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Research Article

CORRELATION OF ANTIOXIDANT ACTIVITY WITH PHENOLIC CONTENT AND ISOLATION OF ANTIOXIDANT COMPOUND FROM *LYGODIUM FLEXUOSUM* (L.) SW. EXTRACTS

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ABSTRACT

In vitro antioxidant activity of different extracts of plant *Lygodium flexuosum* (L.) Sw were evaluated using models of DPPH radical scavenging activity, total antioxidant capacity, nitric oxide scavenging activity, ion chelating activity, hydroxyl radical scavenging activity. Phenolic contents of extracts were estimated as gallic acid equivalents by Folin- Ciocalteu method and correlated with antioxidant activity. Highest phenolic content (6.24 %) was found in methanolic extract with highest antioxidant activity. The fractionation and characterization of methanolic extract showed presence of quercetin.

Keywords: *Lygodium flexuosum*, phenolic content, antioxidant activity, quercetin.

INTRODUCTION

Lygodium flexuosum (L.) Sw. (Schizaeaceae) is climbing or scrambling fern with glabrous, pinnately lobed leaves. The rhizomes, roots and leaves are ethnomedicinally useful in the treatment of jaundice[1,2,3]. Antheridiogens[4], Lygodinolide[5], O- P-coumaryldryocrassol, tectoquinone, kaempferol, kaempferol-3-beta-D-glucoside, beta-sitosterol and stigmasterol were main chemical constituents studied from *Lygodium flexuosum*[6].

Many properties of plant products are associated with the presence of phenolic compounds, which are essential for plant development and play an important role in their defense mechanisms. These compounds, present in the regular diet, might be beneficial to human health by lowering incidence of diseases. Active oxygen molecules such as (O2-, OOH), hydroxyl (OH*) and peroxyl (ROOH*) radicals play an important role in oxidative stress related to the pathogenesis of various important diseases. In healthy individuals, the production of free radicals is balanced by antioxidative defense system[7].

Because of increased safety concerns about synthetic antioxidants, exploitation of cheaper and safer sources of antioxidants based on natural origin is the focus of research nowadays. Recently there has been growing interest in oxygen containing free radicals in biological systems and their implied roles as causative agents in the etiology of a variety of chronic disorders. Currently available synthetic antioxidants like butylated hydroxy anisole (BHA), butyrate hydroxy toluene (BHT), tertiary butylated hydroquinone and gallic acid esters, have been suspected to cause or prompt negative health effects. Hence, strong restrictions have been placed on their application and there is a trend to substitute them with naturally occurring antioxidants. Moreover, these synthetic antioxidants also show low solubility and moderate antioxidant activity[8]. Hence to find natural sources of antioxidants was the focus of this study.

Various antioxidant activity methods have been used to monitor and compare the antioxidant activity of foods and herbal drugs. These methods need special equipment and technical skills for the analysis. These analytical methods measure the radical-scavenging activity of antioxidants against free radicals like l, l-diphenyl-2-picrylhydrazyl (DPPH) radical, the superoxide anion radical, hydroxyl radical (OH), or peroxyl radical. Antioxidant activity methods using free radicals are fast, easy and simple. Some methods are summarized as malondialdehyde (MDA) or thiobarbituric acid-reactive-substances (TBARS) assays[9], Solid-phase spectrophotometry using tetrabenzo-(b, f, j, n) (l,5,9,13)-tetraazacyclohexadecine-Cu (II) complex immobilized10, hydroxyl radical scavenging capacity[11], fluorometric analysis[12], bioassay-guided ractionation, high-performance liquid chromatography and spin trapping electron spin resonance (ESR) method[13].

Plant phenolics and flavonoids are especially common in leaves, flowering tissues and woody parts such as the stem and bark[14]. Phenolics compounds are a large, heterogeneous group of secondary plant metabolites that are widespread in plant kingdom[15,16]. Phenolics contain different chemical classes tannins, catechins, flavonoids, steroids etc. The phenolic content is estimated by various methods like Folin Ciocalteu Method[17], Chromatographic response function (HPLC)[18] Reversed phase HPLC[19], Protein dye binding[20]. On the basis availability of the facilities, we referred Folin-Ciocalteu method to estimate phenolic content of the extracts.

