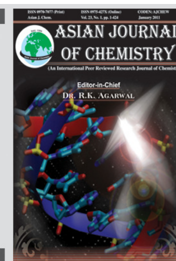
Asian Journal of Chemistry; *Vol. 23, No. 5 (2011), 2207-2211*

ASIAN JOURNAL OF CHEMISTRY

www.asianjournalofchemistry.co.in



Evaluation of Antioxidant Activity of Different Fractions of *Melothria maderaspatana*

ASHOK KUMAR BALARAMAN*, JAGADISH SINGH, SASMITA DASH, ARUP NASKAR and TAPAN KUMAR MAITY

Department of Pharmaceutical Technology, Jadavpur University, Kolkata-700 032, India

*Corresponding author: E-mail: ashokkumarbalaraman@gmail.com

(*Received*: 13 September 2010; *Accepted*: 28 January 2011) AJC-9527

In the present investigation, fractions of *Melothria maderaspatana* were tested for their *in vitro* antioxidant activity. Protocols, such as DPPH radical scavenging assay, lipid peroxidation assay, hydroxy radical scavenging assay, reducing ability, SO radical scavenging assay, were used for evaluation. The strength of fractions and standard drug to scavenge free radicals was inter-compared. The fraction with most promising antioxidant activity was taken for the determination of content of total phenolics and flavanoids. Butylated hydroxy toluene (BHT) and ascorbic acid were used as standard on various occasions in the study. Among all fractions tested, ethyl acetate fraction showed a most significant antioxidant activity. The estimation of total phenolic content and flavonoid content suggested the cause of observed beneficial antioxidant effect associated with the ethyl acetate fraction of *M. maderaspatana*. The study could lead to a conclusion that the antidiabetic, antilipidemic and antihypertensive activities of this plant could be due to the major contribution of high content of polyphenolics and flavanoids present in ethyl acetate soluble fraction of the plant.

Key Words: Antioxidant ability, Free radicals, *Melothria maderaspatana*, Fractions.

INTRODUCTION

Free radicals are capable of attacking the healthy cells of the body, causing them to lose their integrity in structure and functions. Cell damage by free radicals appears to be a major contributor of aging and degenerative diseases such as diabetes, cancer, cardiovascular diseases, cataracts and brain dysfunction[1]. Therefore the occurrence and consequences of free radicals induced cell damages during any pathological conditions have gained much attention among researchers. Epidemiological studies have confirmed that intake of exogenous antioxidants, especially from food supplements, is significantly effective in preventing or suppressing many chronic diseases including diabetes. Therefore, there is a notable interest in antioxidants that are specifically rich in poly phenols and flavanoids and that could help in preventing or decreasing oxidative damages without exerting harmful side effects to normal cells[2].

During diabetes, reactive oxygen species (ROS) are generated from various reactions in various tissues play major role in the development of diabetes associated complications[3]. The expression of bodily antioxidant enzymes, such as superoxide dismutase, catalase and glutathione peroxidase, are known to be very low in pancreatic tissues compared with other tissues in the body. Although animals have their own antioxidant defense systems, the defense can be strengthened externally. This could be especially true for the pancreas, since it has a relatively weak intrinsic defense system against oxidative stress[4]. The increased incidence of vascular damage in diabetic patients always associated with increased free radical activity during diabetes[5,6].

Because of the alleged outcome, decreased or nil side effects or toxic manifestation and comparatively cost effective than synthetic, herbals are widely recommended as drug of choice in many chronic conditions even without their scientific evidences. The substantial role of plant derived medicines as potent antioxidant urged the research world to focus more on their scientific evaluation both in phytochemical and pharmacological aspects.

Melothria maderaspatana (Linn) Cogn. Syn. *Mukia maderaspatana*, *Cucumis maderaspatana* or *Mukia scabella* (family: Cuccurbitaceae) is a monoecious plant having scandent or prostrate stems, very hispid and the leaves are variable in size, densely covered with white hairs. This plant is used as drug in the compound preparation for chronic diseases in which cough is a predominant symptom[7]. Folklores claim this plant as good diuretic, stomachic, gentle aperients, antipyretic and anti-flatulent[8,9]. This plant is recommended in southern part of Sri Lanka for the alleviation of various forms of liver disorders[10]. The plant also reportedly found to exhibit antiinflammatory[11] and anticancer[12] activities.

