A report on the antioxidant activity of the powder of the entire plant of *Phyllanthus reticulatus* Poir

V. Maruthappan, K. Sakthi Shree[1]
Department of Zoology, Unit of Endocrinology, Bharathiar University, [1]PG and Research Centre, Government Arts College (Autonomous), Coimbatore, Tamil Nadu, India

In the present study, we determined the antioxidant activity of the entire plant of *Phyllanthus reticulatus* by performing different *in vitro* antioxidant assays, including 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) radical scavenging, β-carotene bleaching, superoxide anion radical scavenging, reducing power and metal chelating assay at different concentrations (100, 200 and 400 µg/ml). The entire plant powder of *P. reticulatus* shows good antioxidant activity of about 90.0% when compared with standard Butylated Hydroxy Toluene (BHT) (85%) at a concentration of 400 µg/ml. Results obtained reveal that methanolic extracts of entire plant of *P. reticulatus* possess higher antioxidant activity when compared with ethanolic extracts. Thus, this study suggests that *P. reticulatus* plant can be used as a potent source of natural antioxidants.

Key words: Antioxidant activity, DPPH, free radical, *Phyllanthus reticulatus*

INTRODUCTION

Free radicals play a significant role in the causation of several diseases such as diabetes, obesity, cirrhosis, cancer and cardiovascular diseases.[1] Antioxidants are compounds that protect cells against the damaging effects of reactive oxygen species (ROS), such as singlet oxygen, superoxide, peroxyl radicals, hydroxyl radicals and peroxynitrite, which result in oxidative stress leading to cellular damage. Thus, compounds or antioxidants that can scavenge free radicals have a vital role in the improvement of these diseased conditions.[2] Plants contain a wide variety of free radical scavenging molecules such as phenols, flavonoids, vitamins and terpenoids, which are rich in antioxidant activity.[3] Many dietary polyphenolic constituents derived from plants are more effective antioxidants *in vitro* than vitamins E or C and thus might contribute significantly to the protective effects *in vivo*.[4]

Phyllanthus reticulatus Poir. (Family: Euphorbiaceae), commonly known as pancoli or karineli, is a large glabrous or pubescent shrub with smooth or lenticellate branches growing from 8 to 10 ft in height. Its leaves are elliptic to oblong or obovate, and the fruit is purplish black berry.[5,6] The plant grows throughout India, in hedges or waste places. Significant chemical as well as biological studies have not been conducted on *P. reticulatus* so far; the plants of this genus are reported to contain lignins, flavonoids, triterpenoids, alkaloids, polyphenolic compounds.[7] The plant is used for a variety of ailments, including astringent, sweet, cooling, diuretic, constipating, smallpox, syphilis, asthma, diarrhoea, bleeding from gums, skin eruption and obesity.[8-10] Moreover, it is also claimed to have antidiabetic and antihyperlipidemic activity in tribal area. Antioxidants play an important role in the alleviation of diabetes and obesity due to oxidative stress.[11] Hence, an ideal antidiabetic plant should have good antioxidant properties. The aim of the present study is to evaluate the antioxidant activity of different extracts of entire plant of *P. reticulatus.*

MATERIALS AND METHODS

Plant

The entire plants of *P. reticulatus*, collected in August–October 2009 from Maruthamalai Hill, Coimbatore, India, were identified and confirmed by the Taxonomist of Department of Botany, Bharathiar Univeristy, Coimbatore, India.

Extraction

The entire plant was shade-dried, powdered, Soxhlet-extracted successively with MeOH and EtOH and concentrated *in vacuo* to give two brownish green residues: MeOH extract (ML, yield: 6.75%) and EtOH extract (EL, yield: 8.7%). The photochemical screening of Methanolic (ML) and (Ethanolic extract) EL revealed the presence of terpenoids, glycosides, protein, carbohydrates and absence of alkaloids and steroids.

