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ANTIOXIDANT FREE RADICAL SCAVENGING PROPERTIES OF A HEMEPARASITIC MISTLETOE PLANT, *DENDROPHTHOE FALCATA* (L.F.) ETTINGSH INFESTED ON *ARTOCARPUS HETEROPHYLLUS* HOST TREE.

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ABSTRACT

Studies on free radical scavenging activity of ethanol extract of *Dendrophthoe falcata* samples collected from *Artocarpus heterophyllus* indicate their potential on DPPH, NO and SO free radical scavenging activity. In general, the ethanol extracts of *Dendrophthoe falcata* samples exhibit less scavenging activity on DPPH, NO and SO radicals when compared to standard. *Dendrophthoe falcata* sample ethanol extracts shows significant variations in the DPPH, NO and SO radical scavenging activities. *Dendrophthoe falcata* tender shoot and bark samples show higher FRAP than the standard at all concentrations tested as compared to leaf sample. The ethanol extracts exhibit significant concentration dependent scavenging effects on free radicals and FRAP. The results indicate that *Dendropthoe falcata* ethanol extracts could be a source of potent natural antioxidants which act as free radical Scavengers and Fe-chelators, and these properties of health benefits are host dependent.

KEYWORDS: Hemiparasite, Mistletoe, *Dendrophthoe falcata*, *Artocarpus heterophyllus*, Free radical scavenging activity, DPPH, NO, SO, FRAP.

INTRODUCTION

The common free radicals such as superoxide, hydroxyl, nitric oxide and superoxide are oxygen reactive species (ROS) which can be internally produced by cellular metabolism, inflammation by immune cells and externally by radiation, pharmaceuticals, hydrogen peroxide, toxic chemicals, smoke, alcohol, oxidized polyunsaturated fats and cooker food. Free radicals can cause damage to parts of cells such as proteins, DNA and cell membranes by stealing their electrons through a process of oxidation. Free radicals may cause heart damage, cancer, and a weak immune system.[1-3]

Dendrophthoe falcata is a climbing woody parasitic plant.[4] It is indigenous to tropical regions and in India; it is widely distributed throughout up to 900m.[5] It has 400 known host species.[6-8] Several reports indicate that *D. falcate*, the parasitic plant has many medicinal properties and used in the treatment of many diseases.

Previous reports [9, 10] indicate that the total phenol and flavonoid content of *D. falcate* plays a major role in controlling antioxidant activity. The methanol extract showed good antioxidant activity as compared to aqueous extract of *D. falcata*.[10] The *D. falcata* ethanol extract possesses potent antioxidant activity by inhibiting lipid peroxidation, reduced glutathione, superoxide dismutase levels and increases the catalase activity.[11] *Dendrophthoe falcata* stem extracts exhibit potent free radical scavenging and the antioxidant activity might be attributed to its polyphenolic content and other phytochemical constituents. The stem of *D. falcata* stem could be a potential source of natural antioxidant that could have great importance as therapeutic agents in preventing or slowing the progress of aging and age associated oxidative stress related degenerative disease and also suggested that the presence of phytoconstituents like quercetin can be attributed to the antioxidant property of the plant.[4] Chandrakasan and Neelamegam[12, 13] reported the free radical scavenging activity of *Loranthus longiflorus* Desr. (Syn.: *Dendrophthoe falcata* (L.f) Ettingsh) leaf and bark samples collected from *Casuarina equisetifolia* and *Ficus religiosa* host trees. The chemical composition of *D. falcata* varies somewhat and depending on the host plant.[14, 15] This study is aimed to investigate the free radical scavenging activity of *Dendrophthoe falcata* (L.f.) Ettingsh collected from *Artocarpus heterophyllus* host tree.

