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Full Length Research Paper

Phytochemical analysis and antioxidant activity of herbal plant *Doronicum hookeri* Hook f. (Asteraceae)

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In this study, *Doronicum hookeri* Hook f. roots were extracted with two solvents of different polarity and evaluated for their *in vitro* antioxidant activities. Of the two extracts, methanolic extract possessed higher phenolic content and thus higher free radical scavenging and reducing activities. In DPPH (2,2-diphenyl-1-picryhydrazy) radical scavenging assay, methanolic extract showed scavenging similar to the standard BHT(butylated hydroxytoluene) (~85%) at concentration 0.5 mg/ml. Methanolic extract also exhibited more than 90% inhibition to ABTS radicals at concentration above 0.3 mg/ml. Reducing power activity of methanolic extract was also higher than dichloromethane extract. Dichloromethane extract was however rich in flavonoids and showed considerable metal chelating (78.684±0.659% at 0.5 mg/ml), nitric oxide (52.232±0.934% at 0.15 mg/ml) and superoxide radical (59.882±0.772% at 0.5 mg/ml) scavenging activities.

Key words: Antioxidant activity, *Doronicum hookeri*, ferric reducing antioxidant power, flavonoids, flavonol, metal chelating activity, nitric oxide radical scavenging, phenols, phytochemistry, superoxide radical scavenging.

INTRODUCTION

Free radicals are atoms or group of atoms that have at least one unpaired electron, making them highly reactive. The potentially reactive derivatives of oxygen are known as reactive oxygen species (ROS) (e.g. superoxide anions, hydrogen peroxide and hydroxyl, nitric oxide radicals), and play an important role in oxidative damage to various biomolecules including proteins, lipids, lipoproteins and DNA, related to the pathogenesis of various important diseases such as diabetes mellitus, cancer, atherosclerosis, arthritis, and neurodegenerative diseases and also in the ageing process (Farber, 1994; Halliwell and Gutteridge, 1999). Antioxidants prevent the oxidative damage by directly reacting with ROS, quenching them and/or chelating catalytic metal ions and also by scavenging free oxygen (Buyukokuroglu et al., 2001; Robak et al., 1995; Shahidi et al., 1992). Since ancient times, many herbs have been potentially used as an alternative remedies for treatment of many infections, diseases and as food preservatives suggesting the presence of antimicrobial and antioxidant constituents (Tatjana et al., 2005).

Doronicum hookeri Hook f. belonging to family Asteraceae is an important medicinal drug and distributed in Himalayas at Lachen and Tungu, and Sikkim, Nepal, Bhutan, Tibet between 12,000 to 14,000 ft (Khare, 2007). Its roots are widely used as folk medicine and are commonly known as “Darunaj-aqrabi” in Unani and as “Leopard’s bane” in English (Figure 1). Roots are traditionally used as a constituent of cardiac and nervine tonics, exhilarant and act as a stomachic and dissolves trapped gases (Khare, 2007). Antibacterial and antifungal activities of *D. hookeri* roots have been previously reported (Kumar et al., 2006; Verma et al., 2008).

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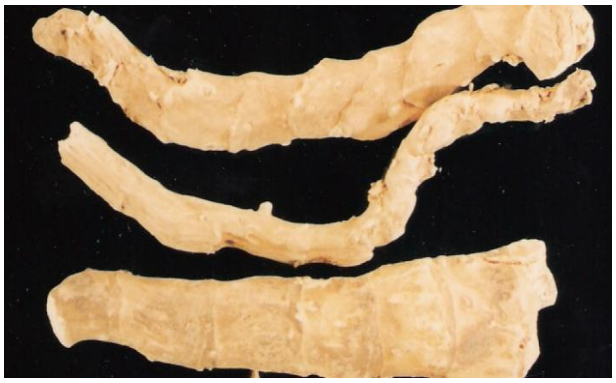

Figure 1. *D. hookeri* Hook. f. roots.

