parallel determination and expressed as quercetin equivalents per gram dry weight.*

Figure 1: Standard curves: A- Standard curves of gallic acid, B- Standard curves of Quercetin.

**RP-HPLC Analysis of phenolic compounds**

In present days standardization and characterization of herbal drugs is a topic of scientific interest especially in the herbal drug industry. Reversed phase HPLC has been used in a number of occasions for the analysis of phenol and flavonoids in plants. In present investigations *L. microcephalum* showed the considerable total phenolic content and therefore considered for RP–HPLC analysis. The HPLC profile in different solvent extracts like methanol, ethanol and acetone were acquired based in the reference standards, in that gallic acid and quercetin was obtained with retention time (t_R) of 9.0 min. & 39.9 min. respectively (Fig. 2 D). Quercetin content in *L. microcephalum* was highest in acetone extract (0.425 ppm) followed by ethanol extract (0.905 ppm) and it was lowest in methanol extract (0.585 ppm) (Fig. 2 A-C) The sample was also screened for their gallic acid content but it was not detected in any studied sample in any solvent extract. They also screened many aromatic plant species from Greece for gallic acid content by using Reversed phase HPLC and noted that some aromatic plants do not detect the gallic acid (Proetos et al., 2005).

Figure 2: RP–HPLC Chromatograms of plant extract- *L. Microcephalum*: A- Acetone extract B- Ethanol extract C-Methanolic extract D - Standard phenolic compound at 20 ppm.
DPPH free radical scavenging activity
The hydrogen atoms or the electron donation ability of the extracts was measured from the bleaching of purple-colored ethanol solution of DPPH. As a very stable organic free radical with a deep violet color, DPPH gives maximum absorption at the range of 515 to 528 nm. DPPH antioxidant property of *L. microcephalum* were studied in different solvent extracts like methanol, ethanol and acetone and it was observed in all the extracts (Sultana et al., 2007).

In the results, *L. microcephalum* showed the highest DPPH free radical scavenging activity 51.27 ± 0.99% in methanolic extracts & lowest in acetone extracts 41.55 ± 0.97% (Fig. 3-A).

Reducing power
Reducing power has been used as an antioxidant capability indicator of medicinal herbs. The reducing power of different solvent extracts was performed and showed solvent and concentration dependant manner (Hsu CL and Chen W, 2003). In the present investigation *L. microcephalum* showed highest reducing power activity (60.34 ± 0.08%) in methanol extract and acetone extracts showed lower reducing power activity (51.42 ± 0.05%). The reducing power activity for the plant sample is shown in (Fig. 3-B).

Hydrogen peroxide free radical Scavenging activity
The extracts were capable of scavenging hydrogen peroxide in a concentration dependant manner. The radical scavenging activity of *L. microcephalum* plant extracts increased with increasing concentration (Fig.3-C). *L. microcephalum* showed highest hydrogen peroxide activity in ethanol extract i.e. 63.24 ± 0.12% and lowest activity for acetone extract i.e. 52.38 ± 0.20%. This indicates that both the plant species exhibited effective antioxidant activity.
CONCLUSION
From the above results we conclude that selected endemic medicinal plants viz., Lamprachaenium microcephalum possessed higher amount of Phytochemicals and a very good antioxidant activity for different assays tested, these plants forms a good base for the further research on the antioxidant ability and justifies its use in predictable medicines. This study also shows that the phenolic and flavonoid content also have an outstanding effect on the antioxidant activity.

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