MATERIALS AND METHODS

Plant material

The hemiparasitic mistletoe plant, *Dendrophthoe falcata* (L.f.) Ettingsh was collected from the host tree of

Artocarpus heterophyllus, at Marthandam area, Kanyakumari district, Tamil Nadu (Plate 1). The plant was identified by BSI, Coimbatore, Tamil Nadu and the voucher specimen is preserved in the Department of Botany, S.T. Hindu College, Nagercoil, Kanyakumari District, Tamil Nadu.

Dendrophthoe falcata infected *Artocarpus heterophyllus* host -Habit.

Plate 01: The Hemiparasite, *Dendrophthoe falcata* infected *Artocarpus heterophyllus* host tree –Habit (Photos -1 to 6).

Preparation of plant extracts for antioxidant free radical scavenging activity

The dry powder of *D. falcata* leaf, bark and tender shoot samples was extracted with ethanol at 20% (w/v) level in Soxhlet, evaporated to dryness and the stored residues were used for analyzing bio-molecule and antioxidants.

Antioxidant Activity Assays

The anti-free radical scavenging activities of *D. falcata* leaf, bark and tender shoot extracts on DPPH,[16] Nitric oxide[17] and Superoxide radicals[18] were assayed in-vitro. Ferric reducing antioxidant power of the *D. falcata* leaf, bark and tender shoot extracts was also performed as described by Pulido *et al*.[19]

Ferric reducing antioxidant power (FRAP) assay[19]

FRAP reagent (900µL), prepared freshly and incubated at 37ºC, was mixed with 90µL of distilled water and 30µL of test sample or methanol (for the reagent blank). The test samples and reagent blank were incubated at 37ºC for 30min in a water bath. The final dilution of test sample in the reaction mixture was 1/34. The FRAP reagent contained 2.5mL 20mMol/L TPTZ solution in 40mMol/L HCL plus 2.5mL of 20mMol/L $FeCl_3$. $6H_2O$ and 25mL of 0.3mol/L acetate buffer (pH 3.6). At the end of incubation, the absorbance reading were taken immediately at 593nm, using a spectrometer. Methanol solutions of known Fe (II) concentration, ranging from 100 to 2000µmol/L, ($FeSO_4.7H_2O$) were used for the preparation of the calibration curve. The parameter equivalent concentration (EC1) was defined as the concentration of antioxidant having a ferric-TPTZ reducing ability equivalent to that of 1mMol/L $FeSO_4.H_2O$. EC1 was calculated as the concentration of antioxidant giving an absorbance increase in the FRAP assay equivalent to the theoretical absorbance value of a 1mMol/L concentration of (II) solution, determined using the corresponding regression equation.

DPPH-radical scavenging activity[16]

Methanol solution of the sample extracts at various concentrations (50-250g) was added to 5ml of a 0.1mM methanol solution of DPPH (1-diphenyl-2-picryl-hydoxyl-) and allowed to stand for 20min at 27ºC. The absorbance of the sample was measured at 517nm. Radical scavenging activity was expressed as the inhibition percentage of free radical by the sample and was calculated using the formula: DPPH radical scavenging activity (%) = (Control OD-Sample OD / Control OD) × 100.

Nitric oxide radical scavenging activity[17]

Three ml of 10mM sodium nitroprusside in 0.2M phosphate buffered saline (pH7.4) was mixed with

different concentrations (50-250µg/ml) of solvent extracts and incubated at room temperature for 150min. After incubation time, 0.5ml of Griess reagent (1% sulfanilamide, 0.1% naphthyl-ethylene-diamine-dihyrochloride in 2% H_3PO_4) was added. The absorbance of the chromophore formed was read at 546nm. The same mixture of reaction without sample extracts was employed as negative control. Percentage radical scavenging activity of the sample was calculated as follows: NO scavenging activity (%) = (Control OD-sample OD / control OD) × 100.

