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Original Article

ANTIOXIDANT ACTIVITES AND PHYTOCHEMICAL ANALYSIS OF METHANOL EXTRACT OF LEAVES OF *HYPERICUM HOOKERIANUM*

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ABSTRACT

Objective: The present investigation was an attempt to evaluate the phytochemical constituents, free radical scavenging activities and antioxidant properties of the methanol extract of leaves of *Hypericum hookerianum*.

Methods: The total antioxidant activity was assayed by DPPH free radical scavenging assay method. The phytochemicals in the methanol extract of leaves of *Hypericum hookerianum* were determined qualitatively and quantitatively using standard methods.

Results: The study indicated that the presence of most of the major phytochemicals *viz.* flavonoids and phenols in the methanol extract of leaves of *H. hookerianum* were 280mg/g and 128mg/g respectively. The DPPH radical scavenging activity of methanol extract of leaves of *H. hookerianum* was recorded as 90.24% and chelating activity of methanol extract was recorded as 62.92% at 21µg/mL concentration. The ferric reducing power assay showed maximum activity of 0.84 and also the phosphomolybdenum reduction assay showed maximum activity of 0.069 at 21µg/mL concentration which were compared with the standards. The IC_{50} of methanol extract of leaves of *H. Hookerianum* for DPPH and Fe^{2+} chelating assay were 3.03 and 13.95 µg/mL respectively and showed significant results when compared with the standards.

Conclusion: The present study revealed that the leaves of methanol extract of *H. hookerianum* showed the highest antioxidant activity as well as phenolic content. This study suggests that the methanol extract of leaves of *H. hookerianum* is a potential source of natural antioxidants.

Keywords: Phytochemical; Methanol extract; Antioxidant; DPPH*; Hypericum hookerianum*

INTRODUCTION

The herbs used in traditional medicine are the goldmine for current therapeutics. According to World Health Organization (WHO) statistics more than three forth of the people in the developing world depends on such medicines. The expensive treatments, adverse effects of several allopathic drugs and development of resistance to existing drugs have forced us to look back into nature as a resource for therapeutics to a wide variety of human ailments. Herbs are turning to be a replacement for synthetics. The herbal products today are considered to be safer to human and environment. *Hypericum hookerianum* belongs to the family of *Hyperiaceae* is a well known plant for treating depression, wound healing and inflammation [1]. The medicinal herb, *Hypericum hookerianum* has a phyto constituent named Has such important constituents as hypericin, a naphthodianthrone and hyperforin, a phloroglucinol derivative which belongs to group of compounds known as napthodianthrones [2]. These secondary metabolites have been used to improve sleep, because it increases the brain's output of melatonin at night. Topically hyperforin has anti-inflammatory properties [3, 4]. Free radicals induce oxidative stress, cause protein and DNA damage. This is the primary cause of ailments.

To protect against this oxidative stress, human body have their own system including various enzymes, proteins and vitamins, which are known as antioxidants. Aging declines the production of such antioxidants hence it requires external source of antioxidants to defend free radicals [5]. Plants are good sources of natural antioxidants which provide protection against harmful free radicals and have been strongly associated with reduced risk of chronic diseases [6]. The antioxidant activity of these natural phyto products is due to their redox properties, which allow them to act as reducing agents, hydrogen donators, singlet oxygen quenchers, and metal chelators [7].

MATERIALS AND METHODS

Chemicals and reagents: All the chemicals used in the study were of analytical grade and procured from Merck India Pvt. Ltd.

Plant material

The plant material in this study was collected from Kodaikanal, Western Ghats of India. The plant was authenticated by Dr. A. G. Pandurangan, Systemic Botanist. The collected plant in Fig. 1 was subjected to shade drying for about 5 weeks. The dried plant material was powdered mechanically and sieved and stored in air tight container for further analysis.

Fig. 1: Habitat of *Hypericum hookerianum*

Preparation of the extract: The shade dried leaves of *Hypericum hookerianum* was extracted with methanol and concentrated at room temperature. The yield of methanol extract was calculated as 6.70 %.