Chemical constituents of *D. hookeri* have not been investigated yet. In view of the limited data on *D. hookeri*, it was the aim of this study to attempt to provide further information on the phenolic content and antioxidant activities of this plant using several antioxidant assays.

MATERIALS AND METHODS

Plant material and chemicals

D. hookeri roots were purchased from supplier of traditional unani medicines, Ballimaran, Delhi, India, in August 2008. Samples were deposited and authenticated at National Institute of Science Communication and Information Resources (NISCAIR), New Delhi, India, (NISCAIR/RHMD/Consult/2008-09/1069/100). All chemicals and reagents used were of analytical grade and obtained mostly from Sigma®. The polyphenolic standards, gallic acid, catechin and rutin were obtained from the same provider.

Preparation of extracts

Finely ground samples (200 g) of roots of *D. hookeri* (DH) were extracted with dichloromethane and methanol using a soxhlet assembly for 6 h for each solvent. Extracts were filtered and concentrated under vacuum in a rotary evaporator. The two extracts were abbreviated as: Dichloromethane extract (DHDCM), methanolic extract (DHME).

Determination of total phenolics, flavonoids and flavonol content

Total phenolic content of extracts were estimated using a modified method of Yu et al. (2002) based on Folin-Ciocalteu reagent. The concentration of total phenolic compounds in different extracts was expressed as mg of gallic acid equivalents (GAE)/ g of dried extract, using a standard curve of gallic acid (concentration range, 0.002 to 0.01 mg/ml), described by the equation $y = 0.0265x$ ($R^2 = 0.9977$).

Measurement of total flavonoid content in the investigated extracts was determined spectrophotometrically according to Zhishen et al. (1999). The flavonoids content was expressed as mg of Rutin equivalents (RE)/g of dried extract, by using a standard graph of Rutin, covering the concentration in between 0.02 to 0.2 mg/ml ($y = 0.0025x$, $R^2 = 0.9974$).

Total flavonols of extracts were estimated as mg Rutin equivalents (RE) /g extract, from the Rutin calibration curve in concentration range, 0.024 to 0.12 mg/ml ($y = 0.0172x$, $R^2 = 0.9979$), using the method of Miliauskas and Venskutonis (2004). Here, y = absorbance and x = concentration. All experiments were done in triplicate.

Antioxidant and free radical scavenging potential determination

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

Scavenging activity on DPPH was assessed according to the method reported by Blois (1958) with a slight modification. Briefly, 100 µl of extracts (0.1 to 0.5 mg/ml) were mixed with 1 ml of methanolic solution of 0.1 mM DPPH. The mixture was shaken well and incubated at room temperature for 30 min and absorbance was measured at 517 nm in a spectrophotometer. BHT was used as standard. Experiment was performed in triplicate and averaged. Percent inhibition was calculated from control using the following equation:

$$\text{Scavenging activity (\%)} = (1\text{-absorbance}_{sample}/\ \text{absorbance}_{control}) \times 100$$

ABTS radical scavenging assay

Trolox equivalent antioxidant capacity (TEAC) was estimated as ABTS (2, 20-azinobis-3-ethylbenzothiazoline-6-sulphonic acid) radical scavenging activity according to the method of Re et al. (1999). Reagent solution consist of 7 mM ABTS and 2.45 mM potassium persulfate in 100 mM phosphate buffer solution (pH 7.4) and was left to stand for 12 to 16 h at laboratory temperature in the dark to form ABTS radical cation. A working solution was diluted to absorbance values 0.7 ± 0.02 at 734 nm with 100 mM phosphate buffer solution (pH 7.4). 10 µl of standards or plant extracts (0.1 to 0.5 mg/ml) were mixed with the working solution (990 µl) and absorbance was measured at 734 nm after 5 min. Trolox was used as a standard. Scavenging activity is calculated as described in DPPH scavenging assay.