Superoxide radical scavenging activity[18]

Each 3ml reaction mixture contained 50mM sodium phosphate buffer (pH 7.6), 20mg riboflavin, 12mM EDTA, 0.1mg NBT and various concentrations (50-250µg) of sample extracts. Reaction was started by illuminating the reaction mixture with sample extract for 90seconds. Immediately after illumination the absorbance was measured at 590nm. The entire reaction assembly was enclosed in a box lined with aluminium foil. Identical tubes with reaction mixture kept in dark served as blank. The percentage inhibition of superoxide anion generation was calculated by using the formula: SO scavenging activity (% -inhibition) = (control OD-sample OD / control OD) × 100.

The results of free radical scavenging activity of *D. falcata* were expressed as mean ± standard deviation. One-way ANOVA was used to analyze the mean. Significance was accepted at $P<0.05$ and $P<0.01$ (5% and 1%) level.

RESULTS AND DISCUSSION

Ferric Reducing Antioxidant Power (FRAP) of *Dendrophthoe falcata*

Ferric Reducing Antioxidant Power (FRAP) of *D. falcata* was evaluated in the leaf, tender shoot and bark samples collected from *A. heterophyllus* host (Table 1; Figure 2a). The result of the present study indicate that the FRAP of *D. falcata* was concentration dependent and ranged from 108.33 to 411.67µg/mg in plant samples and 120 to 303.33µg/mg in the standard. When compared to control, the pant samples shows less FRAP in leaf extract at 10µg and 20µg, while it was increased slightly at 30µg. On the other hand, the tender shoot and bark sample extracts shows more FRAP than the standard. Among the *D. falcata* sample ethanol extracts, tested, the bark extract of *D. falcata* shows maximum activity than the standard and leaf and tender shoot samples. The variations in the values of FRAP at different concentrations was significance at 5% level (p=0.05). The tender shoot sample extract shows more activity of FRAP as compared to leaf sample of *D. falcata* al all concentrations tested. The FRAP of *D. falcata* samples is arranged in the following order: bark > tender shoot > leaf.

Table 1: Estimation of ferric reducing antioxidant power of *Dendrophthoe falcata* leaf, tender shoot and bark samples obtained from *Artocarpus heterophyllus* host tree.

Concentration of ethanol extract used (µg)	Ferrous Sulphate (Standard)	Ferric reducing antioxidant power (mMol Fe(11)/mg extract) of *Dendrophthoe falcate* samples			One-way ANOVA (between plant samples extract) F-value
		Leaf	Tender shoot	Bark	
10 (µg)	120.00±4.08	108.33 ±6.24 *(-9.73)*	230.00 ±14.14 *(+241.67)*	410.00 ±08.16 *(+91.67)*	728.15**
20 (µg)	149.33±0.94	121.67 ±6.24 *(-18.52)*	280.00 ±04.08 *(+175.68)*	411.67 ±10.27 *(+87.50)*	1316.33**
30 (µg)	303.33±4.71	308.33 ±6.24 *(+1.65)*	311.67 ±10.27 *(+35.17)*	410.00± 08.16 *(+2.75)*	135.05**
One-way ANOVA (between conc. of extracts) F-value	2197.69**	963.34**	47.38**	0.04NS	

**-Significance at1% level (p=0.01); NS –Non-significance; (n=3);
Values within parenthesis indicate the percent change (increase or decrease) over control.

DPPH Free Radical Scavenging Activity

The DPPH radical scavenging activity of ethanol extracts of *D. falcata* leaf, tender shoot and bark samples was assayed and the data are presented in table 2; figure 2b. The ethanol extracts of *D. falcata* samples had significant scavenging effects on the DPPH radicals and the effects increased with increasing concentrations in the range from 10 to 30µg. In general, all the extracts show reduced DPPH free radical scavenging activity as compared to BHT standard. This reduction in DPPH-FRS activity was ranged from 5.56% (in tender shoot extract at 30µg) to 14.19% (in leaf extract at 10µg). The DPPH-FRS activity of BHT standard and tender shoot extract between concentrations was non-significant, whereas the activity was significant between leaf and bark extracts and their concentrations at 5% / 1% level. Among tested plant extract, bark and tender shoot samples had maximum activity than leaf sample. The result indicates the efficacy of extracts on DPPH radical scavenging activity was noted in the following order: bark > tender shoot > leaf at 10µg level; bark > leaf > tender shoot at 20µg level; and bark > leaf > tender shoot at 30µg level of extract concentration.