Qualitative phytochemical analysis

Chemical tests for screening and identification of bioactive chemical constituents present in the methanol extract of leaves of *H. hookerianum* were carried out using the standard procedures [8-10].

Quantitative phytochemical estimations

1. **Estimation of total phenol by Folin-Ciocalteu reagent method**

Total phenolic compound contents were determined by the Folin-Ciocalteau method [11] with slight modifications. The extract samples (1 mL of different dilutions) were mixed with Folin Ciocalteu reagent (1 mL, 1:10 diluted with distilled water) for 5 min and aqueous Na_2CO_3 (1 mL, 1 M) were then added. The mixture was allowed to stand for 15 min and the phenols were determined by colorimetric method at 765 nm. Gallic acid was used to prepare standard curve. The total phenolic content was expressed in terms of gallic acid equivalent (mg/g of dry mass), which is a common reference compound.

2. **Estimation of total flavonoid by $AlCl_3$ method**

Aluminium chloride colorimetric method was used to determine the total flavonoid [12] with slight modifications. 1 mL of methanol extract of leaves of *H. hookerianum* was mixed with 0.5 mL of 10 % aluminium chloride, 0.5 mL of 1M potassium acetate and 1 mL of distilled water. It was incubated at room temperature for 30 min. The absorbance of the reaction mixture was measured at 415 nm with Dual 40- Beckmann spectrophotometer. The total flavonoid content was expressed as in terms of quercetin equivalent which is common reference standard.

Antioxidant activites

1. DPPH radical scavenging assay

The antioxidant activity of methanol extract of leaves of *H. hookerianum* was measured on the basis of the scavenging activity of the stable 1, 1- diphenyl 2-picrylhydrazyl (DPPH) free radical according to the method described by Brand-Williams *et al.,* (1995) with slight modifications [13, 14]. 1 mL of 0.1 mM DPPH solution in methanol was mixed with 1mL of various concentrations (3-21 µg/mL) of methanol extract of leaves of *H. hookerianum*. Quercetin was used as the reference standard. Mixer of 1mL methanol and 1mL DPPH solution was used as the control. The decrease in absorbance was measured at 517nm after 30 minutes in dark using UV-Vis spectrophotometer. The inhibition % was calculated using the following formula.

% of inhibition = Control-Sample/Control×100

2. Fe^{2+} chelating assay

The chelation of Fe^{2+} by methanol extract of leaves of *Hypericum hookerianum* was estimated by the method of Dinis *et al*. (1994) [15,16]. Methanol extract was added in different concentrations (3 to 21 µg/mL) to a solution of 1mM $FeCl_2$ (0.05 mL). The reaction was initiated by the addition of 1 mM Ferrozine (0.1 mL) and the mixture was shaken vigorously and left standing at room temperature for 10 min. After the mixture had reached equilibrium, the absorbance of the solution was then measured spectrophotometrically at 562 nm. EDTA was used as the standard reference.

% of inhibition = Control-Sample/Control×100

3. Ferric (Fe^{3+}) reducing power assay

The reducing power assay of methanol extract of leaves of *H. hookerianum* was determined by slightly modified method of Oyaizu, 1986 [17]. 1 mL of each plant extract concentration (3-21 mg/mL) was mixed with phosphate buffer (1 mL, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (1 mL, 1 %). The mixtures were then incubated at 50°C for 20 min. 1 mL of trichloroacetic acid (10 %) was added to each mixture, which were then centrifuged for 10 min at 1036 x g. The upper layer of the solutions (1 mL) were mixed separately with distilled water (1 mL) and $FeCl_3$ (0.5 mL, 0.1 %), and the absorbance were measured at 700 nm using a spectrophotometer. Ascorbic acid was used as the standard reference.

4. Phosphomolybdenum reduction assay

The total antioxidant capacity of the methanol extract of leaves of *H. hookerianum* was evaluated by the phosphomolybdenum reduction assay method according to the procedure described by Prieto *et al*. 1999 [18]. The assay is based on the reduction of Mo (VI) to Mo (V) by the methanol extract of leaves of *H. hookerianum* and subsequent formation of green phosphate/Mo (V) complex at acid pH. 1mL of various concentrations (3-21 µg/mL) of extract was combined with 1 mL of reagent solution (0.6M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) and incubated at 95°C for 90 min. The absorbance of the reaction mixture was measured at 695 nm using a spectrophotometer.

