Free radical scavenging activity of bark of Hypericum mysorense Heyne

Sornalakshmi V[1], Tresina PS[2], Mohan VR[3]

[1] Department of Botany, A.P.C. Mahalaxmi College for Women, Tuticorin.

[2] Ethnopharmacology unit, Research Department of Botany, V.O. Chidambaram College, Tuticorin.

Abstract- **The genus Hypericum is a large genus of herbs or shrubs with more than 450 species dispersed worldwide. The plants grow generally in temperate regions and are used in traditional medicine in many parts of the world. In this study, antioxidant activity of petroleum ether, benzene, ethyl acetate, methanol and ethanol extract of bark of Hypericum mysorense was determined. Antioxidant activity was evaluated using four different reactive oxygen species (ROS) scavenging assays containing DPPH (1,1-diphenyl-2-pricrylhydrazyl) free radical, hydroxyl radical, superoxide anion, ABTS radical and reducing power assay. Ethanol extract of Hypericum mysorense (800µg/ml) exhibited the maximum DPPH (113.64%) and Superoxide (113.27%) radical scavenging activity. Methanol extracts showed higher Hydroxyl (106.31%) and ABTS (116.22%) radical scavenging activity. It also exhibited higher reducing activity. Hence, these extracts could be considered as natural antioxidants and may be useful for treating diseases arising from oxidative deterioration.**

KEYWARDS: Hypericum mysorense bark, DPPH, free radical, reducing power.

INTRODUCTION

A free radical is a molecule with one or more unpaired electrons in the outer orbital. Most of these free radicals are in the state of reactive oxygen and nitrogen species [1]. These free radicals are generated at the end of metabolic pathways in the human cellular system. It is dangerous to human and animal system, damage the membrane of the cells [2]. Many diseases are caused by lipid oxidation in biological membranes and ROS are the important initiators of these reactions [3]. Enzymatic scavengers such as catalase, glutathione peroxide and superoxide dismutase are present in the eukaryotic system. These scavengers have a number of cellular defence systems. As the ROS are implicated in several diseases, antioxidants play an important role in preventing the interaction of reactive oxygen species with biological system [4]. Many synthetic antioxidants are currently in use, on the other hand, there is a rising evidence of consumer preference for natural antioxidants because of their potentially lower toxicity [5].

The genus Hypericum is a large genus of herbs or shrubs with more than 450 species distributed worldwide. The plants grow widely in temperate regions and are used in folklore medicine in many parts of the world. It has been reported that it contains some antiviral prenylated phloroglucinol derivatives [6], and variety of compounds such as flavonoids [7], xanthones [8], chromenyl ketones [9], hyperforins derivatives [10], n-alkanes [11, napthodianthrones [12] and essential oil [13]. The phytochemistry of Hypericum has attracted the attention of scientists mainly for the two marker compounds, hypericin and hyperforin due to their potent biological activities. Hypericum mysorense belongs to the family Hypericaceae. Hypericum mysorense is a plant native of the Nilgiri Hills in India. It is closely related to Hypericum perforatum.

Recently interest has been increased considerably in finding natural occurring antioxidants that are used in foods or medicinal materials to replace synthetic antioxidants which are being restricted due to their side effects such as carcinogenicity [14]. Hence, the present study aims to examine the antioxidant capacity of bark extracts of H. mysorense for their in vivo antioxidant activity.

MATERIALS AND METHODS

Collection of plant material

Hypericum mysorense Heyne was collected from Kothagiri, Nilgiri Biosphere Reserve, Western Ghats, Tamil Nadu. The plant samples were identified with the help of local flora and authenticated by Botanical Survey of India, Southern Circle, Coimbatore, Tamil Nadu, India. A voucher specimen of collected plants was deposited in the Ethnopharmacological Unit, PG & Research Department of Botany, V.O. Chidambaram College, Thoothukudi District, Tamil Nadu.