Nitric oxide radical scavenging assay

The nitric oxide radical scavenging activity of extracts was determined using the method of Sreejayan and Rao (1997). Sodium nitroprusside in aqueous solution at physiological pH spontaneously generate nitric oxide which interacts with oxygen to produce nitrite ions determined by the Griess reagent. 2 ml of 10 mM sodium nitroprusside dissolved in 0.5 ml phosphate buffer saline (pH 7.4) was mixed with 0.5 ml of extract at various concentrations (0.05 to 0.25 mg/ml). The mixture was incubated at 25ºC. After 150 min, 0.5 ml of incubation solution was withdrawn and mixed with 0.5 ml of Griess reagent. The mixture was incubated at room temperature for 30 min. The absorbance was measured at 540 nm. BHT was used as standard. The amount of nitric oxide radicals scavenged was calculated as described in DPPH assay.

Table 1. Total phenol, flavonoid, flavonol contents and yields of the extracts from *D. hookeri* roots.

Extract	Total phenol (mg GAE/g extract)	Total flavonoids (mg RE/g extract)	Total flavonol (mg RE/g extract)	Yield (%)
DHDCM	7.233±0.286	138.667±7.379	4.864±0.187	1.463±0.0375
DHME	45.535±1.153	53.467±5.22	4.379±0.089	19.65±0.35

Superoxide radical scavenging activity

Superoxide anion scavenging activity was estimated by modified method of Robak and Gryglewski (1988). The reaction mixture consisting of 250 µl of 150 µM nitroblue tetrazolium (NBT), 250 µl of 468 µM nicotinamide adenine dinucleotide (NADH) and 250 µl of extract (0.1 to 0.5 mg/ml) was mixed in sodium phosphate buffer (100 mM, pH 7.4). The reaction was initiated by adding 250 µl of 60 µM phenazine methosulfate (PMS) to the mixture. The reaction mixture was incubated at 25ºC for 5 min, and the absorbance was measured against the corresponding blank solution. Ascorbic acid was used as positive control. The superoxide radical scavenging activity was calculated using the formula as given in DPPH assay.

Metal ion chelating activity

The chelating activity of extract on Fe^{2+} was measured according to the method of Dinis et al. (1994). 1 ml of extracts (0.1 to 0.5 mg/ml) was incubated with 50 µl of 2 mM ferrous chloride. The reaction was started by the addition of 200 µl ferrozine (5 mM). After 10 min, the absorbance of ferrous ion-ferrozine complex at 562 nm was read. Na_2EDTA served as positive control. Triplicate samples were run for each set and averaged. The ability of extracts to chelate ferrous ion was calculated using the following equation:

$$\text{Chelating activity (\%)} = (1\text{-absorbance}_{sample}/\ \text{absorbance}_{control}) \times 100$$

Ferric reducing antioxidant power assay (FRAP)

The assay was based upon the methodology of Benzie and Strain (1996). The FRAP reagent consisted of 10 mM 2,4,6-tripyridyl- 2-triazine (TPTZ) in 40 mM HCl, 20 mM ferric chloride and 250 mM sodium acetate buffer (pH 3.6). FRAP reagent was freshly prepared by mixing TPTZ solution, $FeCl_3$ solution and acetate buffer in a ratio 1:1:10. A 100 µl of extract solution containing 0.1 mg extract was mixed with 900 µl of FRAP reagent. After the mixture stood at 37ºC for 4 min, the absorbance at 593 nm was determined against blank. Trolox was used as calibration standard in concentration range, 0.002 to 0.01 mg/ml ($y = 0.160x$, $R^2 = 0.981$). FRAP values were calculated as mg of Trolox equivalents (TE)/g extract from three determinations and are averaged.

Reducing power assay

The reducing power of extracts was determined as per the method of Oyaizu (1986). 1 ml of extracts (0.25 to 1 mg/ml) was mixed with 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml potassium ferrocyanide (1 %). After incubating the mixture at 50ºC for 20 min., 2.5 ml of 10% trichloroacetic acid was added, and then mixture was centrifuged at 3000 rpm for 10 min. 2.5 ml of supernatant was mixed with 2.5 ml of distilled water and 0.5 ml $FeCl_3$ (0.1%) and the absorbance was measured at 700 nm and compared with standard ascorbic acid.