RESULTS

Table 1: Qualitative estimation of phenols and flavonoids of methanol extract of leaves of *Hypericum hookerianum*

S.No	Phytoconstituents	Chemical reagents	Results
1.	Alkaloids	1. Dragendorff's Reagent	++
		2. Wagner's reagent	++
2.	Terpenoids	$CHCl_3$ + conc. H_2SO_4	++
3.	Steroids	Libermann-Burchard's reagent	++
4.	Phenols	$FeCl_3$ solution	++
5.	Flavonoids	1.Shinoda (NaOH solution) test	++
		2. Magnesium filaments+ HCl test	
6.	Glycosides	1. Fehling's test	++
		2. Molish's test	++
7.	Saponins	Foam test	++

++ Strongly present

Table 2: Quantitative estimation of phenols and flavonoids of methanol extract of leaves of *Hypericum hookerianum*

S. No.	Components	Concentration, mg/g
1.	Phenols	280
2.	Flavonoids	128

Antioxidant-based drug formulations are used for the prevention and treatment of complex diseases like atherosclerosis, stroke, diabetes, Alzheimer's disease and cancer [19].The methanol extract of leaves of *H. hookerianum* was subjected to various qualitative chemical tests [20]. The methanol extract showed the presence of phytochemical constituents such as alkaloids, terpenoids, steroids, phenolic compounds, flavonoids, glycosides and saponins as depicted in Table 1. The qualitative analysis showed the major

presence of flavonoids and Phenols 280mg/g and 128mg/g respectively as depicted in Table 2. The antioxidant activity of methanol extract of leaves of *H. hookerianum* was evaluated by DPPH free radical scavenging assay, Fe^{2+} chelating assay, Ferric (Fe^{3+}) reducing power assay and Phosphomolybdenum reduction assay methods and the results were presented in Fig 3-6.

The increase in percentage of DPPH radicals scavenging activity as well as Fe^{2+} chelating activity increases with increase in concentrations of methanol extract of leaves of *H. hookerianum*. The maximum DPPH radical scavenging activity showed the % of inhibition was 90.24 and the standard quercetin was 95.27 at 21µg/mL concentration. The maximum chelating activity of methanol extract of leaves of *H. hookerianum* showed the % of inhibition was 62.92and the standard EDTA was 89.48 at 21µg/mL concentration.

The ferric reducing power assay showed maximum activity of 0.84 and also the phosphomolybdenum reduction assay showed maximum activity of 0.069 at 21µg/mL concentration, which were compared with the standards showed the activity of 0.52 and 0.082 respectively. The IC_{50} of methanol extract of leaves of *H. hookerianum* for DPPH and Fe^{2+} chelating assays were 3.03 and 13.95 µg/mL respectively, which were given in Fig 7.

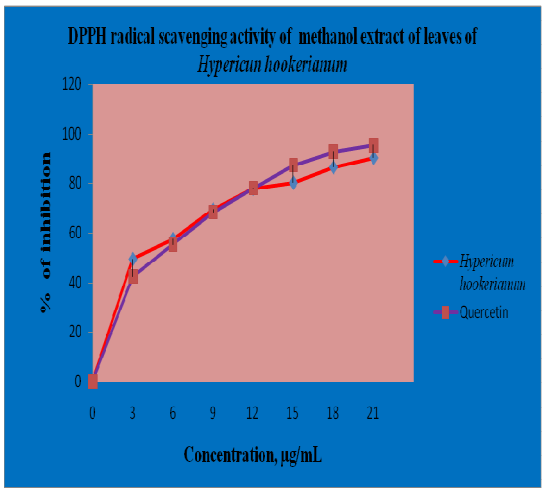

Fig. 2: DPPH radical scavenging assay of methanol extract of leaves of *Hypericum hookerianum*