The ethanol extract obtained from aerial parts of this plant exhibited a significant antidiabetic and lipid lowering activities in STZ-diabetic rats at our laboratory[13]. *M. maderaspatana* has also been proved to be a powerful antioxidant against various *in vitro* models and suggested the plant as an accessible source of natural antioxidants and potential food supplements[14]. The strong association between diabetes and oxidative cell damage prompted us to conclude that the presence of antioxidant principles such as poly phenols and flavonoids, beside other pharmacophores in this plant, could account for possible antidiabetic and hypolipidemic activities. The observed antidiabetic, hypolipidemic and antioxidant activities of the plant led us to optimize its bioactive fraction. In continuation, the current study was designed to evaluate different fractions (chloroform (ChF), ethyl acetate (EaF) and methanol (MeF)) of *M. maderaspatana* using various *in vitro* models and the results were inter compared among fractions and standard drug used in the study.

EXPERIMENTAL

2,2-Diphenyl-1-picryl hydrazyl (DPPH), ascorbic acid, trichlorocaetic acid (TCA), thiobarbituric acid (TBA), sodium dodecyl sulphate (SDS), hydrogen peroxide, 2-deoxyribose, nitroblue tetrazolium (NBT), nicotinamide adenine dinuleotide (NADH), phenazine methosulphate (PMS), potassium ferricyanide.

Aerial parts of *M. maderaspatana* were collected from Koothiramedu village, Kanchipuram district (Tamil Nadu, India) during October-December. The plant was authenticated by Prof. Jayaraman, Director, National Institute of Herbal Science, PARC, Chennai and a specimen voucher (Ref. No. PARC/2009/260) has been kept there for future reference. Washed and shade dried plant materials were pulverized using a mechanical grinder and kept separately in an air tight container till used further.

Preparation of the fractions: Pulverized plant materials of *M. maderaspatana* were extracted with the help of Soxhlet extractor with pet ether (40-60 ºC), chloroform, ethyl acetate and methanol successively to obtain the fractions of respective solvent. The fractions thus obtained were passed through cotton to filter and concentrated separately using a rotary vacuum evaporator at 50 ºC.

DPPH radical scavenging assay: 1,1-Diphenyl-2-dipicryl hydrazyl (DPPH) produce a purple colour stable free radical in solution. When free radical scavengers are added to this solution then the purple colour radical is reduced to yellow colour, based on the antioxidant ability of the agent used. A 100 µM solution of DPPH in methanol was added to the various concentration of the fraction (15.625-250 µg/mL). The absorbance was measured at 515 nm after 10 min incubation in dark. The change in absorbance with respect to the control containing DPPH only in methanol, expressed as percentage scavenging ability of the test samples used[15].

Lipid peroxidation assay: Thiobarbituric acid (TBA) test: lipid peroxidation induced by Fe^{2+}-ascorbate system in rat liver homogenate was estimated by standard method. The reaction mixture (0.5 mL) consisting rat liver homogenate 0.1 mL (25 % W/V in *tris* HCl buffer), 0.1 mL KCl (30 mM), 0.1 mL $FeSO_4$ (0.16 mM), 0.1 mL ascorbic acid (0.06 mM) and 0.1 mL of various concentration (2.5-25 µg/mL) of the fractions were incubated at 37 ºC for 1 h. The reaction mixture was treated with 0.2 mL of SDS (8.1 %), 1.5 mL of TBA (0.8 %) and 1.5 mL of TCA (10 %; pH 3.5). The total volume is made-up to 4 mL by distilled water and then kept in water bath at 90-100 ºC for 0.5 h. After cooling, 1.0 mL of distilled water and 5.0 mL of *n*-butanol and pyridine mixture (15:1 v/v) were added to the reaction mixture, shaken vigorously and centrifuged at 4000 rpm for 10 min. The organic layer was removed and its absorbance was measured at 532 nm[15].