Evaluation of Antioxidant Activity

DPPH free radical scavenging assay

The radical scavenging activity was determined as described by Mensor and Menezes[12] with suitable

Address for correspondence: Dr. V. Maruthappan, Department of Zoology, Unit of Endocrinology, Bharathiar University, Coimbatore, Tamil Nadu India. E-mail: zoomaruthu@yahoo.co.in
Received: 29-12-2009; **Accepted:** 12-08-2010; DOI: 10.4103/0973-8258.74136

modifications by Jayasri *et al*.[13] Briefly, 1 ml of 0.3 mM alcoholic solution of DPPH was added to 2.5 ml of the samples extracted with solvents like methanol and ethanol at different concentrations (100, 200 and 400 μg/ml). The samples were kept in the dark for 30 minutes after which the optical density (OD) was measured at 518 nm. The radical scavenging activity was determined by the following formula:

$$AA\ \% = 100 - \{[(Abs_{sample} - Abs_{empty\ sample})] \times 100/Abs_{control}\},$$

where empty sample = 1 ml methanol + 2.5 ml of various concentrations of the plant extracts and control sample = 1 ml of 0.3 mM DPPH + 2.5 ml methanol. The ODs of the samples, control and the empty samples were measured against methanol as blank. The synthetic antioxidant Butylated Hydroxy Toluene (BHT) was used as positive control.

β-carotene linoleic acid assay

β-carotene linoleic acid assay was carried out as described by Miller.[14] A solution of β-carotene was prepared by dissolving 2 mg of β-carotene in 10 ml $CHCl_3$. Two milliliters of this solution was pipetted into a 100-ml round bottomed flask after $CHCl_3$ was removed under vacuum. Purified linoleic acid (40 mg), 400 mg of Cween 40 emulsifier and 100 ml of aerated distilled water were added and vigorously shaken. Aliquots (4.8 ml) of this emulsion were added to test tubes containing 100 μl of different extracts. BHT was used for comparison. As soon as the emulsion was added to each tube, zero time absorbance was measured on UV-VIS spectrophotometer (Jasco make, USA) at 470 nm. A solution with 100 μl of solvent in the place of extract and 4.8 ml of the above emulsion was used as negative control. The tubes were then placed in a water bath at 50°C and the measurement of absorbance was continued until the colour of the β-carotene disappeared.

$$\text{Absorbance} = \frac{\beta\text{-carotene content after 2 hour assay}}{\text{Initial } \beta\text{-carotene content}}$$

Reducing Power

The reducing power was quantified by the method described by Yen and Chen,[15] with minor modifications by Jayasri *et al*.[13] Reaction mixture containing test samples at different concentrations (10–100 μl) in phosphate buffer (0.2 mM, pH 6.6) was incubated with potassium ferricyanide (1%, w/v) at 50°C for 20 minutes. The reaction was terminated by the addition of tricholoroacetic acid (TCA) solution (10%, w/v) and the mixture was centrifuged at 3000 rpm for 20 minutes. The supernatant was mixed with distilled water and ferric chloride (0.1%, w/v) solution and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power.

Metal Chelating Activity

The chelating effect of ferrous ions by *P. reticulatus* extracts was estimated by the method of Dinis and Madeira,[16] with minor modifications by Jayasri *et al*.[13] Briefly, 200 μl of different concentrations of the extracts (100, 200 and 400 μg/ml) and 740 μl methanol were added to 20 μl of 2 mM $FeCl_2$. The reaction was initiated by the addition of 40 μl of 5 mM ferrozine to the mixture, which was then shaken vigorously and left standing at ambient temperature of 10 minutes. The absorbance of the reaction mixture was measured at 562 nm. Three replicates were made for each test sample. The ratio of inhibition of ferrosine–Fe^{2+} complex formation was calculated as follows:

% inhibition = [(absorbance of control – absorbance of test)/absorbance of control] × 100.