Chemicals

All the chemicals and reagents used in the experiments were of analytical grade and were obtained from BDH (England and India), E. Merck (Germany), Sigma Chemical Company (U.S.A.), Sarabhai, M. Chemicals (India) and LOBA–Chemie Indo Austranol Co., (India). Whenever necessary, the solvents were redistilled before use.

Preparation of plant extract

H. mysorense bark was cut into small pieces, washed and dried at room temperature; the dried bark was powdered in a

Wiley mill. The coarse bark powder (100g) of H. mysorense 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 estimation of total phenolic and flavonoid.

Estimation of total phenolic content

Total phenolic contents were estimated using Folin-Ciocalteau reagent based assay as previously described [15] with little modification. To 1mL of each extract (100µg/mL) in methanol, 5mL of Folin-Ciocalteau reagent (dluted ten-fold) and 4mL (75g/L) of Na2CO3 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 . [16] An aliquot of 0.5ml of sample (1mg/mL) was mixed with 0.1mL of 10% aluminium chloride and 0.1mL of potassium acetate (1M). In this mixture, 4.3ml of 80% methanol was added to make 5mL volume. This mixture was vortexed and the absorbance was measured spectrophotometrically at 415nm. 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.

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 [17]. Briefly, an 0.1mM solution of DPPH in methanol was prepared, and 1mL of this solution was added to 3 mL of the solution of all extracts at different concentration (50,100,200,400 & 800µg/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 to scavenging the DPPH radical was calculated by using the following formula. DPPH scavenging effect (% inhibition) = {(A0 – A1)/A0)*100}

Where, A0 is the absorbance of the control reaction, and A1 is the absorbance in presence of all of the extract samples and reference. All the tests were performed in triplicates and the average of the results were calculated.

Hydroxyl radical scavenging activity

The scavenging capacity for hydroxyl radical was measured according to the modified method of Halliwell [18]. Stock solutions of EDTA (1mM), FeCl3 (10 mM), Ascorbic Acid (1mM), H2O2 (10mM) and Deoxyribose (10 mM) were prepared in distilled deionized water.

The assay was performed by adding 0.1mL EDTA , 0.01mL of FeCl3, 0.1mL H2O2, 0.36mL of deoxyribose, 1.0mL of the extract of different concentration (50,100,200,400 &800µg/mL)dissolved in distilled water,0.33mL of phosphate buffer (50mM , pH 7.9), 0.1mL of ascorbic acid in sequence . The mixture was then incubated at 370C for 1 hour. 1.0mL portion of the incubated mixture was mixed with 1.0mL of 10%TCA and 1.0mL of 0.5% TBA (in 0.025M 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. [19]. 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 (0.3mM), 0.5 mL NADH (0.936mM) solution, 1.0 mL extract of different concentration (50,100,200,400 & 800µg/mL), and 0.5 mL Tris – HCl buffer (16mM, PH 8.0). The reaction was started by adding 0.5 mL PMS solution (0.12mM) 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. [20]. ABTS radical cation (ABTS+) was produced by reacting 7mM 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 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 [21]. 1.0 mL of solution containing 50,100,200,400 &800µg/mL of extract was mixed with sodium phosphate buffer (5.0 mL, 0.2 M, pH6.6) 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 read at 700 nm. The experiment was repeated thrice and the average of the results was calculated.

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

RESULTS AND DISCUSSION

Total phenolic and total flavonoid contents

The total phenolic and total flavonoid contents of the methanol extract of H. mysorense bark was found to be 1.14g 100g-1 and 1.26g 100g-1 respectively.

The close relationship between total phenolic content and high antioxidant activity was reported in many studies and several researchers demon¬strated that phenolic compounds were one of the most effective antioxidants [22,23,24].