Hydroxy radical scavenging assay: The incubation mixture of total 1 mL was made to contain 0.1 mL of phosphate buffer containing various concentration of fraction (20-100 µg), 0.2 mL ferric chloride (500 µM), 0.1 mL ascorbic acid (1 mM), 0.1 mL EDTA (1 M), 0.1 mL hydrogen peroxide (10 mM) and 0.02 mL 2-deoxyribose. The mixture mixed thoroughly and incubated at room temperature for 1 h. 1 mL of TBA (1 % w/v in 0.05 M NaOH) and 1 mL of 20 % TCA were added to this solution. All the test tubes were kept on a boiling water bath for 0.5 h. The absorbance were measured at 535 nm. Ascorbic acid was used as standard in the study. The percentage of radical scavenging was calculated by the formula, percentage hydroxyl radical scavenged = $(A_0 - A_1)/A_0 \times 100$, where A_0 = absorbance of control and A_1 was absorbance of the test fractions. Control in the study contained water instead of the fraction[15].

Reducing ability: Various concentrations of the fractions (20-200) in phosphate buffer (5 mL, 0.2 M, pH 6.6) were added to 5 mL of potassium ferricyanide (1 % w/v) and incubated at 50 ºC for 20 min. After incubation, TCA solution (10 %) was added to the above mixture. The content was vortexed and centrifuged at 650 × g for 10 min at 23 ºC. The upper layer of supernatant was mixed with distilled water (5 mL) and ferric chloride solution (1 mL, 0.1 % w/v) for 5 min and the absorbance was measured at 700 nm. Higher absorbance of the reaction mixture indicates the stronger reducing ability[16].

Superoxide radical scavenging assay: The superoxide radicals were generated in *tris*-HCl buffer (2.5 mL, 16 mM, pH 8.0) containing NBT (0.5 mL, 300 µM) solution, NADH (0.5 mL, 468 µM) and various concentration of the fractions (25-125 µg/mL). The reaction was started by the addition of PMS (0.5 mL, 60 µM) solution to the mixture. The whole content was incubated at room temperature for 5 min and measured for their absorbance at 560 nm. Butylated hydroxy anisole was used as standard in the study. The ability of the fractions to scavenge the superoxide radicals was estimated using the formula, percentage of superoxide radical scavenged = $\{(A_0 - A_1)/A_0$, where A_0 = absorbance of the control and A_1 = absorbance of test samples[17].

Determination of total phenolic compounds in the extracts: The reaction mixture was composed of 1.0 mL of ethyl acetate fraction (10 mg), 10.0 mL of distilled water and 1.5 mL of the Folin-Ciocalteu reagent. After a period of 5 min, 4.0 mL of 20 % sodium carbonate solution was added and made up to 25 mL with distilled water. This mixture were shaken and allowed to stand for 0.5 h. The absorbance was measured at 765 nm. The percentage of total phenolics was

calculated from the calibration curve of gallic acid plotted and total phenolics were expressed as mg GAE (gallic acid equivalents)/g dry fraction[18].

Determination of total flavonoid content in the extracts: The total flavonoid content was determined spectrophotometrically according to standard protocol. The reaction mixture was composed of 0.5 mL of 2 % aluminum chloride ($AlCl_3$) ethanol and 0.5 mL of ethyl acetate fraction (1 mg/mL). Absorbances at 415 nm were taken after 1 h against blank (ethanol). The total flavonoid content of fractions was expressed as mg of quercetin equivalents/g of dry fraction)[19].

Statistical analysis: Statistical analysis was done using Graph Pad Prism 5 version. All the data expressed as Mean, ± SD of the triplicate assays. Data was analyzed by one way ANOVA. The obtained results were considered statistically significant when *p*-values is < 0.05. The amount of fraction needed to inhibit the 50 % (IC_{50}) free radical concentration was represented graphically by the linear regression method.

RESULTS AND DISCUSSION

Free radicals are the main causative agents in diabetes mellitus and in pathogenesis of many other diseases. Therefore antioxidants, scavenging free radicals, are of increasing interest both in the food and drug discovery fields. Antioxidants can break radical chain reaction that gives out excess free radicals produced in the body during stress or disease conditions. Many plant derived supplements (especially those rich in poly phenols and flavonoids) or drugs show remarkable antioxidant effect gains much importance in health care system. In the present study, the antioxidant activity of different fractions and standard drug was determined on different *in vitro* models. It was observed that the fractions of this plant scavenged the free radicals significantly in a dose dependant manner.