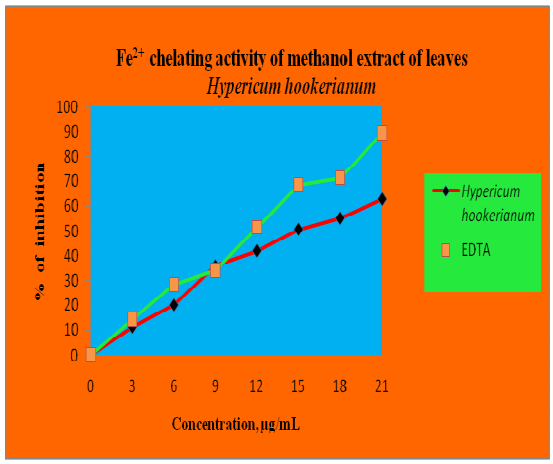

Fig. 3: Fe^{2+} chelating assay of methanol extract of leaves of *Hypericum hookerianum*

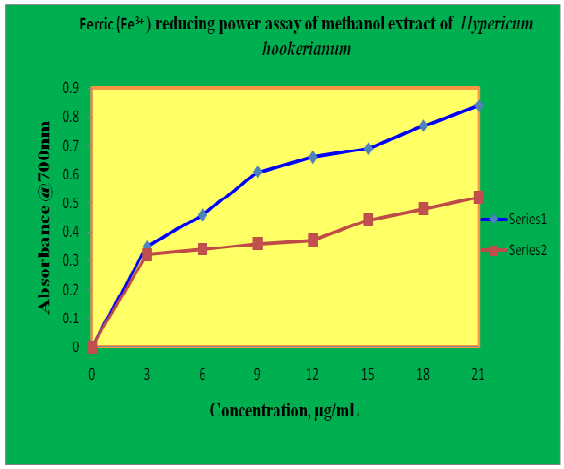

Fig. 4: Reducing power assay of methanol extract of leaves of *Hypericum hookerianum*

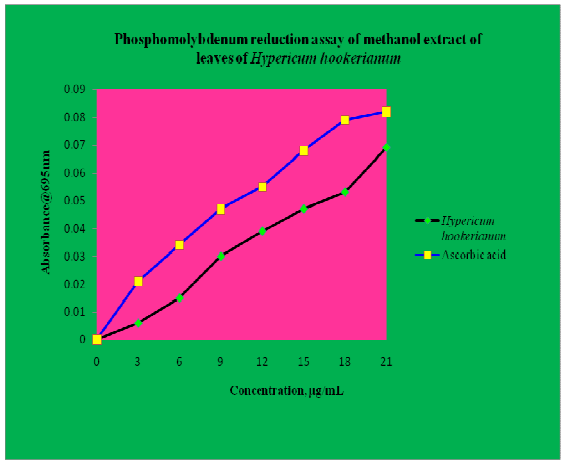

Fig. 5: Phospho molybdenum reduction assay of methanol extract of leaves of Hypericum *hookerianum*

DISCUSSION

Phenolic substances and flavonoids are associated with antioxidant activity and play important role in stabilizing lipid peroxidation [21, 22] by adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides [23,24]. Present study entailed that methanol extract of leaves of *H. Hookerianum* showed higher phytochemicals. Phenolic compounds and flavonoids are widely distributed in plants. The total phenolic content was estimated by Folin-Ciocalteu method and gallic acid was used as the standard reference. The total flavonoid content was estimated by $AlCl_3$ method and quercetin was used as the standard reference. DPPH radicals scavenging assay is widely used to investigate the total antioxidant activity in plants along with Fe^{2+} chelation assay, ferric reducing power assay and phosphomolybdenum reduction assay.

DPPH radical scavenging activity increased with increasing phenolic components such as flavonoids, phenolic acids and phenolic diterpenes. These phenolic components possess many hydroxyl groups including o-dihydroxy group which have very strong radical scavenging effect and antioxidant power. In DPPH assay, the antioxidants present in methanol extract of leaves of *H. Hookerianum* was able to reduce the violet coloured stable 1, 1-diphenyl-2-picryl hydrazyl radical to the yellow coloured 1, 1-diphenyl-2-picryl hydrazine. The molecule of 1, 1-diphenyl-2-picrylhydrazine is characterised as a stable free radical by virtue of the delocalisation of the spare electron over the molecule as a whole.