The aim of this study was to determine in vitro antioxidant activity of the solvents extracted material of *Lygodium flexuosum* and its correlation with the total phenolic content and characterization of potent extract for its main active constituents.

Experimental

Chemicals

All chemicals and solvents used in this study were of analytical grade and obtained from HiMedia Chemicals Mumbai, India.

Solvent extraction

L. flexuosum was collected from the forest of Bahuligaon, Igatpuri District-Nashik, Maharashtra, India. The entire plant was sent to Botany Dept, University of Pune, Pune for authentication and obtained certificate of authentication. A voucher specimen was deposited in herbarium of our laboratory. The shade dried plants were pulverized and subjected to extraction. The method of extraction was successive extraction by Soxhlet apparatus. The solvents used in sequence for extractions were petroleum ether (60:80), n-hexane, benzene, ethyl acetate, chloroform, acetone, methanol and water.

In vitro antioxidant activity

DPPH radical scavenging activity

DPPH scavenging activity was measured by spectrophotometric method[21]. To 1 ml of various concentrations of extract, 1 ml solution of DPPH (0.1 mM) was added. An equal amount of methanol and DPPH served as control. After 20 min of incubation in dark, absorbance was recorded at 517 nm. The experiment was performed in triplicate and the percentage inhibition calculated by using the formula[22]:

$$\text{Inhibition \%} = \frac{(\text{Control-Test})}{\text{Control}} \times 100$$

Total antioxidant capacity

Total Antioxidant Capacity was measured by spectrophotometric method. 0.1 ml of the extract (10 mg/ml) dissolved in water was combined in ependorff s tube with 1 ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate).The tubes were capped and incubated in a thermal block at 95 °C for 90 min. After cooling to room temperature, the absorbance of aqueous solution was measured at 695 nm against blank. Ascorbic acid was used as the standard and total antioxidant capacity is expressed as equivalents of ascorbic acid[23].

Scavenging of nitric oxide radical

Nitric oxide was generated from sodium nitroprusside and measured by Griess' reaction[24]. Sodium nitroprusside (5 mM) in standard phosphate buffer saline solution (0.025M pH 7.4) was incubated with different concentrations (2-1000 µg/ml) of the test extract dissolved in phosphate buffer saline (0.025 M, pH 7.4) and the tubes were incubated at 25°C for 5 hr. Control experiments without the test compounds but equivalent amounts of buffer were conducted in identical manner. After 5 hr, 0.5 ml of incubation was removed and diluted with 0.5 ml of Griess' reagent (1% sulphanilamide, 2 % O-phosphoric acid and 0.1 % napthyl ethylenediamine dihydrochloride).The absorbance of the chromophore formed during diazotization of nitrite with suphanilamide and its subsequent coupling with napthyl ethylene diamine was read at 546 nm. The experiment was repeated in triplicate.

Ion chelating activity

Ion chelating activity was performed by colorimetric method. The reaction mixture containing 1 ml 0.05% O-phenathroline in methanol, 2 ml ferric chloride 200 mM and 2 ml various concentrations of the test compound was incubated at ambient temperature for 10 min and the absorbance of the same was measured at 510 nm. The experiment was performed in triplicate.

Scavenging of hydroxyl radical

Extracts of different concentrations were prepared in 2% alcohol were taken in different test tubes and evaporated on a water bath. To these, 1 ml of Iron-EDTA solution (Iron was added as 2.0 mg Fe (FeSCU) mixed with Na2EDTA as an aqueous solution in a 1:1 molar ratio), 0.5 ml of 0.018 % EDTA and 1 ml of DMSO (0.85% v/v in 0.1 M phosphate buffer, pH 7.4) were added and the reaction was initiated by adding 0.5 ml of 0.22 % ascorbic acid to each of the test tubes. Test tubes were capped tightly and heated on water bath at 80°-90° C for 15 min. Then the reaction was terminated by the addition of 1 ml of ice-cold trichloroacetic acid (17.5%, w/v) to all the test tubes, kept aside for 2 min and the formaldehyde formed was determined by adding 3 ml of Nash reagent (75 g of ammonium acetate, 3 ml glacial acetic acid, 2 ml acetyl acetone in to 1 L with distilled water) after 10-15 min for colour development[25]. Intensity of yellow colour formed was measured spectrophotometrically at 412 nm against reagent blank. Percentage scavenging of hydroxyl radical was calculated by comparison of the results of the samples with that of the blank[26].

