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Research Article

Research Article

Estimation of Total Phenolics and Flavonoid Content and *In-vitro* Antioxidant Activity of *Drypetes sepiaria* Stem (Wight & Arn.) Pax & K. Hoffm.

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ABSTRACT

The total phenolics, flavonoids and *in vitro* antioxidant activity of petroleum ether, benzene, ethyl acetate, methanol and ethanol extracts of stem of *Drypetes sepiaria* were determined using various antioxidant model systems viz, DPPH, hydroxyl, superoxide, ABTS and reducing power. Total phenolic content was estimated by Folin-Ciocalteau method. Flavonoids were determined by Aluminium chloride method. The total phenolics and flavonoids contents were found to be 0.81 g 100 g^{-1} and 1.12g 100 g^{-1} respectively in the methanol extract. Among the solvent tested, methanol and ethanol extracts of *D. sepiaria* stem showed potent *in vitro* antioxidant activities. This investigation explored that *D. sepiaria* stem is a potential source of natural antioxidant.

Keywords: *Drypetes sepiaria,* Gallic acid, Antioxidant, Methanol, DPPH, ABTS.

INTRODUCTION

Generation of free radicals or reactive oxygen species (ROS) during metabolism and other activities beyond the antioxidant capacity of a biological system gives rise to oxidative stress[1]. Oxidative stress plays a role in heart diseases, neurodegenerative diseases, cancer and in the ageing process[2]. This concept is supported by increasing evidence that oxidative damage plays a role in the development of chronic, age-related degenerative diseases and that dietary antioxidants oppose this and lower risk of diseases[3]. Antioxidants are the substance that when present in low concentrations compared to those of an oxidisable substrate significantly delays or prevents oxidation of that substance[4]. Apart from their role of health benefactors, antioxidants are added in foods to prevent or delay oxidation of food, initiated by free radicals formed during their exposure to environmental factors such as air, light and temperature[5]. At present, most of the antioxidants are manufactured synthetically. They belong to the class of synthetic antioxidants. The main disadvantage with the synthetic antioxidants is the side effects when taken *in vivo*[6]. Hence, considerable interest has been given to the use of natural antioxidants which may also have nutritional properties[7]. Several medicinal plants have been extensively used for the treatment of number of diseases. Some of these plants have shown strong antioxidant activity. However, majority of plants have not yet been monitored for such activity.

Medicinal plants have been playing a vital role on the health and healing of man since down of human civilization. In spite of tremendous development in the field of allopathic medicines during 20th century, plants still remain one of the major sources of drugs in modern as well as in traditional system of medicine. Medicinal plants are sources of certain bioactive molecules which act as antioxidants and antimicrobial agents[8-11].

Drypetes sepiaria (Wight & Arn.) Pax & k. Hoffm belongs to the family Putranjivaceae (Euphorbiaceae). This plant is used in folk medicine by tribal people of Western Ghats to treat pain and inflammation. The seeds of this plant are used as a wild edible food by Palliyars (A tribal Group) of Western Ghats, India[12]. Recent report on *D. sepiaria* has been stated that the paste of the roots can be used as an antidote for scorpion bite. The decoction of leaves and seeds of this plant is also given for reducing rheumatic inflammation [13]. So our present study is focussed on D. Sepiaria stem to determine their free radical scavenging properties. The literature survey showed very little information on this plant and thus prompted us to analyze this plant.

MATERIALS AND METHODS

Collection and processing: The stem of *Drypetes sepiaria* (Wight & Arn.) Pax & K. Hoffm. were collected from Kolamkode, Muppuram, Sacred forest of Kanyakumari District, Tamil Nadu, India. The plant specimen was identified and authenticated in Botanical Survey of India,