Table 2: DPPH free radical scavenging (DPPH-FRS) activity of *Dendrophthoe falcata* leaf, tender shoot and bark extract collected from *Artocarpus heterophyllus* host tree.

Concentration of Ethanol extract used (µg)	BHT Standard	DPPH free radical scavenging activity (%) of *Dendrophthoe falcate* samples			One-way ANOVA (between plant sample extract) F-value
		Leaf	Tender shoot	Bark	
10 (µg)	90.20 ±1.63	77.40 ±0.16 *(-14.19)*	81.10 ±0.82 *(-10.09)*	84.70 ±0.16 *(-6.10)*	105.56^{**}
20 (µg)	93.10 ±4.49	85.10 ±0.08 *(-8.59)*	84.70 ±1.63 *(-9.02)*	86.30 ±0.33 *(-7.30)*	8.07^{**}
30 (µg)	94.10 ±1.25	86.59 ±0.16 *(-7.98)*	88.87 ±4.89 *(-5.56)*	86.45 ±0.04 *(-8.13)*	6.01^{*}
One-way ANOVA (between extract Conc.) F-value	1.52^{NS}	3801.20^{**}	4.50^{NS}	62.22^{**}	

**-Significance at1% level (p=0.01); *-Significance at 5% level (p=0.05); NS –Non-significance; (n=3);
Values within parenthesis indicate the percent change (increase or decrease) over control.

Nitric Oxide Free Radical Scavenging (NO-FRS) activity

Nitric Oxide free radical scavenging activity was assessed in the ethanol extracts of *D. falcata* leaf, tender shoot and bark samples and the data are presented in table 3; figure 2c. The result reveals that nitric oxide radical generated from sodium nitroprusside at physiological pH is found to be initiated by the extracts and the percentage of NO-FRS activity is concentration dependent. The NO-FRS activity was reduced from 63.17% to 93.67% in the plant sample extracts as compared to BHT standard. Further, the variations noted in the values of NO-FRS activity were significant between the plant extracts and their concentrations, tested, at 1% level. The NO-FRS activity of plant extracts generally ranged from 1.33% (in leaf extract at 10µg) to 26.76% (in bark extract at 30µg). Among plant samples, bark extract of *D. falcata* shows higher activity than other extracts of *D. falcata* at all concentration tested. Maximum NO-FRS activity (26.76%) noted in the bark sample (at 30µg) of *D. falcata*. NO-FRS activity increased with increasing concentrations in the range from 10 to 30µg. Among plant extracts tested, leaf sample had minimum activity (6.10% at 30µg) bark and tender shoot samples of *D. falcata*. The efficacy of NO-FRS activity in the plant samples of *D. falcata* is found in the following order: bark > tender shoot > leaf at all concentrations tested.

Table 3: Nitric oxide free radical scavenging (NO-FRS) activity in the extracts *Dendrophthoe falcata* leaf, tender shoot and bark samples collected from *Artocarpus heterophyllus* host tree.

Concentration of ethanol extract used (µg)	Standard	Nitric oxide free radical scavenging activity (%) of *Dendrophthoe falcate* samples			One-way ANOVA (between plant sample extracts) F-value
		Leaf	Tender shoot	Bark	
10 (µg)	20.81 ±0.75	1.33±0.04 *(-93.61)*	04.44 ±0.47 *(-78.66)*	06.05 ±0.01 *(-70.93)*	1146.16^{**}
20 (µg)	44.99 ±5.95	2.85±0.02 *(-93.67)*	13.52 ±0.46 *(-69.95)*	16.57 ±0.25 *(-63.17)*	108.90^{**}
30 (µg)	74.45 ±4.23	6.10 ±0.12 *(-91.81)*	20.00 ±0.01 *(-73.14)*	26.76 ±0.78 *(-64.06)*	571.22^{**}
One-way ANOVA (between extracts Conc.) F-value	120.59^{**}	3258.46^{**}	1270.98^{**}	1438.33^{**}	