Estimation of Phenolic content

Preparation of sample

Extract solution prepared 2.5 mg/ml in ethanol, mixed, sonicated and volume was made up to 100 ml with HPLC grade water. Solution was filtered with No.l Whatman paper. Folin-Ciocalteu Phenol reagent (0.5 ml) was added to aliquot of sample and mixed again. After 5 min, 1.5 ml sodium carbonate solution (20 %w/v) was added. The volume was adjusted to 10 ml with HPLC grade water, mixed and filtered after 2 hours. The absorbance at 760 nm was recorded. The same solution without the extract solution was used as blank solution. This procedure was repeated for each extract.

Preparation of standard

Gallic acid 0.5mg/ml solution prepared in ethanol and volume was made up to 100 ml with HPLC grade water. Again 1ml resultant solution diluted to 10 ml with water. Remaining procedure was done as mentioned in previous section.

Calculation of phenolic content

Calculation of percent Total Phenols content was based on GAE gallic acid equivalents

$$\text{Total Phenolic content \%} = \frac{\text{A sample} \times \text{W std.}}{\text{A std} \times \text{W sample}} \times 100$$

Characterization of methanolic extract

Isolation of compounds

The dried samples were separately Soxhlet extracted in 80% methanol (100 ml/gm dry weight) on a water bath for 24 hrs[27]. Each of the extracts was defatted in petroleum ether (40°-60°C) (fraction-I), ethyl ether (fraction-II) and ethyl acetate (fraction-III) in succession. Each of the steps was repeated three times to ensure complete extraction in each case. Each reaction was analyzed for the free flavonoids in each of the samples. Fraction III was hydrolyzed by refluxing with 7% H2SO4 (10 ml/gm residue) for 5 hours. The mixture was filtered; the filtrate was extracted with ethyl acetate in a separating funnel. The ethyl acetate layer was washed with distilled water till neutrality and dried in vacuum. The residues were taken up in small volumes of ethanol separately and then subjected to preliminary phytochemical screening. Fraction I, II and III were analyzed for antioxidant activity.

Preparative thin layer chromatography

Glass plates (20 x 20 cm) thickly coated (0.4-0.5 mm) with silica gel 'G' (45 gm/80 ml water) and activated at 100°C for 1 hour and cooled at room temperature were used for preparative thin layer chromatography. The fraction III was applied on separate plates and developed in n-butanol, acetic acid and water (4: 1 : 5, upper layer v/v). Developed plates were air dried and visualized under UV light. Each of the fluorescent spots coinciding with those of standard reference compound of quercetin was marked. The marked spots were scrapped and collected separately along with the silica gel G and eluted with ethanol. Each of elute was then crystallized with chloroform. The purified material was subjected to spectral studies.

Fraction III Compound was obtained as a yellow powder of melting point 300° C and EIMS m/z: 302 [M]+. IR (K Br, nmax in cm-1) Fig. 1: 674, 827, 942, 1220, 1001, 1210, 1382, 1408, 1466, 1660, 1662, 3407. IR absorption band at 3407, 1660, 1408 and 1210 cm-1 were consistent with the presence of hydroxyl, carbonyl and aromatic ring and ether groups respectively.

RESULTS

The anti-oxidant activity of petroleum ether (60:80), n-hexane, benzene, ethyl acetate, chloroform, acetone, methanol and aqueous extracts of *L. flexuosum* were measured by different methods like DPPH scavenging activity, Total Antioxidant Capacity, Scavenging of nitric oxide radical, Ion chelating activity, Scavenging of hydroxyl radical. The aim was to evaluate the activity and isolate possible activity responsible compound. The results obtained by these studies are mentioned in Table 1.

According to DPPH assay, methanol extract showed maximum activity with IC50 = 5.1 µg/ml and the activity was comparable to that observed with standard ascorbic acid having IC50 = 2.76 µg/ml. DPPH is relatively stable free radical. The assay based on measurement of scavenging ability of antioxidants towards stable DPPH radical. From the present result it may be postulated that some hydrogen donors in antioxidant principles of *L. flexuosum* reduces the radical when it react with hydrogen donors in antioxidant principles. DPPH radicals react with suitable reducing agents, the electrons become paired off and solution looses colour stochiometrically depending on number of electrons taken up[28].