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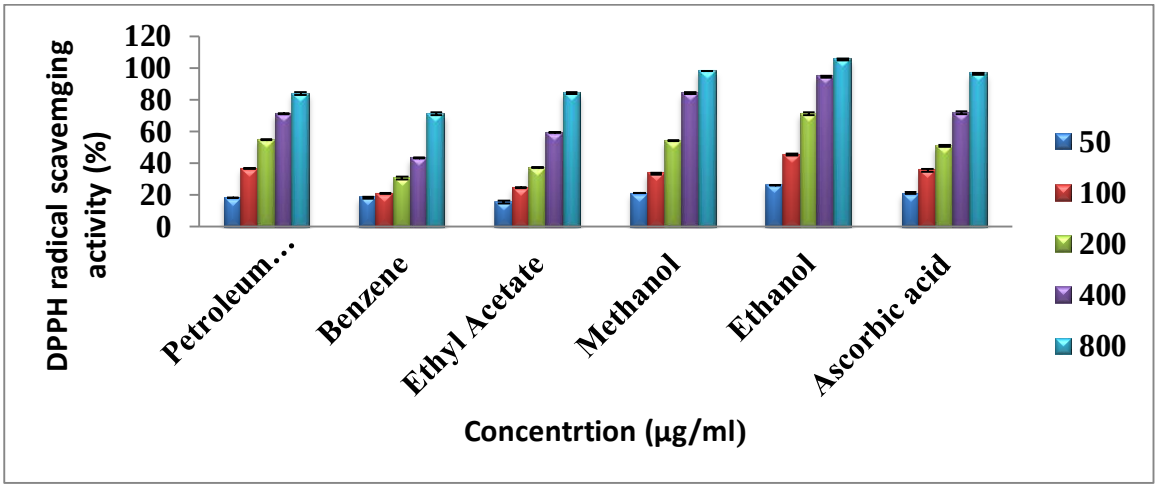

Fig. 1: DPPH radical scavenging activity of different extracts of D. sepiaria stem

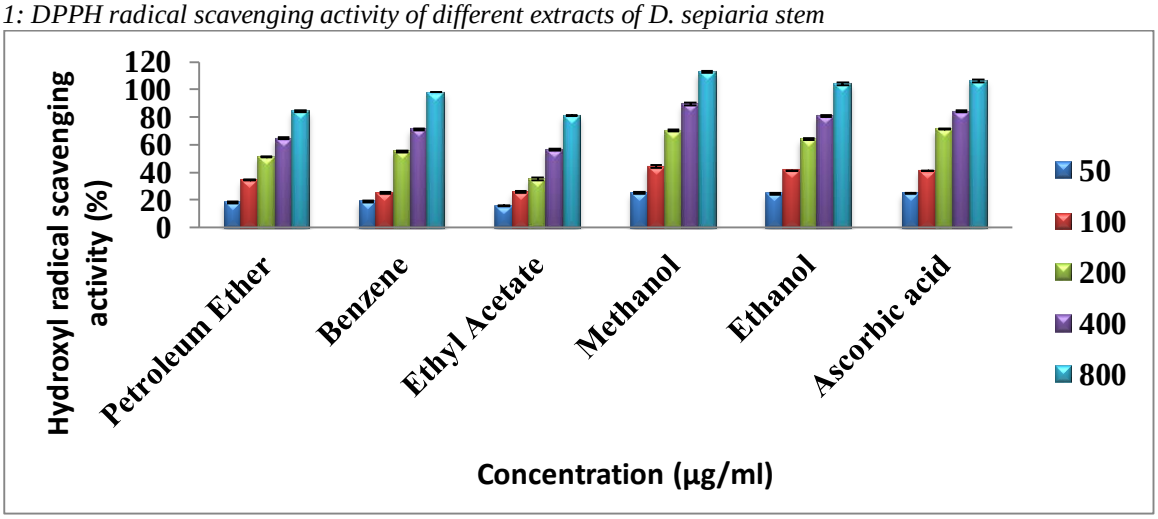

Fig. 2: Hydroxyl radical scavenging activity of different extracts of D. Sepiaria stem

