Evaluation of anti-oxidant activity \textit{(in vitro)} of \textit{Heckeria subpeltata} (Willd.) Kunth. leaves

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\textit{Heckeria subpeltata} (Willd.) Kunth. the fresh bark, leaves and root of the plant are extensively used by the kurichia, adiya, kuruma and kattunaika tribes of Wayanad for curing piles, dysentery, diarrhea, arthritis, jaundice, kidney problems, leucorrhoea, menstrual problems, ear pain, blood clot etc. The objective of the present study is to examine the antioxidant activity of methanolic and water extracts of leaves of \textit{H. subpeltata} (Willd.) Kunth. The DPPH radical scavenging activity, Phosphomolybdenum assay, Fe (III) to Fe (II) reducing activity and the estimation of total phenol content of plant material are the methods used for the present study to determine the antioxidant activity. The DPPH radical scavenging activity 107.05 obtained at 517 nm for the methanolic leaf extract of \textit{H. subpeltata} (Willd.) Kunth is significant when compared to the commonly used butylated hydroxytoluene (BHA) (175 at 517 nm). The maximum value for phenol estimation, reducing power and antioxidant capacity of phosphomolybdenum was shown by the methanolic leaf extract of \textit{H. subpeltata} (Willd.) Kunth, which forms a potential source of natural antioxidant.

\textbf{Key words}: Antioxidants, reducing power, radical scavenging, piperaceae, traditional.

\section*{INTRODUCTION}

\textit{Heckeria subpeltata} (Willd.) Kunth is belonging to the family Piperaceae which has the common name Attanari (Sasidharan, 2004). The tribal people of Wayanad district Kerala called it as Pannippaperapill. The roots, stem and leaves are important ingredients of their medicines used for curing piles, dysentery and diarrhea, arthritis, jaundice, kidney problems, leucorrhoea, menstrual problems, ear pain, blood clot etc. It occurs in damp locations in evergreen forest undergrowth, swamp forest or river banks etc. This is a perennial scrambling shrub or woody herb that reaches 1-2.5 m tall. The stem is numerous, succulent, ribbed forming a dense clump with rooting at the nodules, while the main roots are woody. The leaves are arranged alternate almost circular to kidney shaped, with its apex shortly acuminate to round. Inflorescence emerges from the axils of the leaf. Brownish fruit is a drupe, obpyramidal. The seed is spherical.

\textit{H. subpeltata} (Willd.) Kunth is a neotropical plant species widely distributed in Mexico, Central America, South America and the West Indian Islands. It has also been introduced to Africa and South-East Asia. Traditional uses for this plant are recorded in 24 countries in three continents, America, Africa and Asia for a wide range of ailments such as kidney, women diseases, diarrhea, skin affections, burns, rheumatism, malaria, intestinal parasites, inflammation and fever. The cross-cultural agreement among traditional uses in different countries and found a high degree of consensus for the indications kidney/diuretic, stomachache and wounds.

Phytochemical studies of \textit{H. subpeltata} (Willd.) Kunth have demonstrated the presence of terpenes (mainly found in the essential oil), alkaloids, flavonoids, sterols and other classes of secondary metabolites. The extracts and pure compounds derived from this plant show a wide spectrum of pharmacological activities including antibacterial, anti-inflammatory, analgesic, antioxidant, cytotoxic, antimalarial, antileishmanial, and antitrypanosomal activity (Carles and Roersch, 2010). Oxidation is essential to many living organisms for the production of energy to fuel biological processes.
However, the uncontrolled production of oxygen derived free radicals is involved in the onset of many diseases such as cancer, rheumatoid arthritis and atherosclerosis as well as in degenerative processes associated with aging (Halliwell and Gutteridge, 1984). Almost all organisms are well protected against free radical damage by enzymes such as super oxide dismutase and catalase or compounds such as ascorbic acid, tocopheroles, and glutathione (Mau et al., 2002). There are some synthetic antioxidant compounds, such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA), commonly used in food processing. However, it has been suggested that these compounds have side effects (Ito et al., 1983). Nowadays, natural antioxidants have become one of the major areas of scientific research (Demo et al., 1998; Sanchez et al., 1999). Therefore, the importance of searching for exploiting natural antioxidants, especially of plant origin, has increased greatly in recent years. “Traditional medical practices are plant based.” The objective of the present study is to examine the antioxidant activity of methanolic and water extracts of leaves of H. subpeltata (Willd.) Kunth.