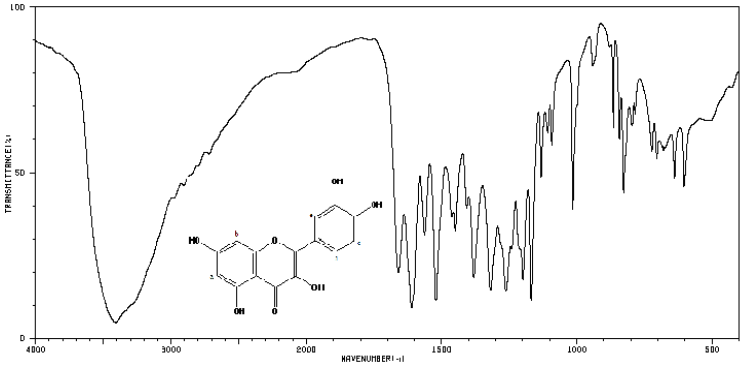

Fig. 1: Infra-red spectrum of fraction III

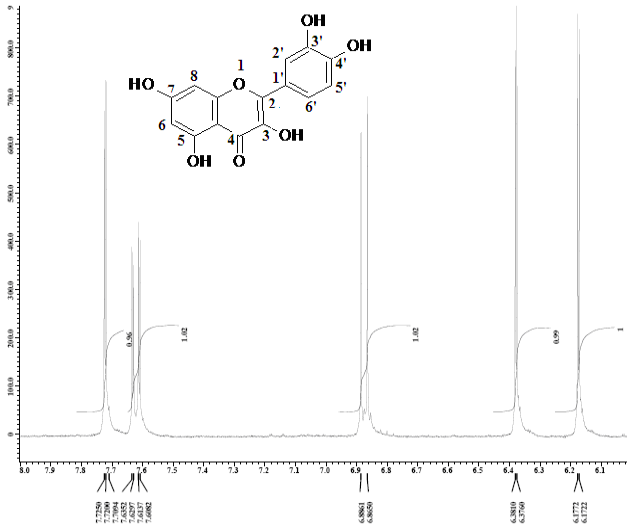

Fig. 2: 1H NMR spectrum of fraction III

1H-NMR (Fig. 2) (400MHz, CDCl3): δ 6.17 (1H, s, H-6, H-8) [C-6, C-8], 6.37 (1H, s, H-8), 6.86 (1H, d, H-5')[C-5'], 7.62 (1H, d, H-6')[C-6'], 7.72 (1H, s, H-2')[C-2']. The 13C-NMR (Fig. 3) (100MHz, CD3OD): δ 94.58 (C-8), 99.41 (C-6), 104.69 (C-10), 116.18 (C-2'), 116.39 (C-5'), 121.85 (C-6'), 124.32 (C-1'), 137.37 (C-3), 146.38 (C-3'), 148.19 (C-4'), 148.93 (C-2), 158.41 (C-9), 162.67 (C-5), 165.72 (C-7), 177.50 (C-4). From these results, fraction III compound may consider as quercetin.

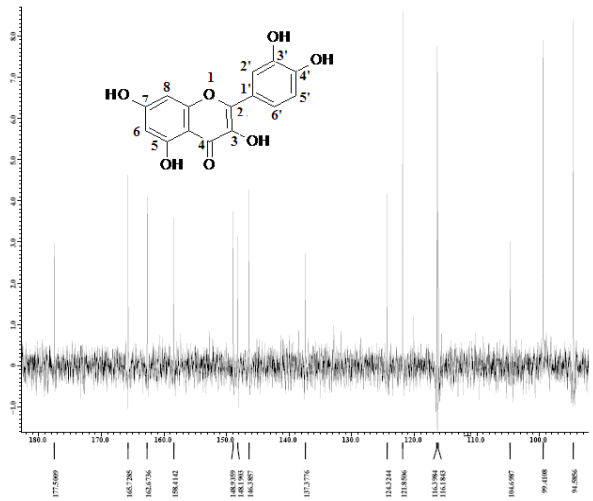

Fig. 3: 13C-NMR spectrum of fraction III

Table 1: Phenolic content of extracts and effect of *L.flexuosum* extracts on various *in vitro* free radicals