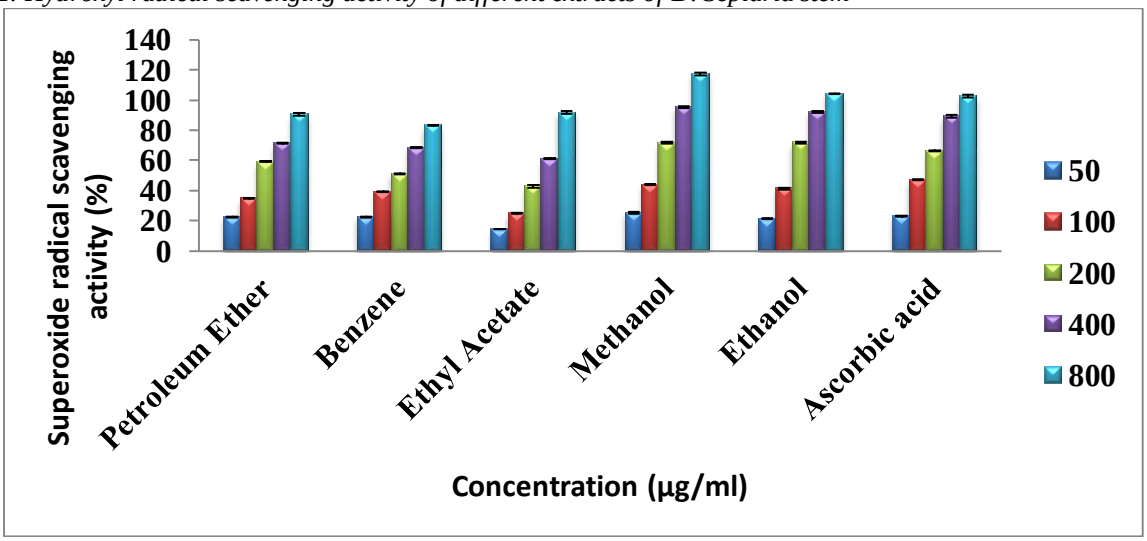

Fig. 3: Superoxide radical cation scavenging activity of different extracts of D. sepiaria stem

Southern Circle, Coimbatore, Tamil Nadu, India. A voucher specimen was deposited in Ethnopharmacology Unit, Research Department of Botany, V. O. Chidambaram College, Tuticorin, Tamil Nadu. The collected samples were cut into small fragments and shade dried until the fracture is uniform and smooth. The dried plant material was granulated or powdered by using a blender and sieved to get uniform particles by using sieve No. 60. The final uniform powder was used for the extraction of active constituents of the plant material.

Preparation of plant extract: The coarse powder (100g) was extracted successively with petroleum ether, benzene, ethyl acetate, methanol and ethanol each 250 ml in a Soxhlet apparatus for 24 hrs. All the extracts were filtered though Whatman No.41 filter paper. All the extracts were

concentrated in a rotary evaporator. The concentrated extracts were used for *in vitro* antioxidant activity. The methanol extract was used for the estimation of total phenolics and flavonoids.

Estimation of total phenolics: Total phenolic content was estimated using Folin-Ciocalteau reagent based assay as previously described[14] with little modification. To 1 mL of each extract (100 µg/mL) in methanol, 5 mL of Folin-Ciocalteau reagent (diluted ten-fold) and 4mL (75g/L) of Na_2CO_3 were added. The mixture was allowed to stand at 20ºC for 30min and the absorbance of the developed colour was recorded at 765nm using UV-VIS spectrophotometer. 1mL aliquots of 20, 40, 60, 80, 100 µg/mL methanolic gallic acid solutions were used as standard for calibration curve. The absorbance of solution was compared with gallic acid calibration curve. The total phenolic content was expressed as gallic acid equivalents (GAE g/100g dry weight of extract).

Estimation of flavonoids: The flavonoids content was determined according to Eom *et al* [15]. An aliquot of 0.5 ml of sample (1 mg/mL) was mixed with 0.1 ml of 10% aluminium chloride and 0.1ml of potassium acetate (1 M). In this mixture, 4.3 ml of 80% methanol was added to make 5mL volume. This mixture was vortexed and the absorbance was measured spectrophotometrically at 415 nm. The value of optical density was used to calculate the total flavonoid content present in the sample.

DPPH radical scavenging activity: The DPPH is a stable free radical and is widely used to assess the radical scavenging activity of antioxidant component. This method is based on the reduction of DPPH in methanol solution in the presence of a hydrogen donating antioxidant due to the formation of the non radical form DPPH-H[16].

The free radical scavenging activity of all the extracts was evaluated by 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) according to the previously reported method[16]. Briefly, an 0.1 mM solution of DPPH in methanol was prepared, and 1mL of this solution was added to 3 ml of the solution of all extracts in methanol at different concentration (50, 100, 200, 400 & 800 µg/mL).The mixtures were shaken vigorously and allowed to stand at room temperature for 30 minutes. Then the absorbance was measured at 517 nm using a UV-VIS spectrophotometer (Genesys 10S UV: Thermo electron corporation). Ascorbic acid was used as the reference. Lower absorbance values of reaction mixture indicate higher free radical scavenging activity. The capability of scavenging the DPPH radical was calculated by using the following formula.

