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Antioxidant Studies on the Methanol Stem Extract of *Coscinium fenestratum*

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Abstract – The methanol extract of *Coscinium fenestratum*, commonly known as tree turmeric, which is widely used in the indigenous system of medicine was studied for its *in vitro* scavenging activity in different methods viz DPPH scavenging, nitric oxide scavenging, iron chelation activity, superoxide scavenging, ABTS radical scavenging and lipid peroxidation. The results were analyzed statistically by regression method. Its antioxidant activity was estimated by IC_{50} value and the values are 57.1 μg/ml for DPPH radical scavenging, 36.5 μg/ml for iron chelating activity, 51.7 μg/ml for nitric oxide scavenging, 53.63 μg/ml for ABTS scavenging, 44.2 μg/ml for superoxide scavenging, and 40 μg/ml for lipid peroxidation. In all the methods, the extract showed its ability to scavenge free radicals in a concentration dependent manner. The results indicate that *C. fenestratum* has potent antioxidant activity.

Keywords – *Coscinium fenestratum*, nitric oxide, ABTS, DPPH, lipid peroxidation, superoxide.

Introduction

Though oxygen is essential for the aerobic process, cells under aerobic condition are threatened with the insult of reactive oxygen metabolites, a threat which is efficiently taken care of by the powerful antioxidant system in human body. Aerobic life is characterized as the continuous production of oxidants balanced by an equivalent synthesis of antioxidants. The improper balance between reactive oxygen metabolite production and antioxidant defense results in "oxidative stress", which deregulates the cellular function leading to various pathological conditions. Antioxidant principles from natural resources possess multifacetedness in their multitude and magnitude of activities and provide enormous scope in correcting the imbalance. Therefore, much attention is being directed to harness and harvest the antioxidant principles from natural resources.

Coscinium fenestratum Colebr. commonly known as tree turmeric is a woody climbing shrub with a cylindrical stem that is externally yellowish brown and internally yellow and longitudinally fluted. The stem is used in the indigenous medicine for treating inflammation, wounds, ulcers, skin diseases, fever, and also for vitiated conditions of kapha and vata. The stem contains up to 3.5% berberine, ceryl alcohol, sitosterol, palmitic acid, oleic acid, and saponin together with resinous material (The Wealth of India, 1950). Antidiabetic activity of *C. fenestratum* was established in STZ-nicotinamide induced diabetic rats in earlier studies by the authors (Shirwaikar *et al.,* 2005). Its hypotensive (Singh *et al.,* 1990) and hepatoprotective (Venukumar & Latha, 2004) actions have also been reported. The *C. fenestratum* stems, studied for its *in vivo* antioxidant activity in carbon tetra chloride rats showed potent antioxidant activity (Venukumar & Latha, 2002). Though widely used in the indigenous system of medicine, there is however no available data with relation to its antioxidant activity in *in vitro* models. The present study therefore aims to assess the antioxidant activity of the methanolic extract of *C. fenestratum* using *in vitro* methods.

Experimental

Chemicals and instruments – All chemicals and solvents used in the study were of analytical grade. Berberine hydrochloride, DPPH (1, 1-diphenyl 2-picryl hydrazyl) and ABTS (2, 2-azinobis-(3-ethylbenzo-thiazoline-6-sulphonate) were obtained from Sigma Chemicals (St.Louis, Mo, USA). Sodium nitroprusside, ferrous sulphate, trichloroacetic acid, dimethyl sulphoxide, ethylene diamine tetra acetic acid (EDTA), sodium hydroxide, potassium chloride, and sulphanilamide were obtained from Ranbaxy Fine Chemicals Ltd., India. NBT (Nitro

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blue tetrazolium chloride) and thiobarbituric acid were obtained from Himedia Laboratories Ltd., Mumbai, India.

UV spectrophotometer (Shimadazu 160 IPC), homogenizer, centrifuge (Remi, India) and pH meter (Elico Ltd., India) were the instruments used for the study.