EXPERIMENTAL

Chemicals

0.004% DPPH in ethanol, 0.6M H₂SO₄, 28 mM ammonium molybdate, ascorbic acid, phosphate buffer, 1% K₂ Fe (CN)₆ potassium hexacyanoferrate, trichloroacetic acid, 0.1% FeCl₃, 20% Na₂CO₃, folin ciocalteau reagent, standard gallic acid and butylated hydroxyanisole. (All chemicals used were analytical grade and obtained from either Sigma –Aldrich or Merck.

Plant material

Fresh leaves of H. subpeltata (Willd.) Kunth. were collected from Mananthavady taluk of Wayanad district, Kerala in the month of April-2008. The designation of plants was confirmed by the taxonomists in M.S Swaminathan Research Foundation Kalpetta. Voucher specimens were deposited in the Environmental Science Department of Mysore University.

Sample preparation

Aqueous and methanol extracts were prepared by homogenizing fresh samples of leaves with a mortar and pestle in the respective solvents to a concentration of 0.01 g/ml. The samples were kept in solvents for 3 days at 4°C. The extracts were centrifuged at 5000 rpm for 10 min and the supernatant were used for the in vitro assay for antioxidant potential.

DPPH assay

Ability of extracts to scavenge DPPH-free radical was determined by the method of Braca et al. (2001). An aliquot of the sample (0.5 ml) is taken and made up to 5 ml with alcohol and then mixed well with 1.0 ml of 0.004% DPPH solution (in ethanol, prepared fresh just before the assay). The tubes are incubated in dark for 30 min and absorbance read at 517 nm.

\[
\text{Calculation: DPPH radical scavenging capacity (%) =} \frac{(\text{ABS of control} - \text{ABS of sample})}{\text{(ABS of control)}} \times 100
\]

Total antioxidant capacity by phosphomolybdenum method

Antioxidant capacities of sample extracts were evaluated by the method of Prieto et al. (1999). An aliquot of each sample is made up to 3 ml with ethanol and combined with 1 ml of reagent solution (0.6 M sulfuric acid, 28 mM Sodium sulphate and 4 mM ammonium molybdate). Ethanol (0.1 ml) is used, in place of sample solution, for the blank. The tubes were capped and incubated in a boiling water bath at 95°C for 90 min. After the samples were cooled to room temperature, the absorbance of the aqueous solution of each was measured at 695 nm against a blank using ascorbic acid as standard. Antioxidant capacity expressed as ascorbic acid equivalents (µmol/g).

Fe (III) to Fe (II) - reducing activity

Fe (III) reducing activity was measured by the method of Oyaizu (1986). An aliquot of each extract dissolved in water, was mixed with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of 1% aqueous potassium hexacyanoferrate solution. After 30 min incubation at 50°C, 2.5 ml of 10% trichloroacetic acid were added, and the mixture was centrifuged for 10 min. a 2.5 ml aliquot of the upper layer was mixed with 2.5 ml of water and 0.5 ml of 0.1% aqueous ferric chloride. The absorbance was measured at 700 nm. Fe (III) reducing activity determined as ascorbic acid equivalents (mmol ascorbic acid/g extract).

Total phenol Estimation

The total phenol content is estimated by the method of Singleton et al. (1999). An aliquot of each extract dissolved in water, was mixed with 250 ml of folin-ciocaltean reagent (undiluted) and allowed to stand for 1 min and added 750 µg of 20% sodium bicarbonate was added and incubated for two hours and the absorbance was measured at 760 nm. Antioxidant capacity is expressed as gallic acid equivalents (µg gallic acid /gm fresh weight).

RESULTS

In recent years, the use of some synthetic antioxidants has been restricted because of their possible toxic and carcinogenic effect (Frankel et al., 1995; Gazzani et al., 1998). This concern has resulted in the investigation of the effectiveness of the naturally occurring compounds with antioxidant properties (Duh et al., 1992; Miyake and Shibamoto, 1997; Yen et al., 1996). Leaves of H. subpeltata (Willd.) Kunth. is widely used by the kuricha tribes of Mananthavady taluk, Wayanad district, Kerala for curing piles, dysentery, diarrhea, worm infections, heart problems and arthritis. It is also used as a vegetable and veterinary medicine to cure digestive problems in cattle. Table 1 shows the mode of application of leaves by the Kuricha tribes in this locality. Due to
Table 1. The mode of application of leaves of *Heckeria subpeltata* (Willd.) Kunth and the therapeutic uses by the kuricha tribes in Mananthavady taluk, Wayanad District, Kerala.