% Inhibition = $\{(A_0 - A_1)/A_0)*100\}$

Where, A_0 is the absorbance of the control reaction, and A_1 is the absorbance in presence of all of the extract samples and reference. All the tests were performed in triplicates and the results were averaged

Hydroxyl radical scavenging activity: The scavenging capacity for hydroxyl radical was measured according to the modified method of Halliwell *et al*[17]. Stock solutions of EDTA (1 mM), $FeCl_3$ (10 mM), Ascorbic Acid (1 mM), H_2O_2 (10 mM) and Deoxyribose (10 mM), were prepared in distilled deionized water.

The assay was performed by adding 0.1 mL EDTA , 0.01 mL of $FeCl_3$,0.1 mL H_2O_2, 0.36 mL of deoxyribose, 1.0 mL of the extract of different concentration (50, 100, 200, 400 & 800 µg/mL) dissolved in distilled water, 0.33 mL of phosphate buffer (50 mM , pH 7.9), 0.1 mL of ascorbic acid in sequence. The mixture was then incubated at 37^0C for 1 hour. 1.0 mL portion of the incubated mixture was mixed with 1.0 mL of 10% TCA and 1.0 mL of 0.5% TBA (in 0.025 M NaOH containing 0.025% BHA) to develop the pink chromogen measured at 532nm. The percentage inhibition was calculated by comparing the results of the test with those of the control using the above formula.

Superoxide radical scavenging activity: The superoxide anion scavenging activity was measured as described by Srinivasan *et al*[18]. The superoxide anion radicals were generated in 3.0 ml of Tris – HCL buffer (16 mM, pH 8.0), containing 0.5 mL of NBT (0.3 mM), 0.5 ml NADH (0.936 mM) solution, 1.0 mL extract of different concentration (50, 100, 200, 400 & 800 µg/mL), and 0.5 mL Tris – HCl buffer (16 mM, pH 8.0). The reaction was started by adding 0.5 mL PMS solution (0.12 mM) to the mixture, incubated at 25ºC for 5 min and the absorbance was measured at 560 nm against a blank sample, ascorbic acid. The percentage inhibition was calculated by comparing the results of the test with those of the control using the above formula

Antioxidant activity by radical cation ($ABTS^+$): ABTS assay was based on the slightly modified method of Huang *et al* [19]. ABTS radical cation ($ABTS^+$) was produced by reacting 7 mM ABTS solution with 2.45 mM potassium persulphate and allowing the mixture to stand in the dark at room temperature for 12-16 h before use. The $ABTS^+$ solution were diluted with ethanol to an absorbance of 0.70+0.02 at 734 nm. After addition of 100 µL of sample or trolox standard to 3.9 mL of diluted $ABTS^+$ solution, absorbance was measured at 734 nm by Genesys 10S UV-VIS (Thermo scientific) exactly after 6 minutes. Results were expressed as trolox equivalent antioxidant capacity (TEAC). The percentage inhibition was calculated by comparing the results of the test with those of the control using the above formula.

Reducing power: The reducing power of the extract was determined by the method of Kumar and Hemalatha[20]. 1.0 mL of solution containing 50, 100, 200, 400 & 800 µg/mL of extract was mixed with sodium phosphate buffer (5.0 mL, 0.2 M, pH 6.6) and potassium ferricyanide (5.0 mL, 1.0%): The mixture was incubated at 50ºC for 20 minutes. Then 5mL of 10% trichloroacetic acid was added and centrifuged at 980 g (10 minutes at 5ºC) in a refrigerator centrifuge. The upper layer of the solution (5.0 mL) was diluted with 5.0 mL of distilled water and ferric chloride and absorbance was read at 700 nm. The experiment was performed thrice and results were averaged.

Statistical analysis: Antioxidant activities like DPPH radical scavenging activity, hydroxyl radical scavenging activity, superoxide radical activity, ABTS radical cation scavenging activity and reducing powers were estimated in triplicate determinations. Data were analyzed using the statistical analysis system SPSS (SPSS software for windows release 17.5; SPSS Inc., Chicago IL, USA).