Superoxide anion scavenging activity

The superoxide scavenging ability was assessed according to the method Nishikimi and Rao[17] with slight modifications by Jayasri *et al*.[13] The reaction mixture contained Nitro Blue Tetrazolium (NBT) (0.1 mM) and Nicotinamide Adenine Dinucleotide (NADH) (0.1 mM) with plant extracts of different concentrations (100, 200 and 400 μg/ml) to be assayed in a total volume of 1 ml of Tris-HCl buffer (0.02 M, pH 8.3). The reaction was started by adding Phenazine Methosulfate (PMS) (10 mM) to the mixture and change in the absorbance was recorded at 560 nm every 30 seconds for 2 minutes. The percent inhibition was calculated against a control without test sample. The inhibition ration (%) was calculated as follows:

%inhibition = [(rate of control – rate of test sample)/rate of control] × 100.

Total phenolics

The content of the total phenolics in plant extract was determined by Folin Ciocalteu method.[18] For the preparation of the calibration curve, 1 ml aliquot of 0.024, 0.075, 0.105 and 0.3 mg/ml ethanolic gallic acid solution was mixed with 5 ml Folin-Ciocalteu reagent (diluted 10-fold) and 4 ml sodium carbonate (75 g/l). The absorbance at 765 nm was measured after 1 hour at 20°C and the calibration curve was drawn. To the same reagent (Folin-Ciocalteu reagent and sodium carbonate), 1 ml methanolic plant extract (10 g/l) was mixed as described above and after 1 hour the absorbance was measured. All determinations were performed in triplicates. Total phenolic content in plant methanolic extracts in Gallic Acid Equivalents (GAE) was measured by the formula:

$C = c \times V/m$,

where C is the total content of phenolic compounds given as mg/g of plant extract, in GAE; c the concentration of gallic acid deduced from the calibration curve in mg/ml; V the volume of extract in ml; and mg the dry weight of the plant material.

RESULTS AND DISCUSSION

DPPH Radical Scavenging Activity

According to Amarowics and Pegg,[19] unlike other free radicals such as the hydroxyl radical and superoxide anion, DPPH has the advantage of being unaffected by certain side reactions such as metal ion chelation and enzyme

inhibition. A freshly prepared DPPH solution exhibits a deep purple colour with absorption maximum at 517 nm. The purple colour generally fades or disappears when an antioxidant is present in the medium. Thus, antioxidant molecules can quench DPPH free radicals and convert them to colourless, resulting in a decrease in absorbance, with the more potent antioxidant activity of the extract. This test is a commonly employed assay in antioxidant studies of specific compounds or extracts across a short time scale. The principle advantage of DPPH is that its reduction can be measured directly in the reaction medium by a continuous spectrophotometric assay. DPPH assay is known to give reliable information concerning the antioxidant ability of the tested compounds.[20,21]

The two extracts of entire plant powder of *P. reticulatus* show free radical scavenging property at all the three concentrations studied. DPPH can be used in determining radical scavenging activity as it forms a stable molecule on accepting an electron or a hydrogen atom.[22] There was a reduction in the concentration of DPPH due to the scavenging ability of extract of entire plant. The results obtained are shown in Figure 1. Plant methanolic extracts show satisfactory effect in inhibiting DPPH. At a concentration of 400 µg/ml, the scavenging effects of different extracts of entire plants on the DPPH radical increased in the order: ethanolic extract (73%) < methanolic extract (82.4%). The results show that among the solvent extracts analysed for DPPH scavenging activity, methanolic extract of the entire plant showed higher radical inhibition activity which is comparable with that of standard BHT (85%) at 400 µg/ml.

β-carotene Bleaching Assay

β-carotene shows strong biological activity and is a physiologically important compound. The β-carotene bleaching test is a convenient test used to measure the ability of a compound or a mixture to inhibit the oxidation of β-carotene. In the β-carotene bleaching assay, linoleic acid produces hydroperoxides as free radicals during incubation at 50°C. The presence of antioxidants in the extract minimises the oxidation of β-carotene by hydroperoxides. Hydroperoxides formed in this system are neutralised by the antioxidants from the extracts. Thus, the degradation rate of β-carotene depends on the antioxidant activity of the extracts. The presence of antioxidants in the extracts can hinder the extent of β-carotene bleaching by acting on the lipid free radicals formed in the system.[23,24]