Table 1. IC50 value of different solvent extract of bark of H. mysorense (µg/ml)

Different solvent extract	DPPH assay	Hydroxyl assay	Superoxide assay	ABTS assay
Petroleum ether	18.79	21.45	22.38	23.88
Benzene	18.21	22.07	22.98	23.03
Ethylacetate	20.07	21.97	22.07	22.11
Methanol	22.78	24.95	23.05	25.69
Ethanol	24.27	21.63	25.56	22.67
Standard (Ascorbic acid)	20.11	19.46	22.18	-
Standard (Trolox)	-	-	-	20.67

1, 1-Diphenyl-2-picryl- hydrazil (DPPH) radical scavenging activity

DPPH radical scavenging method was used to evaluate the antioxidant capacity of the seaweed extracts, because the use of DPPH radical provides an easy, rapid and convenient method to evaluate the anti¬oxidants and radical scavengers [25]. The radical-scavenging activity of petroleum ether, benzene, ethyl acetate, methanol and ethanol extracts of A. spicifera are shown in Fig. 1 and expressed as percentage reduction of the initial DPPH absorption by the tested compound. The scavenging activity increases with the increasing concentration of the standard and sample. The percentage of inhibition was highest (113.6%) for ethanolic extract followed by methanol, ethyl acetate, petroleum ether and benzene. The IC50 values of ethanol extract of bark of H. mysorense and standard ascorbic acid were 24.27.µg/ml and 20.11µg/ml respectively (Table 1). Chandrashekar et al. [26] studied antioxidant activity of H.perforatum, H.japonicum, and H. patulum. Extract of H.mysorense was found to scavenge DPPH radicals more efficiently than other Hypericum species

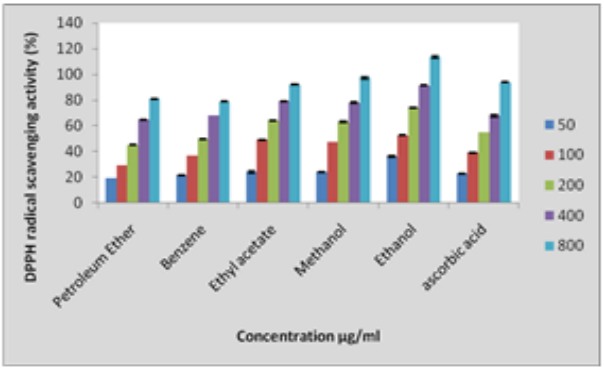

Fig. 1 DPPH radical scavenging activity of different solvent extracts of bark of H. mysorense

Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity of enzymatic extracts from seaweed was measured as the percentage of inhibition of hydroxyl radicals generated in the Fenton reaction mixture. Hydroxyl radical scavenging activity of petroleum ether, benzene, ethyl acetate, methanol and ethanol of H. mysorense is shown in Fig 2. Methanol extract showed a very high potent activity. The methanol extract of H. mysorense displayed a scavenging behaviour of 106.31% at 800 µg/ml concentration in hydroxyl radical. The IC50 values of methanol extract and standard ascorbic acid were 24.95µg/ml and 19.46µg/ml respectively (Table 1). As the strongest of free radicals, the ability of hydroxyl radical to damage cells extensively is well known [27]. All the bark extracts of H. mysorense scavenge hydroxyl radicals in a concentration dependent manner. The scavenging of the hydroxyl radicals may be due to the presence of phenolic compounds in the extracts [28].

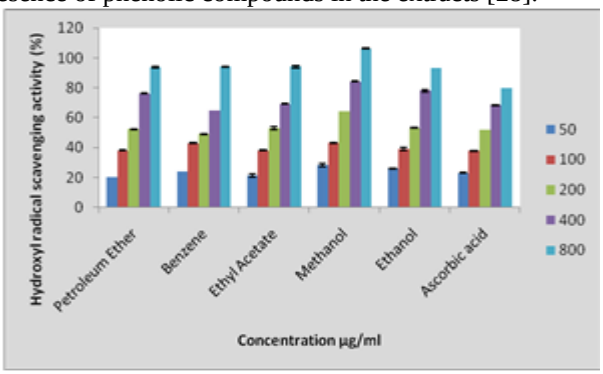

Fig. 2 Hydroxyl radical scavenging activity of different solvent extracts of bark of H. mysorense