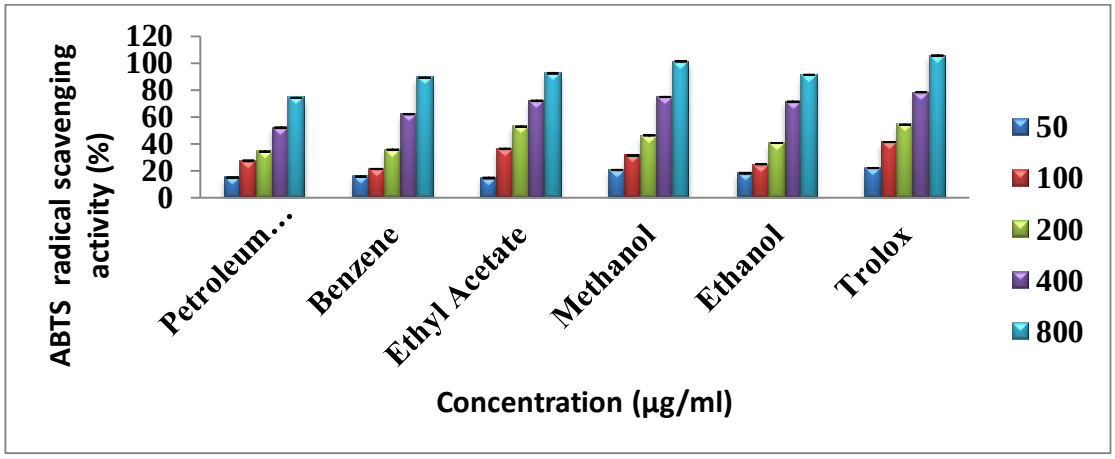

Fig. 4: ABTS radical cation scavenging activity of different extracts of D. sepiaria stem

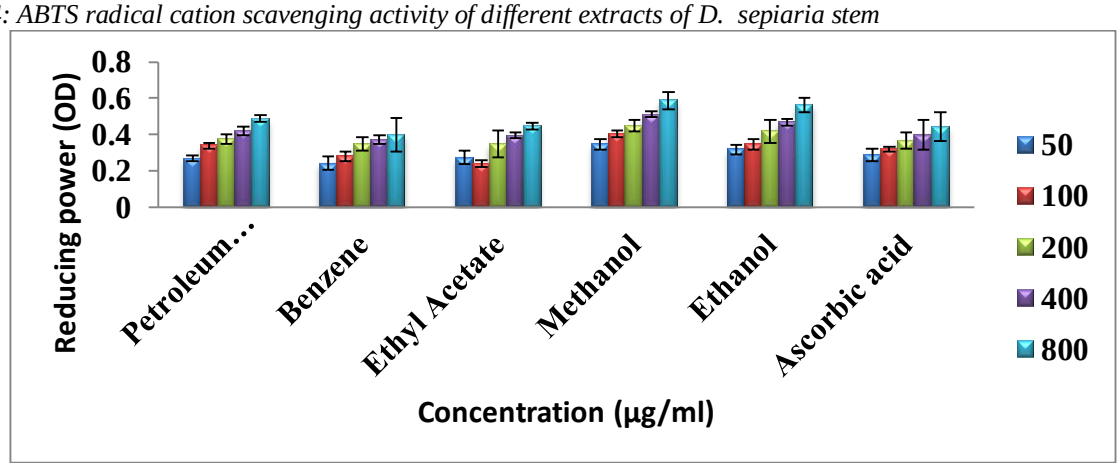

Fig. 5: Reducing power ability of different extracts of D. sepiaria stem

Estimates of mean, standard error for aforesaid parameters were calculated.

RESULT AND DISCUSSION

Total phenolic and flavonoid content**:** The total phenolic and flavonoid content of methanol extract of *D. sepiaria* were found to be 0.81 g 100 g^{-1} and 1.12 g 100 g^{-1} respectively. Plants have been the basis of traditional medicines throughout the world for thousands of years and continue to provide new remedies to human kind; a great deal of effort has therefore focussed on using available experimental techniques to identify natural antioxidants from plants [21]. Phenolic compounds are one among the major classes of antioxidant compounds. The phenolic compounds of plants fall into several categories such as simple phenolic, phenolic acids (derivatives of cinnamic acid and benzoic acids), coumarins, flavonoids stilbenes, tannins and lignans. Nitrogen compounds such as alkaloids also present antioxidant activity[22]. Flavonoids are a group of polyphenolic compounds, which exhibit several biological effects such as antiinflammatory, antihepatotoxic, antiulcer, antiallergic, antiviral and anticancer activities. They also inhibit enzymes such as aldose reductase and xanthine oxidase. They are able of effectively scavenging the reactive oxygen species because of their phenolic hydroxyl groups and are potent antioxidants [23]. The presence of high phenolic and flavonoid contents in the extracts have contributed directly to the antioxidant activity by neutralizing the free radicals [24]. The free radicals are chemical species which contains one or more unpaired electrons. They are highly unstable and cause damage to other molecules by extracting electrons from them in order to attain stability. Radical scavenging activities are very important due to deleterious role of free radicals in biological systems.