β-carotene in the system undergoes rapid discolouration in the absence of antioxidants, and their presence produces the opposite effect. The linoleic acid free radical formed by the abstraction of a hydrogen atom from one of its diallylic methylene groups attacks the highly unsaturated β-carotene molecules. The compound loses its chromophore as β-carotene molecules lose their double bond by oxidation and the loss in orange colour intensity is measured spectrophotometrically.[25] The antioxidant activity of plant extracts and BHT was measured using β-carotene which is represented in Figure 2. The decrease in the absorbance of various organic solvent extracts tested using the β-carotene bleaching method goes in the order: BHT > methanol extract > ethanol extract. The methanolic extract of *P. reticulatus* shows greater inhibitory effect at a concentration of 400 µg/ml.

Reducing Power Assay

The capacity of a compound to reduce Fe^{3+}/ferricyanide complex to the ferrous form may serve as a significant indicator of its antioxidant capacity.[26,27] The existence of reductones is the key of the reducing power, which exhibit their antioxidant activities through the action of breaking the free radical chain by donating a hydrogen atom.[13] The reduction of the Fe^{3+}/ferricyanide complex to the ferrous form occurs due to the presence of reductants

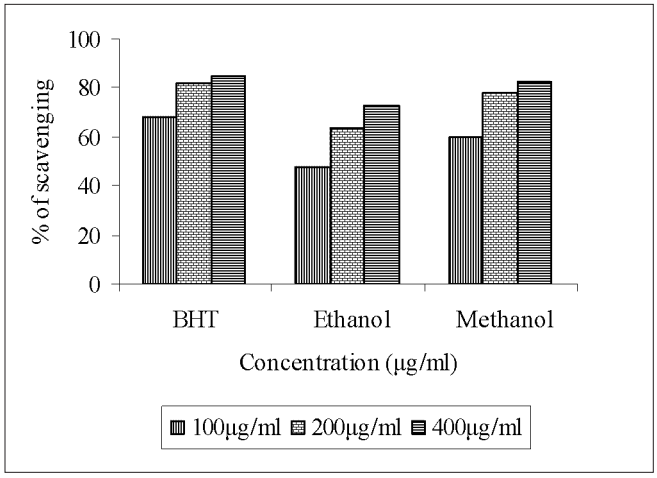

Figure 1: Scavenging effects of extracts of the powder from the entire plant of *P. reticulatus* on DPPH radical

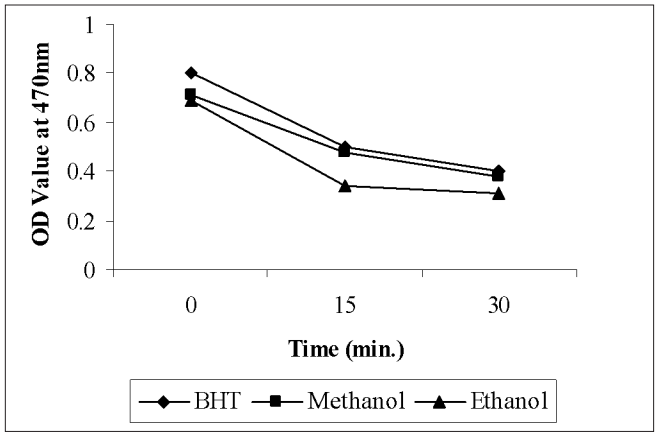

Figure 2: Reduction in absorbance by β-carotene method affected by the extracts of *P. reticulatus* entire plant powder

in the solution. Absorbance of Fe^{2+} can be observed by measuring the OD values at 700 nm.[28] As shown in Figure 3, the reducing power of extracts increased with increase in concentration. At the concentration of 400 µg/ml, the reducing power of plant extract was in the decreasing order: methanolic extract (2.7) > ethanolic extract (2.1).