Extracts	Extractive value	Phenolic content	DPPH activity	Total antioxidant capacity	Nitric oxide activity	Ion chelating activity	Hydroxyl radical activity
Petroleum Ether	2.30	0.62	11.43±0.005*	34.76±0.98*	13.6±.0.85*	11.31±0.51*	12.28±0.41*
n-hexane	3.14	0.67	11.34±0.24*	23.29±0.84*	20.07±0.94*	14.08±0.34*	13.05±0.42*
Benzene	2.84	1.54	19.63±0.78*	19.55±0.64*	21.94±0.87*	19.24±0.45*	17.09±0.37*
Ethyl acetate	2.01	1.50	20.31±0.65*	22.59±0.54*	24.21±0.64*	26.48±0.86*	24.57±0.34*
Chloroform	3.65	1.14	15.92±0.46*	18.14±0.67*	21.83±0.35*	18.32±0.67*	23.56±0.58*
Acetone	4.53	0.66	19±0.78*	18.43±0.87*	28.8±0.82*	20.15±0.74*	19.26±0.21*
Methanol	7.86	6.24	5.1±0.25*	6.14±0.54*	4.5±0.21*	8.79±0.28*	7.91±0.20*
Aqueous	9.68	4.38	6.92±0.36*	6.71±0.21*	6.18±0.34*	5.72±0.26*	8.91±0.40*
Standard(Ascorbic acid)	--	--	2.76±0.12	3.10±0.20	2.87±0.21	4.50±0.34	2.76±0.30

a % Phenolic Content represents Gallic Acid Equivalents

*Values are in the Mean ± S.D., n = 3 for each experiment, data were analyzed by one way ANOVA followed by Turkey test using Graph pad Instat sotware, * P > 0.001 compared with standard ascorbic acid

In the total antioxidant capacity experiment it was seen that methanol and aqueous extract showed maximum activity with IC50 values 6.14 µg/ml and 6.71 µg/ml respectively. The total antioxidant capacities of extracts were calculated based on formation of phosphomolybdenum complex which was measured spectrophotometrically at 695 nm.

Nitric oxide radical scavenging activity was maximum for methanol extract with IC50 = 4.5 µg/ml in the experiment of ion chelating activity as compared with ascorbic acid IC50 = 6.9 µg/ml. In the assay of hydroxyl radical scavenging activity again methanol extract showed maximum activity with IC50 = 7.91 µg/ml compared with 2.76 µg/ml of ascorbic acid. Nitric oxide is free radical produced in biological cells, involved in regulation of various physiological processes. Nitric oxide is very unstable species under aerobic conditions. It reacts with oxygen to produce stable product nitrate and nitrite through intermediates NO2, N2O4 and N3O4. In present study nitrite produced by incubation of solution of sodium nitroprusside in standard phosphate saline buffer at 25°C was reduced by extracts of *L. flexuosum*. This may be due to antioxidant principles in extracts which compete with oxygen to react with nitric oxides and thus inhibit generation of nitrite[29].

Ortho substituted phenolic compounds may exert pro-oxidant effect by interacting with iron. O-phenanthroline quantitatively forms complex with Fe which get disrupted in the presence of chelating agent[30]. The studied extract interfered with the formation of ferrous-O-phenanthroline complex, thereby suggesting that extract had metal chelating activity.

Hydroxyl radical scavenging activity of extracts was assayed by generating hydroxyl radicals using ascorbic acid-iron-EDTA[31]. The hydroxyl radical formed by the oxidation react with DMSO to yield formaldehyde. The formaldehyde production from DMSO provides a convenient method to detect hydroxyl radicals formed during oxidation of DMSO by Fe+3/ ascorbic acid system which was used to detect hydroxyl radical.

The total phenolic content was obtained and was mentioned in Table 1. It was revealed that methanol extract had the maximum phenolic content 6.24 % The correlation coefficients for phenolic content and antioxidant activity of different extracts were studied and it was found that methanol extract showed good correlation coefficient (r2) of 0.87 to 0.97 for all antioxidant methods. Among all the extracts analyzed, a significant phenolic content and antioxidant activity were found for methanolic extract so it can be predicted that the antioxidant activity may be due to the total phenolic content in the plant. Previously it was revealed that the antioxidant activity of phenolics is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers[32]. We estimated antioxidant activity of extracts of L. *flexuosum*; the activity maybe due to redox properties of the phenolic content. In characterization of methanolic extract three fractions were collected and screened for preliminary phytochemical screening with phenolic content. Fraction III showed maximum phenolic content with presence of flavonoids. IC50 values for Fraction I, II and III are mentioned in Table 2.