Superoxide radical scavenging activity

Superoxide anion radical is generated by four electron reduction of molecular oxygen into water. This radical also formed in aerobic cells due to electron leakage from the

electron transport chain. Superoxides are generated from molecular oxygen of oxidative enzymes and as well as non enzymatic reactions such as auto oxidation by catecholamines [29]. Superoxide anion radical scavenging activity of bark extracts of H. mysorense was denoted in Fig.3. It indicates that, ethanol extract of H. mysorense exhibited the maximum superoxide radical scavenging activity of 113.27% which is higher than the standard ascorbic acid whose scavenging effect is 93.51%. IC50 values of ethanol extract of bark of H. mysorense and andard ascorbic acid are 25.56µg/ml and 22.18µg/ml respectively (Table 1).

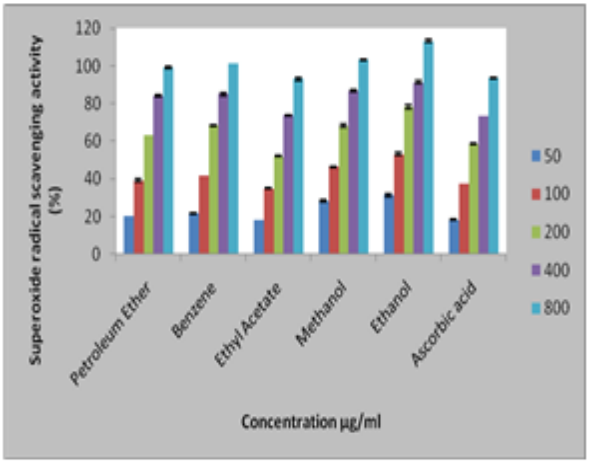

Fig. 3 Superoxide radical scavenging activity of different solvent extracts of bark of H.mysorense

ABTS radical cation scavenging activity

ABTS radical scavenging activity involves a more drastic that is chemically produced and it is a recent one. It is increasingly utilised to screen complex antioxidant mixtures like plant extracts, beverages and biological fluids. Its efficacy in both the organic and aqueous media and the stability in a wide pH range have caused an interest in the use of ABTS in the estimation of antioxidant activity [30]. Hagerman et al. [31] reported that the high molecular weight phenolics (tannins) have more ability to quench free radicals (ABTS). H. mysorense extracts were subjected to ABTS radical cation scavenging activity and the results were shown in Fig 4. The methanol extract exhibited potent ABTS radical cation scavenging activity in concentration dependent manner. At 800 µg/ml concentration, methanol extract of H. mysorense possessed 116.22 % scavenging activity on ABTS which is higher than the standard trolox whose scavenging activity is 79.16%. IC50 values of methanol extract of H. mysorense and standard trolox were 25.69µg/ml and 20.67µg/ml respectively (Table 1).

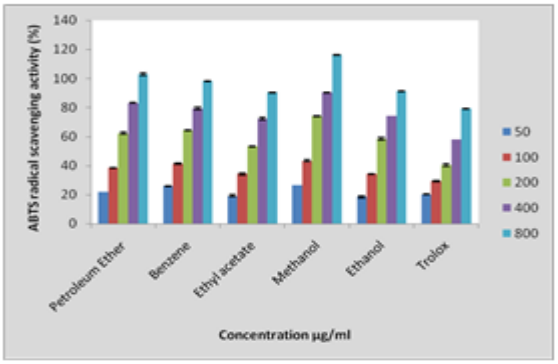

Fig. 4 ABTS radical cation scavenging activity of different solvent extracts of bark of H.mysorense

Reducing power

Figure 5 shows the reducing ability of different solvent extracts of H. mysorense comparision to ascorbic acid. The absorbance of the solution increased as the concentration increased. A higher rate of absorbance is an indicator of an increased reducing power. Several reports indicated that the reducing power of bioactive compounds was associated with antioxidant activity [32]. In the Reducing power assay, antioxidants in the sample reduce ferric to ferrous in a redox-linked colourimetric reaction that involves single electron transfer [33]. The reducing power shows that the antioxidant compounds are electron donars and reduce the oxidized intermediate of the lipid peroxidation process, so that they can act as primary and secondary antioxidants [34].