In vitro antioxidant activity of the petroleum ether, benzene, ethyl acetate, methanol and ethanol extract of *D. sepiaria* stem have been investigated in the present study by DPPH, hydroxyl, superoxide radical scavenging and ABTS radical cation scavenging activity. It is probably due to the presence of respective phytochemicals like flavonoids, phenolics, etc [25-26]. The main function of the antioxidants is to neutralize the free radicals, which is routinely produced in the biological system. Free radicals are known to play a vital role in a wide variety of pathological manifestations. Antioxidants fight with free radicals and protect us from various disease. They exert their action either by scavenging the reactive oxygen species or protecting the antioxidant defence mechanism.

DPPH radical scavenging activity: DPPH is a stable, organic free radical extensively used to evaluate scavenging activity of antioxidants because it is sensitive enough to detect active ingredients at low concentrations [27]. When an antioxidant is mixed with any concentration of the free radical forming sample such as DPPH, it reduces the free radical formation of DPPH[28]. DPPH has a purple colour which is reduced to yellow coloured diphenylpicryl hydrazine. The switch in colour (that is, from deep-violet

Table 1: IC_{50} values of different solvent extracts of stem of *D. sepiaria*

Solvent	IC_{50} (µg/mL)			
	DPPH	Hydroxyl	Superoxide	ABTS
Petroleum ether	21.14	24.39	24.46	24.82
Benzene	24.07	21.19	26.17	23.84
Ethyl acetate	21.12	24.54	23.37	20.19
Methanol	16.24	17.09	17.14	19.44
Ethanol	16.12	20.44	19.14	20.74
Ascorbic acid	17.68	18.48	19.38	-
Trolox	-	-	-	16.74

to light-yellow) can be measured spectrophotometrically. Figure 1 illustrates the DPPH radical scavenging activity of petroleum ether, benzene, ethyl acetate, methanol and ethanol extracts of *D. sepiaria* stem. All the extracts of *D. sepiaria* stem showed potent DPPH radical scavenging activity depending upon the increasing concentration. Among all the solvents tested, ethanol extract at 800 µg/mL concentration showed highest scavenging activity (105.41%) against DPPH radical with lowest IC_{50} value (16.12 µg/mL) which is nearly equal to the standard ascorbic acid (IC_{50} value=17.68 µg/mL) (Table-1). In the present study, the DPPH radical scavenging activities of different extract of stem of *D. sepiaria* increased gradually in a concentration dependent manner. Smaller IC_{50} value corresponds to a higher antioxidant activity of the plant extract [29]. It is evident from the observations that ethanol extract of *D. sepiaria* had the lowest IC_{50} value, which indicated its powerful free radical scavenging ability.

Hydroxyl radical scavenging activity: The present study shows the abilities of the extracts to inhibit hydroxyl radical deoxyribose degradation in a concentration dependent manner. The extract had significant scavenging effects on the hydroxyl radical, which was increasing with the increase in concentration from 50-800 µg/mL. In the present exploration, scavenging effect of different extracts of *D. sepiaria* stem against hydroxyl radical increased as the concentration increased. The highest percentage scavenging effect on hydroxyl radicals was observed in methanol extract of *D. sepiaria* stem which had lowest IC_{50} value. The radical scavenging capacity may be attributed to phenolic compounds in plant extracts with the ability to accept electrons, which can combine with free radical competitively to decrease hydroxyl radical[30].

Hydroxyl radical scavenging activity of different extracts of *D. sepiaria* stem was shown in figure 2. Hydroxyl radical scavenging activity of different extracts of *D. sepiaria* stem increased with the concentration of extract. At 800 µg/mL concentration, methanol extract which has lowest IC_{50} value (17.09µg/mL) showed highest scavenging activity (112.74%) against hydroxyl radical. Hydroxyl scavenging activity of methanol extract is more or less similar to standard ascorbic acid.