Total antioxidant capacity (TAC)

Total antioxidant capacity of extracts was estimated as described by Prieto et al. (1999). An aliquot of 0.1 ml of extract was mixed with 1 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate) in an eppendorf tube. The tubes were capped and incubated at 95°C for 90 min. After the samples had cooled to room temperature, the absorbance of each was measured at 695 nm against a reagent blank. Gallic acid was used as standard (0.02 to 0.1 mg/ml) and TAC was estimated as mg GAE/g dried extract from calibration curve given by equation $y = 0.006x + 0.102$ ($R^2 = 0.93$).

RESULTS AND DISCUSSION

Total phenolics, flavonoids and flavonol determination

Phenolic compounds are secondary metabolites of plants and can act as antioxidants by many potential pathways such as free radical-scavenging, oxygen radical absorbance, and chelating of metal ions (Halliwell et al., 1995). In this study, two solvents of varying polarity were used to extract and separate the medium and high polarity compounds from the non-polar compounds in the roots of *D. hookeri*. Roots were extracted with dichloromethane and methanol using a Soxhlet apparatus. The results of total phenols, total flavonoids, total flavonols and percent yields of extracts are presented in (Table 1). The DHME extract exhibited the total phenolic content approximately 6 fold more than the DHDCM extract. The differences in total phenolic content of extracts were significant ($p < 0.05$).

Although phenolic compounds from plant sources are found to be responsible for antioxidant activities (Cai et al., 2004), antioxidant effects do not always correlate with the presence of large quantities of phenolics. The flavonoids, which contain hydroxyl groups, are found to be responsible for the radical scavenging effect in the plants (Das et al., 1990; Younes, 1981). Therefore, flavonoid and flavonol content of the extracts were also analyzed. The flavonoid and flavonol content of the extracts were in order: DHDCM>DHME, which is reverse

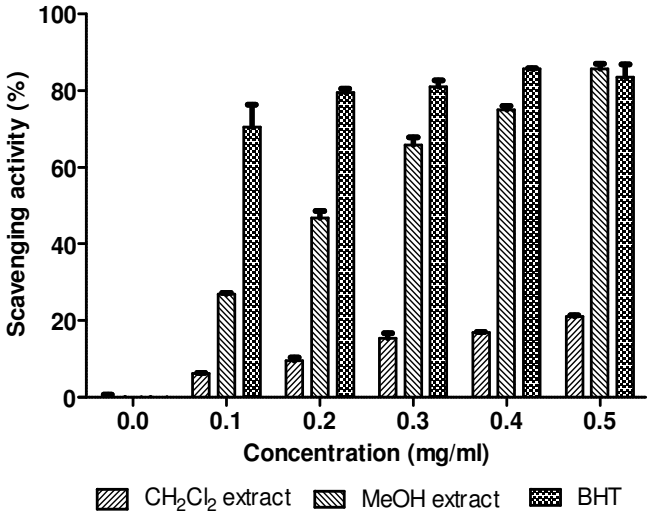

Figure 2. Effect of *D. hookeri* root extracts and standard BHT on DPPH radical scavenging study. The data is expressed as percent scavenging of DPPH radicals.