Metal Chelating Activity

Antioxidants inhibit interaction between metal and lipid by forming insoluble metal complexes with ferrousion.[29] The iron-chelating capacity test measures the ability of antioxidants to compete with ferrozine in chelating ferrous ion.[30] Figure 4 shows the metal chelating effect of different extracts of *P. reticulatus* plant. Ferrozine can quantitatively form complexes with Fe^{2+}. In the presence of samples possessing chelating activity, the formation of complexes is decreased. Therefore, measurement of the rate of colour reduction helps to estimate the chelating activity of the samples.[13] As shown in Figure 4, chelating capacity of the extracts increased with increase in concentration. The order of metal chelating effect of extracts of *P. reticulatus* plant is: methanolic extract (81.4%) > ethanolic extract (54.2%).

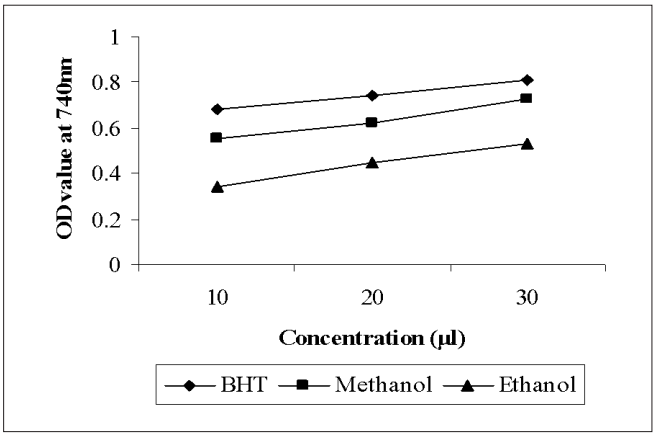

Figure 3: Reducing power of extracts of the powder from entire plant of *P. reticulatus*

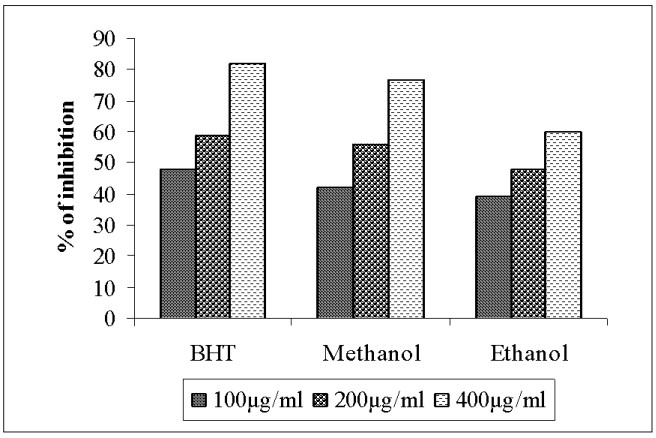

Figure 4: Metal chelating ability of extracts from entire plant of *P. reticulatus*

Superoxide Anion Activity

Numerous biological reactions generate superoxide radical which is a highly toxic species. Although they cannot directly initiate lipid oxidation, superoxide radical anions are potential precursors of highly reactive species such as hydroxyl radical, and thus, the study of the scavenging of this radical is important. Superoxide radicals were generated in a PMS–NADH system and assayed by the reduction of NBT.[31] Results of superoxide anion scavenging activities of *P. reticulatus* plant measured by the PMS–NADH superoxide generating system are shown in Figure 5. Superoxide anion plays an important role in the formation of ROS such hydrogen peroxide, hydroxyl radical and singlet oxygen, which induce oxidative damage in lipids, proteins and DNA.[32,33] The extracts demonstrate a dose–response inhibition of the superoxide anion radicals. The methanolic extracts of *P. reticulatus* plant exhibit good superoxide anion radical scavenging activity at higher concentrations. The % inhibition of superoxide generation by methanolic extract of *P. reticulatus* plant was found to be 79.4 and 72.8%, at a higher concentration of 400 µg/ml. Ethanolic extract of entire plant showed 58.3 and 49.5% of inhibition. Among the two tested extracts of entire plant for superoxide anion scavenging activity, higher activity was shown by methanolic extracts when compared with ethanolic extracts.