Values within parenthesis indicate the percent change (increase or decrease) over control;
**-Significance at1% level (p=0.01); (n=3);

Superoxide Free Radical Scavenging (SO FRS) activity

The SO-FRS activity was determined in ethanol extracts of *D. falcata* leaf, tender shoot and bark samples and the data are presented in table 4; figure 2d. The result of the present study indicates that the ethanol leaf, bark and tender shoot extracts possess SO-FRS activity and was concentration dependents. When compared to standard, the extracts of plant samples shows less SO-FRS activity and the reduction in activity was ranged from 59.94% (in to tender shoot at 10µg) 95.20% (in leaf extract at 10µg). From this result, maximum SO-FRS activity (17.5%) was noted in the tender shoot sample of *D. falcata* (20mg). Leaf sample of *D. falcata* had maximum activity than bark and tender shoot samples. Among tested concentration, maximum activity was noted at 30µg of leaf bark, tender shoot samples of *D. falcata*. The efficacy of plant extracts on SO-FRS activity is arranged

in following order: tender shoot > bark > leaf at all concentrations. Further, the differences in the values of SO-FRS activity between plant sample extracts and between their concentrations were significant at 5% level.

Table 4: Superoxide free radical scavenging (SO -RSA) activity in the extracts of *Dendrophthoe falcata* leaf, tender shoot and bark samples collected from *Artocarpus heterophyllus* host tree.

Concentration of Ethanol extract Used (µg)	Standard	Superoxide free radical scavenging activity (%) of *Dendrophthoe falcata* samples			One-way ANOVA (between plant sample extracts) F-value
		Leaf	Tender shoot	Bark	
10 (µg)	23.54 ±0.87	1.13 ±0.09 *(-95.20)*	9.43 ±0.39 *(59.94)*	2.03 ±0.05 *(-91.38)*	1401.09**
20 (µg)	39.69 ±0.91	4.48 ±0.04 *(-88.91)*	9.59 ±0.05 *(-75.84)*	6.81 ±0.08 *(-82.94)*	3894.73**
30 (µg)	62.64 ±4.24	5.42 ±0.08 *(-91.35)*	17.50 ±0.01 *(-72.06)*	9.67 ±0.05 *(-84.56)*	463.84**
One-way ANOVA (between extracts Conc.) F-value	177.61**	2842.57**	1238.38**	11762.84**	

Values within parenthesis indicate the percent change (increase or decrease) over control.
**-Significance at1% level (p=0.01); (n=3);