DPPH radical scavenging assay: Various concentrations (15.625, 31.23, 62.5, 125, 250 μg/mL) of chloroform fraction, ethyl acetate fraction and methanol fraction were used in DPPH radical scavenging assay. Butylated hydroxy toluene (BHT) was used as standard. The efficacy of individual fraction in scavenging formed DPPH radicals was determined by changes in absorbance of test solution at 517 nm (Fig. 1). The IC_{50} (50 % inhibition of free radical activity) values of ChF, EaF and MeF were found to be 90.32, 2.95 and 58.65 μg/mL, respectively. The IC_{50} value of ethyl acetate fraction was significantly lower than the standard (24.14 μg/mL).

DPPH assay is one of the most frequently used tools to testing the radical scavenging ability of any bio-active component(s). A redox mechanism involved with DPPH radical is used to determine the antioxidant capacity of the drug in interest. DPPH is a stable free radical because of its spare electrons delocalized over the whole molecule. This delocalization of spare electrons causes a deep violet colour with λ_{max} around 517 nm. When a solution of DPPH is mixed with a substrate acting as a hydrogen atom donor, a stable neutralized form of DPPH is obtained with simultaneous change in violet colour to yellow one. The colour change can be followed to find the anti oxidative potential of a substance[20]. All tested fractions could reduce the stable, purple coloured

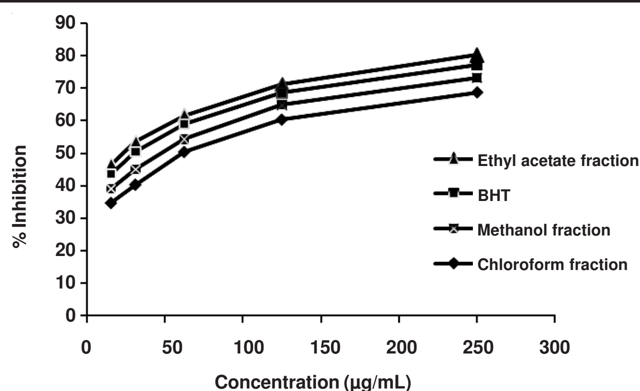


Fig. 1. DPPH free radical scavenging activity of fraction of *M. maderaspatana* at different concentration (15.625-250 μg/mL) and reference compound (BHT). The absorbance was measured at 515 nm with a UV spectrophotomer. Values are expressed as mean ± SEM of three replicates

DPPH radical reaching 50 % of reduction at different concentration. The high content of total phenolics and flavonoids associated with ethyl acetate fraction could account for its significant scavenging ability.

Lipid peroxidation assay: The lipid peroxidation assay performed to test the antioxidant capability of different fractions and standard drug is illustrated in Fig. 2. Addition of fraction of *M. maderaspatana* to the reaction mixture observed to significantly inhibit the lipid peroxidation in a marked dose dependant manner. Among all fractions and standard tested, ethyl acetate fraction has exhibited a lowest IC_{50} concentration (7.70 μg/mL) due to its highest anti oxidant potential where the standard (BHT) could show only 16.31 μg/mL IC_{50}. Lipid peroxidation is a well-established mechanism of cellular damage in both plants and animals, thus used as an indicator of oxidative stress in cells and tissues. The unstable peroxides produced decompose to form a series of reactive compounds such as malondialdehyde (MDA). This product of decomposition, especially MDA, form coloured complex with thiobarbituric acid in the reaction. Inclusion of antioxidant in the reaction mixture reduces the formation of above complex exhibiting which is observed as decrease in absorption[21].