Phenolic Content

Phenols, flavonoids and lignin precursors are considered to be beneficial antioxidants as they exhibit scavenging activity of harmful active oxygen species.[34] The content of phenolic compounds of methanolic extracts was determined from regression equation of calibration curve (Y=0.396x–0.676, R^2=0.963) and expressed in GAE. The total phenolic content was 4.56 mg/g of entire plant extract in *P. reticulatus*. The antioxidant property of this plant may be due to the present of phenolic substances.

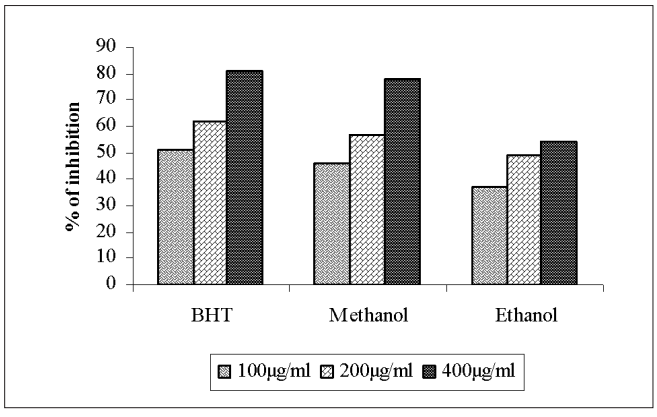

Figure 5: Superoxide radical scavenging activity of extracts of powder from the entire plant of *P. reticulates*, measured using NBT assay

CONCLUSION

The present study revels that the entire plant of *P. reticulatus* exhibits satisfactory scavenging effect in all the radical scavenging assays. This is the first report on the antioxidant property of this plant. The results obtained by these methods provide some insight into the important factors responsible for the antioxidant potential and the mechanism of action. However, methanolic extract of the entire plant possesses good antioxidant activity. Further studies on isolating and characterizing the antioxidant substances and their potential as antidiabetic and antihyperlipidemic agents are in progress.