Table 2: Effect of *L.flexuosum* methanolic extract fractions on various *in vitro* free radicals

Fractions of methanolic extract	DPPH activity	Total antioxidant capacity	Nitric oxide activity	Ion chelating activity	Hydroxyl radical activity
I	47.88±0.85*	57.40±0.87*	60.33±0.76*	52.54±0.86*	65.22±0.78*
II	69.20±0.94*	69.25±0.78*	66.65±0.68*	68.05±0.67*	68.86±0.26*
III	3.53±0.78*	5.65±0.64*	3.01±0.56*	3.96±0.68*	3.29±0.67*
Standard (Ascorbic acid)	2.76±0.12	3.10±0.20	2.87±0.21	4.50±0.34	2.76±0.30

Values are in the Mean ± S.D., n = 3 for each experiment, data were analyzed by one way ANOVA followed by Turkey test using Graph pad Instat sotware, * P > 0.001 compared with standard ascorbic acid.

Fraction III showed maximum antioxidant activity with IC50 values 3.53, 5.65, 3.01, 3.96, 3.29 µg/ml for DPPH, total antioxidant capacity, nitric oxide activity, ion chelating activity, hydroxyl radical activity assays respectively. Further Fraction III was characterized by spectral studies. It resembled presence of quercetin in Fraction III. Phenolic components phytochemically associated with quercetin like anthocyanins, anthocyanidines, catechins and myricetins may be responsible for the observed activity.

CONCLUSION

The antioxidant activity of *L.flexuosum* extracts were evaluated by five different *in-vitro* testing systems. The results showed that methanolic extract was rich in polyphenol and flavonoid content and had significant antioxidant activity which is in correlation with phenolic content. The fractionation of methanolic extract gave biologically active fraction which on characterization by spectral studies found to be quercetin.

Thus this study gives support for expanding future investigations of pharmacological activities associated with free radicals.