Plant material – The plant material of *C. fenestratum* was purchased from Jogappa Shanbag Ayurvedic store, Udupi, Karnataka, India in August 2003 and was authenticated by Dr. Gopalakrishna Bhat, Professor, Department of Botany, Poorna Prajna College, Udupi, Karnataka, India. A voucher specimen (PP 526) has been deposited at the Department of Pharmacognosy, Manipal College of Pharmaceutical Sciences, Manipal, India.

Preparation of methanol stem extract – About 160 g of the stem powder was taken in a soxhlet extractor and extracted with methanol for 72 hours. The solvent was recovered by distillation *in vacuo* and the residue (yield 18 g), stored in the dessicator was used for subsequent experiments.

Preliminary phytochemical screening – Preliminary phytochemical screening (Kokate, 1986) revealed the presence of alkaloids, carbohydrates, phytosterols, and phenolic compounds.

Preparation of *C. fenestratum* stock solution – *C. fenestratum* stock solution was prepared in the concentration of 1000 µg/ml in methanol. From the stock solution different concentrations viz 2, 4, 8, 16, 32, 64, 128, 256, and 512 µg/ml were prepared in methanol and used for antioxidant studies.

Preparation of Ascorbic acid stock solution – Ascorbic acid was used as a standard. Ascorbic acid stock solution was prepared in the concentration of 1000 µg/ml. From the stock solution different concentrations viz 2, 4, 8, 16, 32, 64, 128, 256 and 512 µg/ml were prepared and used for antioxidant studies.

DPPH radical scavenging – To 1 ml of various concentrations of berberine, 1 ml solution of DPPH 0.1 mM was added. An equal amount of methanol and DPPH served as control. After 20 minutes incubation in the dark, absorbance was recorded at 517 nm. The experiment was performed in triplicate. The percentage scavenging of berberine was calculated (Williams *et al.,* 1995, Molyneux, 2004, Schlesier *et al.,* 2002).

Iron chelating activity – The reaction mixture containing 1 ml 0.05% o-phenathroline in methanol, 2 ml ferric chloride 200 µM 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 (Benzie and Strain, 1996; Benzie and Szeto, 1999).

ABTS radical scavenging – ABTS 2 mM and potassium per sulphate 70 mM were prepared in distilled water. 200 µl of potassium persulphate and 50 ml of ABTS were mixed and used after 2 hrs. This ABTS radical cation solution was used for the assay. To 500 µl of various concentrations of test compound, 300 µl of ABTS radical cation and 1.7 ml phosphate buffer pH 7.4 was added. For the control, methanol was used instead of the test compound. The absorbance was measured at 734 nm. The experiment was performed in triplicate (Re *et al.,* 1998, Blois 1958).

Nitric oxide radical scavenging – Sodium nitroprusside 5 mM was prepared in phosphate buffer pH 7.4. To 1 ml of various concentrations of test compound, sodium nitroprusside 0.3 ml was added. The test tubes were incubated at 25 °C for 5 hr after which, 0.5 ml of Griess reagent was added. The absorbance of the chromophore formed during diazotization of nitrite with sulphanilamide and its subsequent coupling with napthyl ethylene diamine was read at 546 nm. The experiment was performed in triplicate (Sreejayan and Rao, 1997 (a), Sreejayan and Rao, 1996 (b).

Superoxide scavenging – Alkaline DMSO was used as a super oxide generating system. To 0.5 ml of different concentrations of the test compound, 1 ml of alkaline DMSO and 0.2 ml of NBT 20 mM in phosphate buffer pH 7.4 was added. The absorbance was measured at 560 nm. The experiment was performed in triplicate (Govindrarajan *et al.*, 2003).

Lipid peroxidation assay

Stock TBA-TCA-HCl reagent

15% w/v tricholoro acetic acid and 0.375% w/v thio barbituric acid were dissolved in 0.25 N hydrochloric acid. The solution was mildly heated to assist the dissolution of TBA.