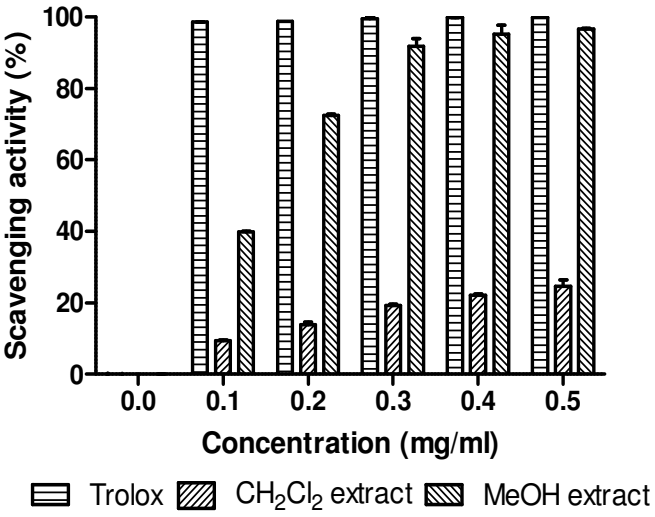

Figure 3. Effect of *D. hookeri* root extracts and standard Trolox on ABTS radical cation decolorization assay. The percent of inhibition was plotted against concentration of sample.

of total phenolic content of the extracts.

Antioxidant and free radical scavenging activity

Because of the complex nature of phytochemicals, the antioxidant activities of plant extracts must be evaluated by combining two or more different *in vitro* assays based on different features of the antioxidant effects, such as the ability to scavenge free radicals, or the metal ion chelation.

DPPH free radical scavenging assay

The DPPH method is an easy, rapid, stable and sensitive way to determine the antioxidant activity of a specific compound or plant extracts (Koleva et al., 2002). In this assay, DPPH free radical accepts hydrogen and gets reduced by an antioxidant. DHDCM and DHME extracts showed steady increase in percentage inhibition of the DPPH radicals with concentration (Figure 2). The radical scavenging activity of DHDCM extract was much lower than the DHME extract suggesting that the antioxidants in the DHDCM extract are weak radical-scavengers and required extremely high concentration to have a significant effect. Overall, the DHDCM and DHME extracts were able to inhibit the formation of DPPH radicals with an IC_{50}, 120.837±10.769 mg/ml and 0.217±0.0138 mg/ml, respectively. Standard BHT and DHDCM extract were significantly different ($p<0.05$) but DHME extract was not significantly different from BHT ($p>0.05$). The two extracts were significantly different ($p<0.05$) in DPPH radical scavenging activity.

ABTS radical scavenging activity

The DHME extract of *D. hookeri* roots was more potent than DHDCM extract in scavenging ABTS radicals (Figure 3). There was a steady increase in the percentage inhibition of the ABTS radicals by the DHME extract of *D. hookeri* roots and maximum inhibition was achieved above 0.3 mg/ml of extract. DHME extract (IC_{50}, 0.131±0.002 mg/ml) was less active than standard Trolox (IC_{50}, 0.002±0.001 mg/ml) and was not significantly different from Trolox ($p>0.05$). The DHDCM extract showed a percentage inhibition of less than 30% at the highest concentration studied (0.5 mg/ml). The DHDCM extract and standard Trolox showed significant difference ($p<0.05$) in ABTS scavenging activity. The two extracts showed significantly different ABTS scavenging ($p<0.05$).

Nitric oxide radical scavenging assay

The percent inhibition of nitric oxide radicals show that both DHDCM and DHME extracts had higher scavenging activity than standard BHT at concentrations up to 0.15 mg/ml (Figure 4). Standard BHT and DHME extract showed consistent increase in scavenging nitric oxide radicals with increase in concentration and were found to

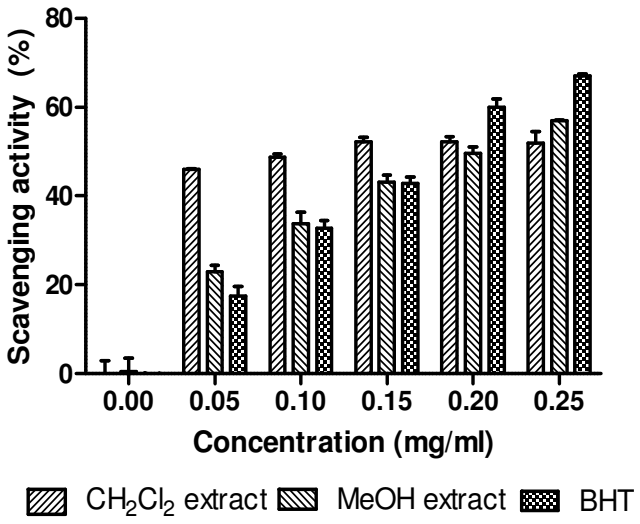

Figure 4. Effect of *D. hookeri* root extracts and standard BHT on nitric oxide scavenging assay. The percent scavenging of NO radicals is plotted against concentration of sample.