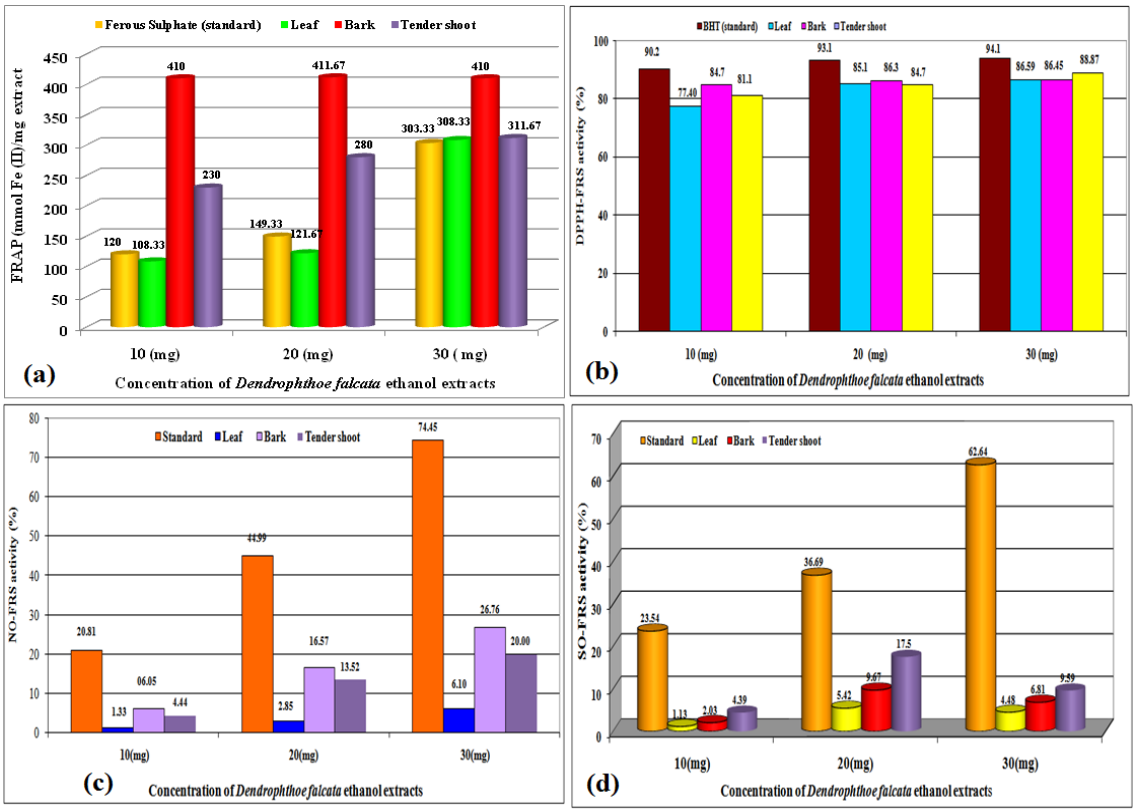

Plate 2: Ferric Reducing Antioxidant Power (a), DPPH (b), NO (c) and SO (d) free radical scavenging activity in the ethanol extracts of *Dendrophthoe falcata* leaf, tender shoot and bark samples collected from *Artocarpus heterophyllus* host tree.

Many synthetic antioxidants reported to have several side effects.[20-23] Therefore, there is a need for more effective, less toxic and cost effective antioxidants. Medicinal plants appear to have these desired comparative advantages, hence the growing interest in natural antioxidants from plants.[24, 25] Several phytochemical surveys have mentioned that the antioxidant property of plants might be due to their phenolic compounds[26, 27] including tannins and flavonoids and they have been reported as promising antioxidants.[28] Antioxidants act as radical scavengers when added to the food products and prevent the radical chain reaction of oxidation that delay or inhibit the oxidation process and increase shelf life by retarding the processes of lipid peroxidation.[29] In recent years, interest in the study of antioxidant activity of plant extracts[30] and isolation of antioxidants from

plants have grown due to the fact that the free radicals have been related to degenerative diseases.[31-32] The antioxidant activities were assessed earlier in the extracts of *D. falcata*[25, 33] and in *Loranthus longiflorus* (Syn.: *Dendrophthoe falcata*) infested on *Casuarina equisetifolia* and *Ficus religiosa*.[12, 13]

In this study, the ferric reducing antioxidant power (FRAP) in the ethanol extracts of *D. falcata* samples, at all concentrations tested, was recorded in the following order: bark > tender shoot > leaf. The FRAP activity was concentration dependent, i.e., it was increased with increasing concentration of extract. The FRAP was higher in the bark extract of *D. falcata* could be due to the presence of more reactive concentration of bioactive constituents and mixture of other compounds in the extract as reported by Pattanayak *et al*.[25] Many studies revealed that only polar extracts of plants had showed effective antioxidant activity and some researches further proved that moderate polarity extracts were more potent even if their total phenolic content did not include all the antioxidant.[34] Vinson *et al*.[35] suggested that the synergism among the antioxidant in the mixture made the antioxidant activity not only dependant on the concentration of antioxidant but also on the structure and interaction among the antioxidant.