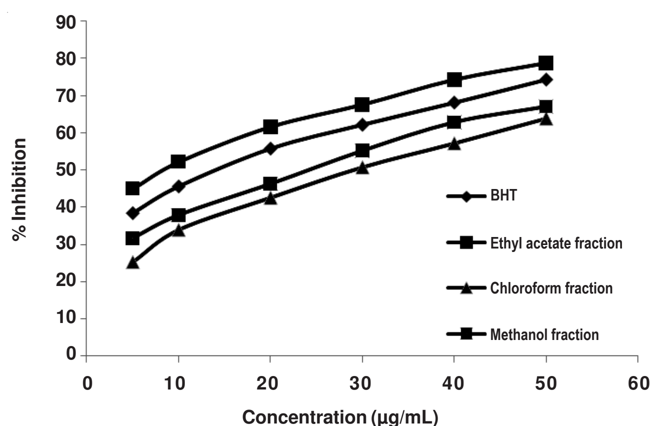


Fig. 2. Antilipid peroxidation ability of fraction of *M. maderaspatana* at different concentration (5-50 μg/mL) and reference compound (BHT). The antioxidant power was estimated based on the concentration of MDA produced. Decrease in absorbance reveals increase in anti oxidant capacity. Values are expressed as mean ± SEM of three replicates

Hydroxy radical scavenging assay: The substrate deoxyribose was disturbed by generation of hydroxylradical by the reaction of Fe^{3+}- EDTA together with H_2O_2 and ascorbic acid. The plant fraction at different concentration was incubated with the above reaction mixture to prevent the damage against the sugar nucleus. The significance of fractions on protection of sugar from hydroxyl free radical damages are shown in Fig. 3. The required concentration for 50 % inhibition for chloroform fraction, ethyl acetate fraction and methanol fraction was found to be 60.09, 29.70 and 47.48 µg /mL, respectively. The IC_{50} value of ascorbic acid was 40.76 µg/mL. The addition of the fraction with the above reaction mixture observed to prevent the damage significantly (Fig. 3) against the sugar unit. The hydroxyl radical scavenging activity of ethyl acetate fraction (IC_{50} 57.52 µg/mL) was more potent than chloroform fraction (IC_{50} 66.99 µg/mL) and methanol fraction (I_{C50} 67.00 µg/mL) including the standard (IC_{50} 66.92 µg/mL)[21].

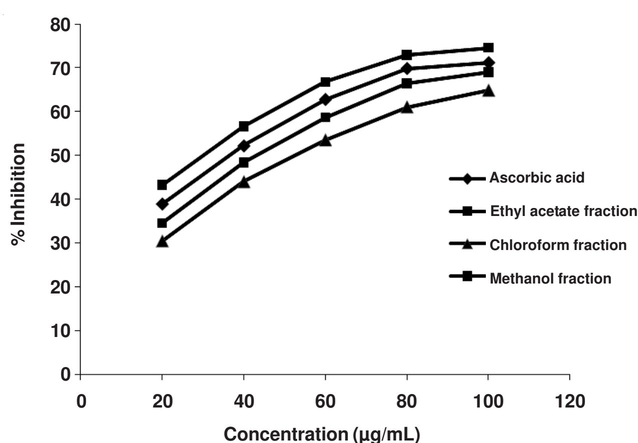


Fig. 3. Hydroxy radical scavenging ability of fraction of *M. maderaspatana* at different concentration (15.625-250 µg/mL) and reference compound (ascorbic acid). The reducing power was estimated based on the absorbance reading at 700 nm with a UV spectrophotomer. Values are expressed as mean ± SEM of three replicates

Reducing ability: The reductive ability of chloroform fraction, ethyl acetate fraction and methanol fraction shown in Fig. 4 reveals that a dose dependent increase in absorbance has been noticed with all the fractions tested and the results were comparable to standard drug (BHT) used in the study. The maximum absorbance shown by EaF (0.726 nm) reveals strong electron donating capacity in the reaction. Ethyl acetate fraction was found to have highest reducing property than BHT (0.676 nm). The maximum reducing property (increase in absorbance) of chloroform fraction and methanol fraction at 200 µg/mL was found to be 0.618 and 0.582, respectively.