The dose response curve of DPPH radical scavenging activity of methanol extract of leaves of *H. Hookerianum* was observed and shown in Figure 2. The IC_{50} of methanol extract of leaves of *H. Hookerianum* for DPPH radical scavenging activity was 3.03 µg/mL concentration.

The transition metal ion, Fe^{2+} possess the ability to move single electrons by virtue of which it can allow the formation and propagation of many radical reactions, even starting with relatively non-reactive radicals[16]. The main strategy to avoid ROS generation that is associated with redox active metal catalysis involves chelating of the metal ions. Ferrozine can quantitatively form complexes with Fe^{2+} ion. However, in the presence of chelating agents, the complex formation is disrupted with the result that the red colour of the complex is decreased. Measurement of colour reduction, therefore, allows the estimation of the chelating activity of the coexisting chelator. *H. hookerianum* the most active extract interfered with the formation of ferrous and ferrozine complex, suggesting that it has chelating activity and captures ferrous ion before ferrozine. The dose response curve of Fe^{2+} chelating activity of methanol extract of leaves of *H. Hookerianum* was observed and shown in Figure 3. The IC_{50} of methanol extract of leaves of *H. Hookerianum* for chelating activity was 13.95 µg/mL. The DPPH radical scavenging activity and Fe^{2+} chelating activity showed significant results.

Reducing power is associated with antioxidant activity and may serve as a significant reflection of the antioxidant activity. Compounds with reducing power indicate that they are electron donors and can reduce the oxidized intermediates of lipid peroxidation processes, so that they can act as primary and secondary antioxidants. In this assay, the yellow colour of the test solution changes to various shades of green and blue depending on the reducing power of methanol extract of leaves of *H. Hookerianum*. Presence of reducers causes the conversion of the Fe^{3+}/ferricyanide complex to ferrous form. Fe^{+3} reduction is often used as an indicator of electron- donating activity, which is an important mechanism of phenolic antioxidant action, and can be strongly correlated with other antioxidant properties [25]. By measuring the formation of Pearl's Prussion blue at 700 nm, it is possible to determine the concentration of ferrous ions. Increase in absorbance of the reaction mixture indicated increase in reducing power of the extract was shown in Figure 4. The reducing power assay showed greater reduction capacity compared with the standard ascorbic acid. Hence the methanol extract of leaves of *H.hookerianum* proved to be potential for the antioxidant phytoconstitutents.

The Phosphomolybdenum reduction assay was based on the reduction of Mo(VI) to Mo(V) in presence of antioxidant compound and subsequent formation of a green phosphate/Mo(V) complex at acidic pH and at higher temperature. The Phosphomolybdenum reduction assay increases with increase in concentration of methanol extract of leaves of *H.hookerianum* was shown in Figure 5.

CONCLUSION

Phenolic compounds are secondary plant metabolites that possess on their structure a benzene ring substituted by, at least, one hydroxyl group [26]. These compounds are ubiquitously distributed in the plant kingdom and exhibit a wide range of pharmacological and medicinal properties, including anti-inflammatory, anti-carcinogenic, vasodilatory actions [27]. These protective effects have been mostly ascribed to their free radical scavenging, and antioxidant action. Antioxidant is one of the vital for preventing *in vivo* oxidative damages. Plants are the good resources for natural antioxidants. The present study indicates the presence of alkaloids, glycoside, saponin, phenols and flavonoids during the phytochemical investigation. The plant *H. hookerianum* was used to evaluate the antioxidant potential using DPPH free radical scavenging assay, Fe^{2+} chelating assay, Ferric (Fe^{3+}) reducing power assay and Phosphomolybdenum reduction assay methods. The methanol extract of *H.hookerianum* showed higher phenolic content as well as flavonoid content and contributes to the higher antioxidant activity. Considering the results obtained it could be concluded that the plant contains essential phytochemical constituents and possess active antioxidant property. Further investigations may be carried out to find active component of the extract and to confirm the mechanism of action. The antidepressant activity in *Hypericum perforatum* has been extensively studied but in *Hypericum hookerianum* the antioxidant activity has not been much explored.

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