CONCLUSION

The results of the antioxidant properties of H. mysorense are certainly highly valuable to promote the use of this plant as natural sources of potential antioxidants. End results can be used in the future to identify, isolate and characterize the specific compounds accountable for antioxidant activity.

REFERENCES

[1] Wong, C.K., V.E.C. Ooi and P.O. Ang 2000. Protective effect of seaweeds against liver injury caused by carbon tetra chloride in rats. Chemosphere., 41: 173-176.

[2] Foon, T.S., L. Ai Ai, P. Kuppusamy, M. M.Yusoff and N. Govindan 2013. Studies on in vitro antioxidant activity of marine edible seaweeds from the east coastal region of Peninsular Malaysia using different extraction methods. J.Coastal Life Med., 1(3):193-198.

[3] Frei, B. 1994. Natural antioxidants in human health and disease. Academic Press, San Diego

[4] Ames, B.N., M.K. Shigenaga and T.M. Hagen 1993. Oxidants, antioxi-dants and degenerative disease of aging. Proc. National Academy of Science, USA 90:7915–7922.

[5] Orcic, D.Z., N.M. Mimica-Dukic, M.M. Franciskovic, S.S. Petrovic and E.D. Jovin 2011.

Antioxidant activity relationship of phenolic compounds in Hypericum perforatum L. Chem Cent J., 5(34).

[6] Tada M, Chiba K, Yamada H, Maruyama H. Phloroglucinol derivatives as competitive inhibitors against thromboxane A2 and leukotriene D4 from Hypericum erectum. Phytochem. 1991; 30: 2559-2562.

[7] Chung MI, Lai MH, Yen MH, Wu RR, Lin CN. Phenolics from Hypericum geminiflorum. Phytochem. 1997; 44: 943-947.

[8] Wu QL, Wang SP, Du LJ, Zhang SM, Yang JS, Xiao PG. Chromone glycosides and flavonoids from Hypericum japonicum. Phytochem. 1998a; 49:1417-1420.

[9] Wu QL, Wang SP, Du LJ, Yang JS, Xiao, PG. Xanthones from Hypericum japonicum and H.henryi. Phytochem. 1998b; 49: 1395-1402.

[10] Decosterd LA, Stoeckli-Evans H, Chapuis JC, Msonthi JD, Sordat B, Hostettmann K. New Hyperforin Derivatives from Hypericum revolutum VAHL with Growth-Inhibitory Activity against a Human Colon Carcinoma Cell Line. Helv Chim Acta. 1989; 72: 464-471.

[11] Brondz I, Greibrokk J, Aasen AJ. n-Alkanes of hypericum perforatum: a revision. Phytochem.1983; 2: 295-296.

[12] Kitanov GM. Hypericin and pseudohypericin in some Hypericum species. Biochem Syst Ecol. 2001; 29: 171-178.

[13] Cakir A, Duru ME, Harmandar M, Ciriminna R, Passannanti S, Piozzi F. Comparison of the volatile oils of Hypericum scabrum L. and Hypericum perforatum L. from Turkey. Flavour Frag J. 1997; 12: 285-287.

[14] Kumaran A, Karunakaran JK. In vivo antioxidant activities of methanol extracts of five Phyllanthus species from India. LWT Food Sci. Technol. 2007; 40:344.

[15] McDonald S, Prenzler PD, Antolovich M, Robards K. Phenolic content and antioxidant activity of olive extracts. Food Chem. 2001; 73:73-84.

[16] Eom SH, Cheng WJ, Hyoung JP, Kim EH, Chung MI, Kim MJ, Yu C, Cho DH. Far infra red ray irradiation stimulates antioxidant activity in Vitis flexuosa Thunb. Berries, Korean J. Med. Crop Sci. 2007; 15:319-323.