Superoxide radical scavenging assay: The superoxide anion derived from dissolved oxygen by PMS/NADH coupling reaction reduces NBT which could be detected by change in the colour. The decrease in absorbance at 560 nm with antioxidants indicates the scavenging of superoxide anion in the reaction mixture. The percentage inhibition (Fig. 5) of superoxide radical generation by various concentrations of fractions (ChF, EaF and MeF) of *M. maderaspatana* and BHT at the same concentrations was calculated in terms of IC_{50}. The drugs show a concentration dependant SOD scavenging activity. Ethyl acetate fraction (41.04 µg/mL) exhibited higher super

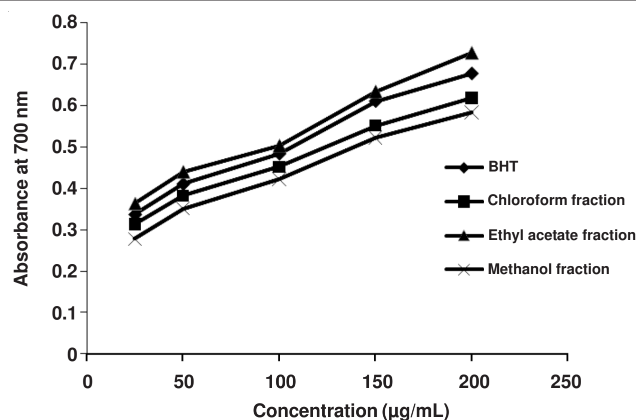


Fig. 4. Reducing ability of fraction of *M. maderaspatana* at different concentration (15.625-250 µg/mL) and reference compound (BHT). The reducing power was estimated based on the absorbance reading at 700 nm with a UV spectrophotomer. Values are expressed as mean ± SEM of three replicates

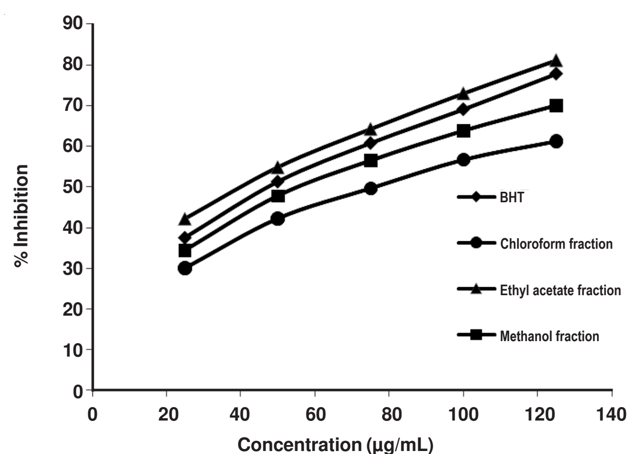


Fig. 5. Superoxide radicals scavenging ability of fraction of *M. maderaspatana* at different concentration (20-125 µg/mL) and reference compound (BHT). The antioxidant power was estimated based on the colour of NBT produced. Decrease in absorbance reveals increase in anti oxidant capacity. Values are expressed as mean ± SEM of three replicates

oxide radical scavenging activity than BHT (51.28 µg/mL). Superoxide anions are a precursor to active free radicals that have potential of reacting with biological macromolecules and thereby inducing tissue damage[22]. In addition, it has been implicated that the superoxide anion could initiate the lipid peroxidation either directly or indirectly by its transformation in to more reactive species such as hydroxyl radical[22,23]. Antioxidant properties of some plant derived lead molecules such as flavanoids are significantly effective in scavenging superoxide anion radicals[24].

Total phenolics and flavanoids content: The antioxidant ability of plant derived medicines is always associated with the bioactive compounds present in it, mainly the content of total phenolics and flavanoids, as they effectively scavenge the free radicals[25,26]. The total phenolics and flavonoids content of ethyl acetate fraction of *M. maderaspatana* is shown in Table-1. The estimated higher amount of total phenolics (10.71 ± 0.16) and flavonoids (7.6 ± 0.15) may be attributed to the significant antioxidant activity of ethyl acetate fraction.

TABLE-1
TOTAL PHENOLICS AND FLAVONOID CONTENT OF ETHYL ACETATE FRACTION (EaF) OF *M. maderaspatana*.
ALL THE VALUES ARE IN MEAN ± SD, (n = 3)

Fraction	Content of total phenolic compounds (mg/g plant fraction in gallic acid equivalents)	Total flavonoids (mg/g plant fraction in quercetin equivalents)
EaF	10.71 ± 0.16	7.6 ± 0.15

Conclusion

The study could lead to a conclusion that the antidiabetic and antilipidemic activities of this plant could be due to the major contribution of high content of polyphenolics and flavanoids present in ethyl acetate soluble fraction of the plant. Further study to isolate a bioactive molecule(s) from the ethyl acetate fraction is under progress.