REFERENCES

1. Hertog MG, Feskens EJ. Dietary antioxidant flavonoids and risk of coronary heart disease: The Zutpher Elderly study. Lancet 1993;342:1007-11.
2. Wilson RL. Free radicals and tissue damage, mechanistic evidence from radiation studies. Biochemical Mechanisms of Liver Injury. New York: Academic Press; 1988. p. 123.
3. Cai YZ, Sun M. Antioxidant activity of betalins from plants of the Amaranthacea. J Agric Food Chem 2003;131:2837-42.
4. Rice-Evans C, Miller N. Antioxidant properties of phenolic compounds. Trends Plant Sci 1997;2:152-9.
5. Gopalakrishna KB. Flora of Udupi. 1st ed. Udupi: Indian Naturalist; 2003. p. 578.
6. Kirtikar KR, Basu BD. Indian Medicinal Plants. 2nd ed. Vol. 3. Dehradun: Bishen Singh and Mahendra Pal Singh; 1991. p. 2219-20.
7. Yoshida T, Seno K, Takama Y, Okanda T. Tannins and related polyphenol of Euphorbiaceous plants. Phytochemistry 1982;21:1180.
8. Nandkarni KM. Indian Materia Medica. Indian Maretia Med 1982;2:948-50
9. Warrier PK, Nambiar VP, Ramankutty C. Indian Medicinal Plants – a compendium of 500 species. Vol. 4. Madras: Orient Longman Ltd; 1995; p. 264-5.
10. Council of Scientific and Industrial Research. The Wealth of India, 2005. Vol. 7. New Delhi: National Institute of Science Communication and Information Resources; 2003. p. 34.
11. Dhanabal SP, Sureshkumar M. Hypoglycemic effect of ethanolic extract of *Musa sapientum* on alloxan induced diabetes mellitus in rats and its relation with antioxidant potential. J Herbal Pharmacother 2005;5:7-19.
12. Mensor LL, Menezes FS. Screening of Brazilian plant extracts for antioxidant activity by the use of DPPH free radical method. Phytother Res 2001;15:127-30.
13. Jayasri MA, Mathew L, Radha A. A report on the antioxidant activity of leaves and rhizomes of Costus pictus D. Don Int J Integ Biol 2009;5:20-6.
14. Miller HE. A simplified method for the evaluation of antioxidant. J Am Oil Chem Soc 1971;48:91-5.
15. Yen GC, Chen HY. Antioxidant activity of various tea extracts in relation to their antimutagenicity. J Agric Food Chem 1995;43:27-32.
16. Dinis TC, Madeira VM. Action of phenolic derivatives (acetaminophen, salicylate and 5-aminosalicylate) as inhibitors of membrane lipid peroxidation and as peroxyl radical scavengers. Arch Biochem Biophys 1994;36:161-9.
17. Nishikimi M, Rao NA. The occurrence of superoxide anion in the reaction of reduced phenazine methosulphate and molecular oxygen. Biochem Biophys Res Commun 1972;48:849-53.
18. Folin O, Ciocalteu V. On tyrosine and tryptophan determination in proteins. J Biol Chem 1927;27:627-50.
19. Amarowicz R, Pegg PB. Free-radical scavenging capacity and antioxidant activity of selected plant species from the Canadian prairies. Food Chem 2004;84:551-62.
20. Gülçin I, Elias R, Gepdiremen A, Chea A, Topal F. Antioxidant activity of bisbenzylisoquinoline alkaloids from *Stephania rotunda*: Cepharanthine and fancchinoline. J Enzyme Inhib Med Chem 2010;25:44-53.
21. Aswatha Ram HN, Shreedhara CS, Gajera FP, Zanwar SB. Antioxidant studies of aqueous extract of *Phyllanthus reticulatus* Poir. Pharmacol 2008;1:351-64.
22. Jun MT, Ujianzhang L. Identification and evaluation of antioxidant activities of bamboo extracts. For Stud China 2004;6:1-5.
23. Jayaprakasha GK, Singh RP. Antioxidant activity of grape seed (*Vitis vinifera*) extracts on peroxidation models *in vitro*. Food Chem 2001;53:1841-56.
24. Suja KP, Jayalekshmy A. Antioxidant activity of sesame cake extract. Food Chem 2007;91:213-9.
25. Singh RP, Murthy KN. Studies on the antioxidant activity of Pomegranate (*Punica granatum*) peel and seed extracts using *in vitro* models. J Agri Food Chem 2002;50:81-6.
26. Meir S, Kanner J. Determination and involvement of aqueous reducing compounds in oxidative defense systems of various senescing leaves. J Agric Food Chem 1995,43:1813-919.
27. Yildirim A, Mavi A. Comparison of antioxidant and antimicrobial activities of Tilia (*Tilia argentea* Desf Ex DC), Sage (*Salvia triloba* L.) and Black Tea (*Camellia sinensis*) extract. J Agric Food Chem 2000;48:5030-4.
28. Zou YP, Lu YH. Antioxidant activity of a flavonoids rich extract of *Hypericum perforatum* L. *in vitro*. J Agric Food Chem 2004;52:5032-9.
29. Hsu CL, Chen W. Chemical composition, physical properties and antioxidant activities of yam flours as affected by different drying methods. Food Chem 2003;83:85-92.
30. Elmastas M, Gulcin I. Radical scavenging activity and antioxidant capacity of bay leaf extracts. J Iran Chem Soc 2006;3:258-66.
31. Kanatt RC. Antioxidant potential of mint (*Mentha spicata* L.) in radiation processed lamb meat. Food Chem 2007;100:451-8.
32. Halliwell B, Gutteridge JM. Free Radicals Biol Med. Oxford: Clarendon Press; 1989. p. 23-30.
33. Pietta PG. Flavonoids as antioxidants. J Nat Prod 2000;63:1035-42.
34. Sakihama Y, Cohen MF. Plant phenolic antioxidant and prooxidant activities phenolics-induced oxidative damage mediated by metals in plants. Toxicology 2002;177:67-80.

Source of Support: Nil, **Conflict of Interest:** None declared.