Preparation of rat brain homogenate

Healthy adult male Wistar albino rats weighing 250 - 300 g were used for the study. The animals were housed in polypropylene cages, maintained under standard conditions (12 h light/12 h dark cycle; 25 ± 3 °C; 35 - 60% humidity), and were fed with a standard rat pellet diet (Hindustan Lever Ltd, Mumbai, India) and water *ad libitum*. The study was approved by the Institutional Animal Ethical Committee of KMC, Manipal, India (IAEC/KMC/03/2003-04).

Randomly selected rats were sacrificed by cervical dislocation, dissected and whole brain tissue was removed carefully and the tissue was homogenated with cold 0.15 M KCl to make 10% homogenate using a teflon homogenizer. The filtered homogenate was used as a source of

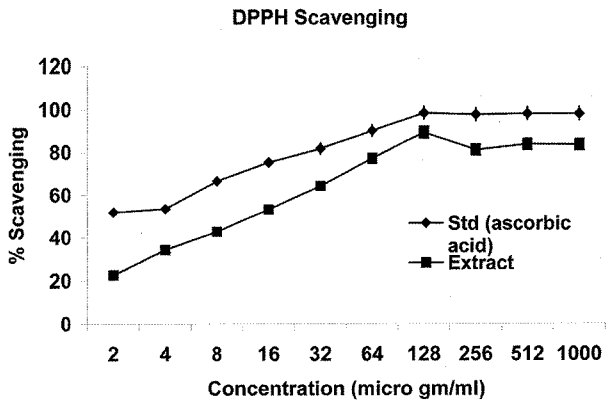

Fig. 1. DPPH radical scavenging activity of different concentrations of *C. fenestratum* and ascorbic acid. The results are expressed in terms of concentration vs % scavenging. Each value represents mean ± S.E.M.

Table 1. IC50 values for the *C. fenestratum* extract and ascorbic acid in different *in vitro* free radical scavenging methods

S. No	model	methanol ext	ascorbic acid
		IC_{50} (μg/ml)	IC_{50} (μg/ml)
1.	DPPH scavenging	57.1	62.4
2.	iron chelating activity	36.5	54.7
3.	nitric Oxide scavenging	51.7	65.5
4.	ABTS scavenging	53.63	74.6
5.	superoxide scavenging	44.2	50.1
6.	lipid peroxidation	40.0	–

polyunsaturated fatty acids for determining the extent of lipid peroxidation (Shirwaikar *et al.,* 2004).

Assay procedure

0.5 ml of tissue homogenate was added to 1 ml of various concentrations of the test compound. The mixture was incubated for 30 min. Peroxidation was terminated by the addition of 2 ml TBA-TCA-HCl reagent. The solution was heated for 15 min in a boiling water bath and then cooled. The flocculent precipitate obtained after cooling was removed by centrifugation at 1000 rpm for 10 min. The absorbance of the supernatant was measured at 535 nm (Yamini and Anil, 2000, Govindrajan *et al.,* 2004, John & Steven, 1984).

Statistical Analysis – All results are expressed as mean ± S.E.M. Linear regression analysis was used to calculate the IC50 values.

Results

Concentrations ranging from 2-1000 μg/ml of the methanol extract of *C. fenestratum* were tested for their

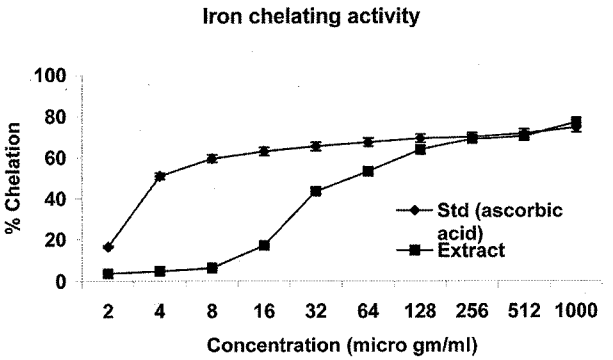

Fig. 2. Reducing power of extract of *C. fenestratum* and ascorbic acid by spectrophotometric detection of the Fe^{+3} - Fe^{2+} transformation. The results are expressed in terms of concentration vs % chelation. Each value represents mean ± S.E.M.