Superoxide radical scavenging activity: Superoxide anion plays an important role in the formation of more reactive species such as hydrogen peroxide, hydroxyl radical and singlet oxygen, which induce oxidative damage in lipids, proteins, and DNA [31]. Therefore, studying the scavenging activity of plant extracts on superoxide radical is one of the most important ways of clarifying the mechanism of antioxidant activity. Superoxide anion derived from dissolved oxygen by PMS–NADH coupling reaction reduces NBT in this system. In this method, superoxide anion reduces the yellow dye (NBT^{2+}) to produce the blue formazan which is measured spectrophotometrically at 560 nm and antioxidants are able to inhibit the blue NBT formation [32]. The decrease of absorbance at 560 nm with antioxidants indicates the consumption of superoxide anion in the reaction mixture. The result of present investigation revealed that, the different extracts of *D. sepiaria* stem exhibited superoxide radical scavenging activity in concentration dependent manner. The IC_{50} value observed for the methanol extract of *D. sepiaria* stem indicated its potent efficacy to scavenge superoxide radical which is higher than the standard ascorbic acid. Superoxide radical scavenging activity of different extract of *D. sepiaria* stem at various concentrations is illustrated in figure 3. All the extracts showed effective scavenging activity against superoxide radical in concentration dependent manner. At 800 µg/mL concentration, methanol extract of *D. sepiaria* seem exhibited maximum scavenging activity (117.29%) against superoxide radical which has lowest IC_{50} value (17.14 µg/mL) than standard ascorbic acid (19.88 µg/mL).

ABTS radical cation scavenging activity: ABTS radical cation scavenging activity is relatively recent one, which involves a more drastic radical, chemically produced and is often used for screening complex antioxidant mixtures such as plant extracts, beverages and biological fluids. The ability in both the organic and aqueous media and the stability in a wide pH range raised the interest in the use of ABTS for the estimation of antioxidant activity[33]. The ABTS chemistry involves direct generation of ABTS radical mono cation with no involvement of any intermediary radical. It is a decolorization assay, thus the radical cation is performed prior to addition of antioxidant test system, rather than the generation of the radical taking place continually in the presence of antioxidant. The results obtained imply the activity of the extract either by inhibiting or scavenging the ABTS radicals since both inhibition and scavenging properties of antioxidants towards ABTS radicals have been reported earlier [34]. In the present study, ABTS radical cation scavenging activity of all the five extracts increase as the concentration increases. IC_{50} value of methanol extract of *D. sepiaria* stem is more or less similar to the IC_{50} value of standard trolox. It might be due to the presence of high level of antioxidant compounds. Different extracts of *D. sepiaria* stem were subjected to ABTS radical cation scavenging activity and

the results were expressed in figure 4. It depicts concentration dependent increased scavenging effect of different extracts of *D. sepiaria* stem and standard trolox. Among the solvent tested, methanol extract at 800 µg/mL concentration, showed highest scavenging activity (101.16%) against ABTS radical with lowest IC_{50} value (19.44 µg/mL) which is almost equal to the standard trolox (16.74 µg/mL).

Reducing power: The reducing power of *D. sepiaria* stem extracts was compared with the standard ascorbic acid. The reducing power increases with the increasing concentration. At 800 µg/mL concentration of methanol and ethanol extracts of *D. sepiaria* stem showed higher reducing power than the ascorbic acid. In reducing power assay, the presence of antioxidants in the sample reduced Fe3+/ ferricyanide complex to the ferrous form. This reducing capacity of compounds could serve as an indicator of potential antioxidant properties and increase in absorbance could indicate an increase in reducing power[35-36]. Among the extracts, investigated methanol extract exhibited higher reducing power activity as compared with ascorbic acid. The reducing power of different extract of *D. sepiaria* stem is depicted in figure 5. Reducing power of different extracts of *D. sepiaria* stem increased with increase in the concentration of extracts. At 800 µg/mL concentration, methanol and ethanol extract of *D. sepiaria* stem showed potent reducing power than standard ascorbic acid.

CONCLUSION

Presence of phenolic and flavonoid contents of *D. sepiaria* stem exhibit antioxidant activity. Natural antioxidants of plant origin have greater application and they also find use as nutraceuticals and phytoceuticals as they have significant impact on the status of human health and diseases prevention. The present study provides scientific basis of the use of these plant extracts in traditional health care system. Detailed work by using different methods will be the aim of further investigation.

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