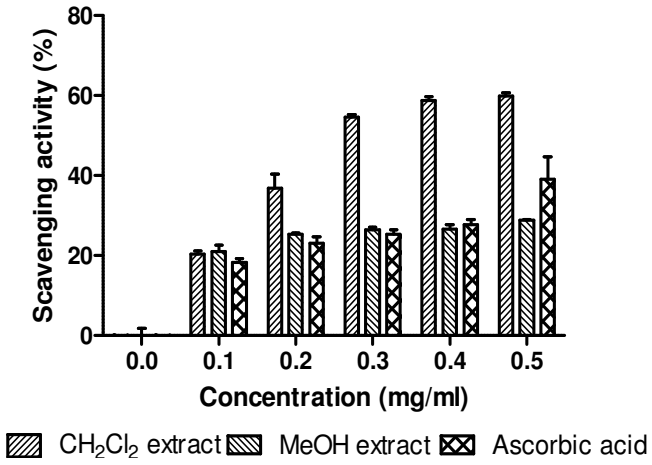

Figure 5. Effect of *D. hookeri* root extracts and standard ascorbic acid on superoxide radical scavenging study. The data is expressed as percent scavenging of superoxide radicals.

show 67.012±0.441 and 56.947±0.145% scavenging, respectively at 0.25 mg/ml. The two extracts were not significantly different with each other as well as from standard BHT ($p>0.05$).

Superoxide radical scavenging assay

DHDCM extract showed significantly higher superoxide radical scavenging activity than DHME extract and standard ascorbic acid (Figure 5). However, DHME extract showed almost scavenging activity similar to that of ascorbic acid up to concentration, 0.4 mg/ml. There was no significant difference in superoxide radical scavenging activity of extracts and standard ascorbic acid ($p>0.05$). The two extracts were also not significantly different ($p>0.05$).

Metal chelation activity

The transition metal ion Fe^{2+} possesses the ability to move single electrons thus allowing the formation and propagation of many radical reactions, even starting with relatively nonreactive radicals (Halliwell, 1994). DHDCM extract showed higher metal chelating activity than DHME extract (Figure 6), with an IC_{50} value of 0.249±0.002 mg/ml but was less potent chelator than standard Na_2EDTA (IC_{50} 0.015±0.001 mg/ml). DHME extract was found to be very less reactive showing a maximum of 30.003±1.101% inhibition at 0.5 mg/ml. The DHDCM extract and Na_2EDTA were significantly not different ($p>0.05$), while DHME and Na_2EDTA were significantly different ($p<0.05$), in metal chelation. The two extracts also showed no significant difference ($p>0.05$). According to the results, the total flavonoid content of DHDCM extract was high which could contribute to its high ferrous ion-chelating activity as the mode of the actions of flavonoids is through scavenging or chelating processes (Cook et al., 1996; Kessler et al., 2003).

FRAP assay

In this assay, reduction of the ferric-tripyridyltriazine to the ferrous complex forms an intense blue colour which can be measured at a wavelength of 593 nm. The intensity of the colour is related to the amount of antioxidant reductants in the samples. FRAP values of DHDCM and DHME extracts were 16.968±0.062 and 17.810±1.091 mg TE/g dried extract and were not significantly different ($p>0.05$). The reductive ability of the samples assessed in this study suggests that the extracts were able to donate electron, hence they should be able to donate electrons to free radicals in biological systems, making the radicals stable and unreactive.