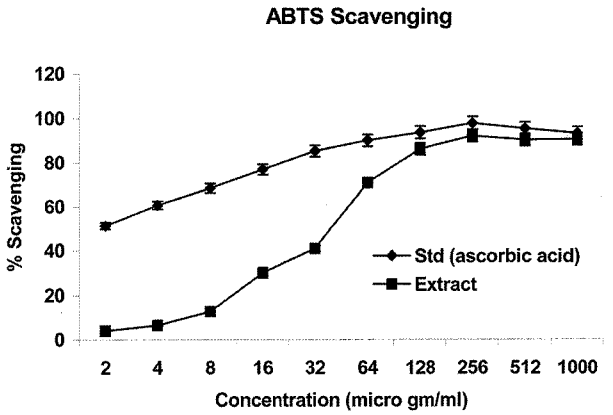

Fig. 3. Scavenging activity of different concentrations of *C. fenestratum* and ascorbic acid in ABTS radical scavenging. The results are expressed in terms of concentration vs % scavenging. Each value represents mean ± S.E.M.

antioxidant activity in different *in vitro* models. It was observed that free radicals were scavenged by the extract in a concentration dependent manner up to the given concentration in all the models. The percentage scavenging and IC50 values were calculated for all models. In the DPPH method, the maximum scavenging activity was found at a concentration 512 μg/ml and the minimum scavenging activity at a concentration of 2 μg/ml (Fig. 1). The IC50 value was found to be 57.1 μg/ml (Table 1). In iron chelating activity, maximum activity was exhibited at 1000 μg/ml with 77.11% scavenging activity (Fig. 2). Even at higher concentration no decline was observed in activity. IC50 value was found to be 36.5 μg/ml. In the ABTS assay, maximum scavenging was found at 1000 μg/ml (Fig. 3) with an IC50 of 53.63 μg/ml. The maximum scavenging activity was observed by the extract in nitric oxide scavenging at 256 μg/ml (Fig. 4). The IC50 value was found to be 51.7 μg/ml. In superoxide scavenging,

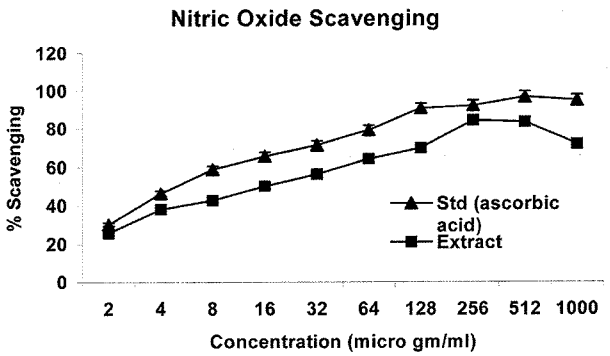

Fig. 4. Nitric oxide scavenging activity of different concentrations of *C. fenestratum* and ascorbic acid. The results are expressed in terms of concentration vs % scavenging. Each value represents mean ± S.E.M.