[17] Shen Q, Zhang B, Xu R, Wang Y, Ding X, Li P. Antioxidant activity in vitro of selenium-contained protein from the se-enriched. Bifodobacterium animalis 01. Anaerobe. 2010; 16:380-386.

[18] Halliwell B, Gutteridge JMC, Aruoma OI. The deoxyribose method: a simple test to be assay for determination of rate constants for reaction of hydroxyl radicals. Anal. Biochem. 1987; 65:215-219.

[19] Srinivasan R, Chandrasekar MJN, Nanjan MJ. Suresh B. Antioxidant activity of Caesalpinia digyna root. J. Ethnopharmacol. 2007; 113:284-291.

[20] Huang MH, Huang SS, Wang BS, Sheu MJ, Hou WC. Antioxidant and anti-inflammatory properties of Cardiospermum halicacabum and its reference compounds ex vivo and in vivo. J. Ethnopharmacol. 2011; 133:743-750.

[21] Kumar RS, Hemalatha S, In vitro antioxidant activity of alcoholic leaf extract and subfractions of Alangium lamarckii Thwaites, J. Chem. Pharm. Res. 2011; 3:259-267.

[22] Pellegrini N, Simonetti P, Gardana C, Brenna O, Brighenti F, Pietta P. Polyphenol content and Total antioxidant activity of Vini Novelli (Young Red Wines). J Agric Food Chem. 2000; 48: 732-735.

[23] Brown JE, Rice-Evans CA. Luteolin-rich artichoke extract protects low density lipoprotein from oxidation In vitro. Free Rad Res. 1998; 29: 247-255.

[24] Halliwell B. Free radicals, antioxidants and human disease: curiosity, cause or consequence. Lancet. 1994; 344: 721-724.

[25] Nickavar, B., M. Kamalinejad and H. Izadpanah 2007. In vitro free radi¬cal scavenging activity of five Salvia species. Pak J Pharm Sci. 20(4):291-4.

[26] Chandrashekhar RH, Venkatesh P, Arumugam M, Vijayan P. Estimation of total phenols with special emphasis to antioxidant potentials of few Hypericum species. Pharmacologyonline. 2009; 1: 680-687.

[27] Koppenaol, W.H. and J.F. Liebman 1984. The oxidizing nature of the hydroxyl radical. A comparison with the ferryl ion (FeO2+). J. Phys.Chem. 88:99–101.

[28] Vadivukkarasi S, Pavithra K. Evaluation of free radical scavenging activity of various leaf extracts from Kedrostis foetidissima (Jacq.) Cogn.Biochem Anal Biochem. 2014; 3:2.

[29] Mustafa, A.G., I.N. Singh, J. Wang and K.M. Carrico 2010. Mitochondrial protection after traumatic brain injury by scavenging lipid peroxyl radicals. J Neurochem., 14(1):271-280.

[30] Huang, M.H., S.S. Huang, B.S. Wang, M.J. Sheu and W.C. Hou 2011. Antioxidant and anti-inflammatory properties of Cardiospermum halicacabum and its reference compounds ex vivo and in vivo. J. Ethnopharmacol., 133:743-750.

[31] Hagerman AE, Riedl KM, Jones GA, Sovik KN, Ritchard NT, Hartzfeld PW, Riechel TL. High molecular weight plant polyphenolics (tannins) as biological antioxidants. J. Agric. Food Chem. 1998; 46:1887–1892.

[32] Siddhuraju P, Mohan PS, Becker K, Studies on the antioxidant activity of Indian Laburnum (Cassia fistula L.): a preliminary assessment of crude extracts from stem bark, leaves flowers and fruit pulp. Food Chem. 2002; 79:61-70.

[33] Li, Y.F., C.J. Guo, J.J. Yang, J.Y. Wei, J. Xu and S. Cheng 2006. Evaluation of antioxidant properties of pomegranate peel extract in comparison with pomegranate pulp extract. Food Chem., 96:254-260.

[34] Yen, G.H. and Chen H.Y. 1995. Antioxidant activity of various tea extract in relation to their antimutagenicity. J. Agri. Food Chem., 43: 27-32.