Reducing power activity

Fe^{3+} reduction, an indicator of electron donating activity, is considered to be an important mechanism of antioxidant activity of phenolics. In the reducing power assay, the presence of antioxidants in the samples would

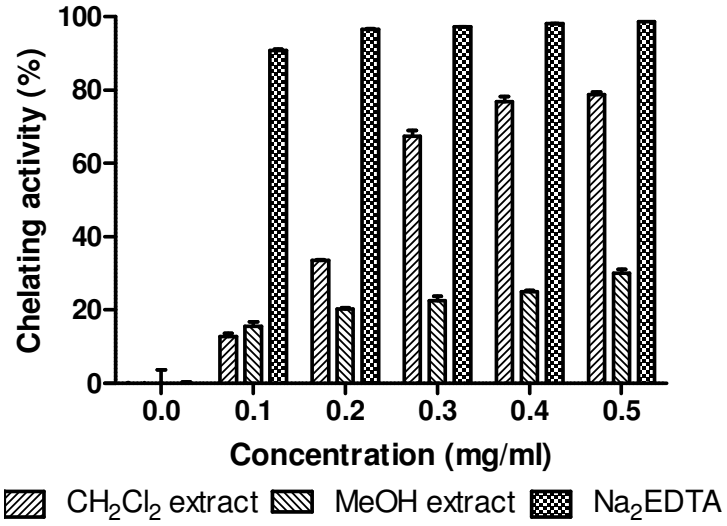

Figure 6. Effect of *D. hookeri* root extracts and standard Na_2EDTA on metal chelation study. The percent chelating activity is plotted against concentration of sample.

Table 2. Reducing power activity of the extracts from *D. hookeri* roots and standard Ascorbic acid.

Extract	Absorbance at 700 nm at different concentration			
	0.25 mg/ml	0.5 mg/ml	0.75 mg/ml	1 mg/ml
DHDCM	0.010±0.000	0.021±0.000	0.030±0.001	0.043±0.001
DHME	0.062±0.001	0.123±0.002	0.184±0.001	0.246±0.001
Ascorbic acid	0.371±0.006	0.431±0.001	0.443±0.002	0.466±0.002

result in the reducing of Fe^{3+} to Fe^{2+} by donating an electron. Amount of Fe^{2+} complex can then be monitored by measuring the formation of Perl's Prussian blue at 700 nm.

Increasing absorbance at 700 nm indicates an increase in the reductive ability. Table 2 depicts the reducing power activity of extracts, indicating that reducing ability correlated well with amount of extracts.

Reducing power of DHME extract was significantly higher than that of DHDCM extract. DHDCM and DHME extracts were found to be significantly different from ascorbic acid ($p<0.05$).

Total antioxidant capacity (TAC*)*

The total antioxidant capacity of the extract was calculated based on the reduction of Mo (VI) to Mo (V) by the extract and subsequent formation of a green phosphate/ Mo (V) complex at acid pH, which was measured spectrophotometrically at 695 nm (Prieto et al., 1999). TAC of the DHDCM and DHME extracts was found to be 1259.692±19.215 and 1370.974±4.7 mg GAE/g extract, respectively. The two extracts were significantly different ($p<0.05$).

Conclusions

The results of our study demonstrated that due to high total phenolics content, DHME extract of *D. hookeri* roots had high free radical scavenging and reducing activities. TAC and FRAP values were also higher in case of DHME extract. However, DHDCM extract was rich in total flavonoid content and showed high metal chelating activity as compared to DHME extract. Thus, methanol seems to be most promising solvent for extraction and isolation of natural antioxidative compounds form *D. hookeri* roots.

Demonstration of the antioxidant potential of the herbs, especially in view of the presence of a rich spectrum of bioactive molecules of therapeutic significance, makes

them likely candidates for bioactivity guided fractionation of useful phytomolecules. Further activity guided isolation and characterization of the extract is in progress to identify the full composition of the extract and the exact compound(s) responsible for its bioactivity.

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