Statement of conflict of interest: There is no conflict of interest found.

ACKNOWLEDGEMENTS

The authors thank University Grants Commission (UGC), New Delhi for the financial assistance in the form of JRF to Mr. Ashok Kumar Balaraman. The authors also thank the authority of Jadavpur University, Kolkata for providing the facility to carry out the project work.

REFERENCES

1. H. Sies, W. Stahl and A.R. Sundquist, *Ann. NY. Acad. Sci.*, **669**, 7 (1992).
2. B.A. Silva, F. Ferreres, J.O. Malva and A.C.P. Dias, *Food Chem.*, **90**, 157 (2005).
3. J.W. Baynes, *Diabetes*, **40**, 405 (1991).
4. M. Tiedge, S. Lortz, J. Drinkgern and S. Lenzen, *Diabetes*, **46**, 1733 (1997).
5. L.W. Oberly, *Free Rad. Biol. Med.*, **5**, 113 (1988).
6. M.C. Garg, S. and D.D. Bansal, *Indian J. Exp. Biol.*, **34**, 264 (1996).
7. K.R. Kirtikar and B.D. Basu, Indian Medicinal Plants, (III) International Book Distributors, New Delhi, India, edn. 2, pp. 1161-1162 (1975).
8. K.N. Nadkarani, Indian Matria Medica, Bombay, Prakashan Pvt. Ltd., p. 820 (1971).
9. The Wealth of India, Publication and Information Directorate, New Delhi: C.S.I.R., p. 336 (1971).
10. K.A.P.W. Jayatilaka, M.I. Thabrew and D.J.B. Perera, *J Ethnopharmacol.*, **30**, 97 (1990).
11. C.H. Ramakrishnamacharya, M.R. Krishanaswamy, R. Bhima Rao and S. Viswanathan, *Clin. Rheumatol.*, **12**, 214 (1996).
12. A.S.M. Hussein and D.G.I. Kingston, *Fitoterapia*, **53**, 119 (1982).
13. A.K. Balaraman, J. Singh, S. Dash and T.K. Maity, *Saudi Pharm. J.*, **18**, 173 (2010).
14. B. Raja and K.V. Pugalendi, *Central Eur. J. Biol.*, **5**, 224 (2010).
15. Y. Chen, M. Wang, R.T. Rosen and C.T. Ho, *J. Agric. Food Chem.*, **47**, 2226 (1999).
16. M. Oyaizu, *J. Nutr. (Japan)*, **44**, 307 (1986).
17. S.-T. Chang, J.-H. Wu, S.-Y. Wang, P.-L. Kang, N.-S. Yang and L.-F. Shyur, *J. Agric. Food Chem.*, **49**, 3420 (2001).
18. R. Siddhuraju and K. Becker, *J. Agric. Food Chem.*, **51**, 2144 (2003).
19. C. Chang, M. Yang, H. Wen and J. Chen, *J. Food Drug Anal.*, **10**, 178 (2002).
20. M.R. Szabo, C. IditOru, D. Chambre and A.X. Lupea, *Chem. Papers,* **61**, 214 (2007).
21. G.-C. Yen, H.Y. Chen and C.-E. Lee, *J. Sci. Food Agric.*, **79**, 1213 (1999).
22. B. Halliwell and J.M.C. Gutteridge, *Biochem. J.*, **219**, 1 (1984).
23. A.P. Wickens, *Resp. Physiol.*, **128**, 379 (2001).
24. G.C. Yen and P.D. Duh, *J. Agric. Food Chem.*, **42**, 629 (1994).
25. E. Casanova, J.M. García-Mina and M.I. Calvo, *Plant Foods Hum. Nutr.*, **63**, 93 (2008).
26. K. Saito, M. Kohno, F. Yoshizaki and Y. Niwano, *Plant Foods Hum. Nutr.*, **63**, 65 (2008).