The DPPH radical is considered to be a model for lipophylic radical. A chain in the lipophilic radical was initiated by the lipid auto-oxidation. DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule.[36] The reduction capability of DPPH radical was determined by the decrease in its absorbance at 517nm which was induced by antioxidants. Hence, DPPH is often used as a substrate to evaluate free radical scavenging activity of antioxidants.[37] Many reports indicate that the use of DPPH radicals provides an easy, rapid and convenient method to evaluate the antioxidant and radical scavenging. It is a sensitive way to survey the antioxidant activity of a specific compound or plant extracts.[38] The DPPH scavenging effect in the ethanol extracts of *D. falcata* leaf, bark and tender shoot samples exhibited dose dependent DPPH radical scavenging activity as reported by and Motalleb *et al*.[27] Pattanayak *et al*.[25] It was noted that all extracts of *D. falcata* samples shows a reduction in the DPPH radical scavenging activities than the control, at all concentrations tested. The scavenging activity of all samples on the DPPH radicals was found to be strongly dependent on the extract concentration as reported by Motalleb *et al*.[27] Among the pant samples tested, the bark extract shows more DPPH-FRS activity than the other sample extracts of *D. falcata*.

Nitric oxide plays an important role in various types of inflammatory processes in the body.[25] It is a potent diffusible free radical involved in a variety of biological functions, including antimicrobial and anti-tumor activity.[39] Despite the possible beneficial effects of nitric oxide, its contribution to oxidative damage is also reported. This is due to the fact that nitric oxide can react with superoxide to form the peroxynitrite anion, which is a potential oxidant that can decompose to produce OH- and NO.[40] The results of nitric oxide free radical scavenging (NO-FRS) activity showed that nitric oxide radical generated from sodium nitroprusside at physiological pH was found to be initiated by the extracts and it was noted that the percentage of NO-FRS activity was concentration dependent in the *D. falcata*. Among the plant samples the bark sample showed higher activity than the other sample extracts at all concentrations tested.

Superoxide is a reactive oxygen species (ROS), which causes damage to the cells and DNA and leads to various diseases. It was, therefore, proposed to measure the comparative interceptive ability of the antioxidant extracts to scavenge the superoxide radical. Several *in vitro* methods are available for generation of superoxide radicals.[41] It is formed by alkaline Dimethyl Sulphoxide (DMSO) which reacts with Nitroblue tetrazolium (NBT) to produce coloured diformazan. It is biologically important as it can form singlet oxygen and hydroxyl radical.[42] Over production of superoxide anion radical contributes to redox imbalance and associated with harmful physiological consequences.[43] From the results of the present study, it was found that the ethanol extracts of *D. falcata* possessed the superoxide free radical scavenging (SO-FRS) activity and was concentration dependent. Maximum activity was recorded at high concentration (30µg/ml) of tender shoot extracts as compared to other extracts.

The results of the present study are in agreement with the report of Ravishankar *et al*.[44] and Mary *et al*.[45] Large number of phytocompounds group was implicated for antioxidants activity.[46] The antioxidant activity is affordable not only by phenolic compound but also has important contributions from other superoxide anion radical scavengers such as essential oils, carotenoids and vitamins.[47] Some variations in the extent of extract in antioxidant activity were observed for each type of assay used in this study. The results of present study suggests that *D. falcata* (leaf, tender shoot and bark) sample extracts could be a potential source of natural antioxidant that could have importance as therapeutic agents in preventing or slowing the progress of aging or age associated oxidative stress related degenerative diseases.[4] This study also indicate that the extracts of *D. falcata* can act as a primary and/or secondary antioxidants, free radical scavengers and plant Fe-chelators, and these properties health benefits are host dependent.[12, 13, 48]

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