<table>
<thead>
<tr>
<th>S/N</th>
<th>The mode of application</th>
<th>Therapeutic use</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>The grinded leaf is applied on the affected area.</td>
<td>Piles</td>
</tr>
<tr>
<td>2</td>
<td>The dried leaf powder is used to prepare massaging oil which is smeared on the affected part. Rubbing of leaf in the affected area.</td>
<td>Arthritis, Dysentery, diarrhea, pinworm infection</td>
</tr>
<tr>
<td>3</td>
<td>Intake of young leaf juice</td>
<td>Jaundice, kidney problems, leucorrhoea, menstrual problems</td>
</tr>
<tr>
<td>4</td>
<td>Intake of leaf decoction</td>
<td>Ear pain, Blood clot</td>
</tr>
<tr>
<td>5</td>
<td>The young leaf juice as ear drop</td>
<td>Good health</td>
</tr>
<tr>
<td>6</td>
<td>An ingredient in the medicine to remove blood clot.</td>
<td>Digestive problems in cattle</td>
</tr>
<tr>
<td>7</td>
<td>Boiled leafy Vegetable</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Simple eating of the fresh leaves.</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>The fresh leaf paste along with required quantity of turmeric paste and calcium carbonate is applied on the affected area.</td>
<td>Maggot wound</td>
</tr>
</tbody>
</table>

Table 2. Showing The DPPH radical scavenging activity, antioxidant capacity by phosphomolybdennum method (Ascorbic acid used as standard), Fe (III) to Fe (II) - reducing activity (Aliquot taken -0.5ml, Ascorbic acid used as standard) and the total phenol content (Aliquot taken -0.5ml, Gallic acid used as standard) of leaf extracts of HSP (*Heckeria subpeltata*) (Sample concentration-0.01 g/ml).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Methanolic leaf extract of (HSP)</th>
<th>Water extract of leaf of (HSP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPPH Radical scavenging</td>
<td>107.05%</td>
<td>53.714%</td>
</tr>
<tr>
<td>Antioxidant Capacity by Phosphomolybdennum method:</td>
<td>743.63 mM ascorbic acid equivalent/g fresh leaf weight</td>
<td>293.5 mM ascorbic acid equivalent/g fresh leaf weight</td>
</tr>
<tr>
<td>Fe (III) to Fe (II) - reducing activity</td>
<td>26.12 mM ascorbic acid equivalent/g fresh leaf weight</td>
<td>19.29 mM ascorbic acid equivalent/g fresh leaf weight</td>
</tr>
<tr>
<td>Total phenol</td>
<td>23.02 mg/g fresh leaf.</td>
<td>12.25 mg/g fresh leaf.</td>
</tr>
</tbody>
</table>

In the present investigation the methanolic and water extracts of leaves of *H. subpeltata* (Willd.) Kunth were subjected to screening for their possible antioxidant activity by performing these four assays.

DPPH radical scavenging activity

Highly reactive free radicals are present in biological system from a wide variety of sources. The free radical scavenging activity of antioxidants in food has been substantially investigated and reported in the literature by Miller et al. (2000). The radical scavenging activities of the extracts were determined by using DPPH stable free radical. The main characteristic of an antioxidant is its ability to trap free radicals. The antioxidant donates protons to these radicals and absorption decreases. The decrease in absorption is taken as the measure of the extend of radical...
scavenging. Here the DPPH radical scavenging activity 107.05% at 517 nm for the methanolic leaf extract of *H. subpeltata* (Willd.) Kunth is significant when compared to the commonly used BHA (175% at 517 nm). But the water extract of the leaf of this plant showed only 53.715% DPPH radical scavenging activity at 517 nm. Methanolic leaf extract of *H. subpeltata* (Willd.) Kunth may be considered as an antioxidant for the preparation of medicine against many diseases and related to this plant further phytochemical investigations needed.

**Total antioxidant capacity by Phosphomolybdenum method**

Antioxidants in food play an important role as a health protecting factor. Scientific evidence suggests that antioxidants reduce the risk for chronic diseases including cancer and heart diseases. Primary source of naturally occurring antioxidants are whole grains, fruits and vegetables. Total quantitative determination of antioxidant capacity of leaf extracts of *H. subpeltata* (Willd.) Kunth were evaluated by Phosphomolybdenum method. The measurement of absorbance of each extracts at 695 nm showed significant values for methanolic extract of this plant (743.6 µg ascorbic acid equivalents/gm fresh leaf weight) (Table 2).