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REFERENCES

1. Jain SK. In: Dictionary of Indian Folk Medicine and Ethnobotany. Deep publications, New Delhi, 1991.
2. Henry AN, Hosagoudar, VB, Ravikumar K. () Ethno-medico-botany of the Southern Western Ghats of India. In: Jain, S.K., (Ed.), Ethnobiology in Human Welfare. Deep Publications, New Delhi, pp. 173-180, *Proceedings of IV International Congress of Ethnobiology*, Lucknow, India, 1996.
3. Kumar K. Notable pertinence of *Lygodium flexuosum* (L.) Sw. in tribal medicine of India: an ethnopharmacognostical investigation. In: Govil JN, Singh VK (Eds.), Recent Progress in Medicinal Plants, vol. I. Ethnomedicine and Pharmacognosy. SCI Tech Publishing LLC, Texas, 2002.
4. Tadayuki Y, Naomi O, Hisakazu Y, Noboru M, Helmut S, Milan P, Mark F, Lewis NM. Identiication of Antheridiogens in *Lygodium circinnatum* and *Lygodium flexuosum. Plant Physiol*, 1996; 11: 741-745.
5. Achari B, Chaudhuri C, Saha CR, Pakrashi SC. X-ray Crystal Srtucture of Lygodinolide: A Novel Spiro Furopyran Perhydrophenanthrene Derivative from *Lygodium flexuosum*, *J. Org. Chem.*, 1990, 55: 4977-4978.
6. Achari B, Basu K, Saha CR, Pakrashi SC. A New Triterpene Ester, an Anthraquinone and Other Constituents of the *Lygodium flexuosum*, *Planta Med*., 1986; 52(4):329-30.
7. Halliwell B. Antioxidants and human diseases: a general introduction. *Nutr. Rev.*, 1997; 55: S 44-S 52.
8. Barlow SM. Toxicological aspects of antioxidants used as food additives. In Food Antioxidants, Hudson BJF, Elsevier, London, 1990.
9. Aruna P. In: Medallion Laboratories Analytical Progress. Plymouth Ave North, Minneapolis, Minnesota, 2001; 19:2, 1-6.
10. Olga A, Olena AK, Natalia AL, Valentina NB. A new test method for the evaluation of total antioxidant activity of herbal products, *J. Agric. Food Chem.*, 2004; 52:21-25.
11. Husain SR, Josiane C, Pierre C. Hydroxyl radical scavenging activity of flavonoids, *Phytochemistry*, 1987; 26: 9, 2489-2491.
12. Stephanson CJ, Stephanson AM, Flanagan GP. Evaluation of hydroxyl radical scavenging abilities of silica hydride, an antioxidant compound, by a Fe 2+ -EDTA-induced 2-hydroxyterephthalate fluorometric Analysis, *J Med Food* 2003; 6: 249-253.
13. Hou WC, Lin RD, Lee TH, Huang YH, Hsu FL, Lee MH. The phenolic constituents and free radical scavenging activities of *Gynura formosana Kiamnra*, *J Sci. Food and Agri.* 2004; 85:615-621.
14. Larson RA. The antioxidants of higher plants. *Phytochemistry* 1988; 27(4): 969- 978.
15. Strube M, Dragstedt LO, Larsen JC. Naturally occurring antitumourigens. I. Plant phenols. The Nordic Council of Ministers, Copenhagen., 1993.
16. Peter BK, Leland JC, Sara W, James AD, Harry LB. Natural Product from Plants, CRC Press, New York, 1999.
17. Mc Donalds, Prenzler PD, Autolovich M, Robards K. Phenolic content and antioxidant activity of olive extracts, *Food Chem.*, 2001; 73:73-74.
18. Martin KL, Choisnard CE, Lamien AM, Wouessidjewe D, Nacoulma OG. Experimental Design Optimization for screening relevant free phenolic acid from various preparation used in Burkina Faso Folk Medicine, *Ar. J. Trad. CAM.*, 2006; 3:115-128.
19. Cheryld LE, David PM. Antioxidant activity and Phenolic contents of Oat Groats and Hulls, *Cereal Chem.*, 1999; 76: 902-906.
20. Bradford MM. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal. Biochem.*, 1976; 72: 248-254.
21. Sreejayan N, Rao MNA. Free radical scavenging activity by curcuminoids, *Drug Res.*, 1996; 46:169.
22. Prasanth Kumar V, Shasidhara S, Kumar MM, Sridhara BY. Effect of Luffechinta on lipid peroxidation and free radical scavenging activity. *J.Pharm. Pharmacol*, 2000; 52 :891-893.
23. Shirwaikar A, Govindrajan R, Rastogi S, Vijaykumar M, Rawat AKS, Ehlotra SM, Puspangandan P. Studies on the antioxidant activities of *Desmodium gangeticum*, *Biol. Pharm. Bull.*, 2003; 26:1424.
24. Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR. Analysis of nitrate and 15N in biological fluids, *Anal. Biochem.*, 1982; 239:131.
25. Bhavani B, Pogozelski WK, Tullius TD. DNA strand breaking by hydroxyl radical is governed by the accessible surface area of the hydrogen atoms of the DNA backbones. *Pro. National Acad. Sci.*, 1998; 95: 9738.
26. Klein SM, Cohen G, Cederbaum AL. Production of formaldehyde during metabolism of dimethyl sulfoxide by hydroxyl radical generating system, *Biochem.*, 1991; 20: 6006.
27. Subramanian SS, Nagarajan S. Flavonoids of the seeds of *Crotlaria retusa* and *C. striata*, *Curr. Sci.*, 1969; 38: 65-68.
28. Blois MS. Antioxidant determination by the use of stable free radicals, *Nature*, 1958 26:1199-1203.
29. Sainani GS, Manika JS, Sainani RG Oxidative stress: a key factor in pathogenesis of chronic diseases, *Med Uptake*, 1997; 1: 1-4.
30. Murthy KNC, Singh RP, Jayprakasha GK. Antioxidant activity of *Vitis vinifera* (Grapes). *J.Agric Food Chem.*, 2002, 50: 5909-5912.
31. Marcocci L, Packer L, Droy-Lefaiz MT, Sekaki A, Gardes-Albert M. Antioxidant action of *Ginko biloba* extract, Meth.Enzymol., 1994; 234: 462-465.
32. Macheix JJ, Fleuriet A. Phenolic acids in fruits. In: Flavonoids in health and disease. Rice-Evans CA and Packer L. Eds. Marcel Dekker Inc., New York, 1998.