**Fe (III) to Fe (II) - reducing activity**

In Fe (III) to Fe (II) - reducing activity experiments the increased absorbance values denote the increased reducing ability of plant extracts by converting Fe³⁺ to Fe²⁺. The methanolic extract of *H. subpeltata* (Willd.) Kunth leaves showed 26.12 mM ascorbic acid equivalents/gm fresh leaf weight. While the water extract of the leaves have comparatively less (19.29 mM ascorbic acid equivalents/gm fresh leaf weight) reducing power (Table 2).

**Total phenol estimation**

Phenolic moieties present in the molecular structure of natural antioxidants often help in enhancing their antioxidant activity (Kakhkonen et al., 1999; Frankel et al., 1995). The phenol content of plant extracts were measured based on the method of Singleton et al. (1999) at 760 nm. The methanolic extract of *H. subpeltata* (Willd.) Kunth has the maximum value (23.02 mg/gm fresh leaf). And water extract of leaves got a value which is 12.25 mg/g fresh leaf. Table 2 showing The DPPH radical scavenging activity, antioxidant Capacity by Phosphomolybdenum method (Ascorbic acid used as standard), Fe (III) to Fe (II) — reducing activity (Aliquot taken -0.5 ml. Ascorbic acid used as standard) and the total phenol content (Aliquot taken -0.5 ml. Gallic acid used as standard) of leaf extracts of HSP (*H. subpeltata*) (Sample concentration-0.01 g/ml).

**DISCUSSION**

The combination of four methods applied in this study gave valuable information in the evaluation of the antioxidant activity of *H. subpeltata* (Willd.) Kunth leaf extracts and could be recommended for other similar investigations. In the present study, the methanolic leaf extracts of this plant exhibited the highest total phenolic content 23.02 mg/g fresh leaf, highest ferric to ferrous reducing activity 26.12 mM Ascorbic acid equivalents/gm fresh leaf weight, highest DPPH radical scavenging activity 107.05% at 517 nm and showed the highest antioxidant capacity, 743.6 µg ascorbic acid equivalents/gm fresh leaf weight at 695 nm in Phosphomolybdenum method. The results revealed that the reducing power, antiradical scavenging activity, and the antioxidant capacity of methanolic leaf extract of this plant increases with the increased total phenolic content present in it and the water extract with low phenolic content showed comparatively less activity to all these parameters. This is in agreement with the recent reports that the antioxidative effect is mainly due to phenolic components, such as phenolic acids, and phenolic diterpenes (Pourmorad et al., 2006).

Previous phytochemical investigations revealed that the essential oil from the aerial parts of *H. subpeltata* (Willd.) Kunth has a high content of β-pinene (27%), α-pinene (18%), (E)-nerolidol (12%) and β-caryophyllene (10%). Other compounds found include safrole, germacrene-D, β-cadinene, δ-cadinene and bicyclogermacrene. The roots and aerial parts contain 4-nerolidylcatechol, a powerful antioxidant with chemo preventative potential. This may explain the traditional use of *H. subpeltata* (Willd.) Kunth in the treatment of skin cancer. Four alkaloids named piperumbellactams A–D were isolated from branches of *H. subpeltata* together with known N-hydroxyaristolom ll, N-p-coumaroyl tyramine, 4-nerolidylcatechol, N-trans-feruloyltyramine, E-3-(3,4-dihydroxyphenyl)-N-2-[4-hydroxyphenylethyl]-2-propenamide, β-amyrin, friedelin, apigenin 8-C-neohesperidoside, acacetin 6-C-β-d-glucopyranoside, β-sitosterol, its 3-O-β-d-glucopyranoside and its 3-O-β-d-[6′-dodecanoyl]-glucopyranoside. Glycosidase inhibition, antioxidant and antifungal activities of these compounds were also evaluated. Studies using methanol extract of the leaves of *H. subpeltata* (Willd.) Kunth showed significant anti-malarial activity against *Plasmodium falciparum in vitro*. (Turibio et al, 2008).

The results of antioxidant activity in this investigation and the various phytochemical studies done by researchers agreed a certain extent with the traditional
therapeutic uses of tribal medicines in Mananthavady taluk, Wayanad district, Kerala. The results form a good basis for the selection of plants for further in vivo investigations and the preparation of medicines for various ailments.

Conclusion

The interesting biological activities of *H. subpeltata* (Willd.) Kunth need further research in in vivo experiments and clinical studies. The outcome of these investigations will determine the possible development of drugs from this plant.

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