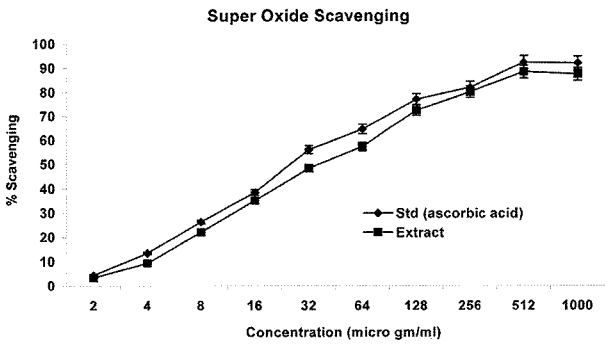

Fig. 5. Superoxide anion radical scavenging activity of different concentrations of *C. fenestratum* and ascorbic acid by NBT method. The results are expressed in terms of concentration vs % scavenging. Each value represents mean ± S.E. M.

the maximum scavenging activity was observed at 512 µg/ml with scavenging of 88.33% and the minimum scavenging activity was observed at 2 µg/ml with scavenging of 3.33% (Fig. 5). The IC50 value was found to be 44.2 µg/ml. Maximum scavenging activity was observed at 512 µg/ml with an IC50 value of 40 µg/ml in lipid peroxidation. In all the models tested the extract exhibited potent antioxidant activity as compared to standard ascorbic acid.

Discussion

There is extensive evidence to implicate free radicals in the development of degenerative diseases (Cross, 1987). Free radicals have been implicated in the causation of ailments such as diabetes, liver cirrhosis, nephrotoxicity, cancer etc (Marx, 1987). Together with other derivatives of oxygen they are inevitable byproducts of biological redox reactions (Arora *et al.,* 2002). Reactive oxygen species such as super oxide anions, hydroxyl radical, and

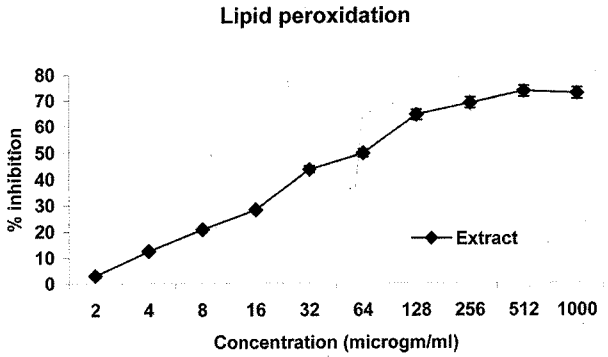

Fig. 6. Antioxidant activity of different concentrations of *C. fenestratum* in lipid peroxidation assay. The results are expressed in terms of concentration vs % inhibition. Each value represents mean ± S.E.M.

nitric oxide inactivate enzymes and damage important cellular components causing tissue injury through covalent binding and lipid peroxidation (Geesin *et al.,* 1990).

DPPH is a stable free radical. When antioxidants react with this stable radical, the electrons become paired off and the bleaching of the color stochiometrically depends on the number of electrons taken up (Badmis *et al.,* 2003). From our findings, it may be postulated that *C. fenestratum* reduces the radical to the corresponding hydrazine when it reacts with the hydrogen donors in the antioxidant principles (Sanchez-Moreno, 2002).

Ortho substituted phenolic compounds may exert pro oxidant effects by interacting with iron. O-phenanthroline quantitatively forms complexes with Fe^{+2} (Mahakunakorn *et al.,* 2004), which get disrupted in the presence of chelating agents. The methanolic extract interfered with the formation of ferrous-O-phenanthroline complex, thereby suggesting that the extract has metal chelating activity. Iron stimulates lipid peroxidation by the Fenton reaction and also accelerates peroxidation by decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals which themselves abstract hydrogen and perpetuate the chain reaction of lipid peroxidation (Chang *et al.*, 2002, Halliwell, 1991). Metal chelating capacity is important since it reduces the concentration of the catalyzing transition metal in lipid peroxidation (Duh *et al.*, 1999). It has been reported that chelating agents, which form bonds with a metal, are effective as secondary antioxidants because they reduce the redox potential thereby stimulating the oxidized form of the metal ion (Gordon, 1990). The observed results demonstrate a marked capacity of the extract for iron binding, suggesting that their action as a peroxidation protector may be related to its iron binding capacity.

ABTS assay is a decolorisation assay, which involves

the direct generation of ABTS radical mono cation, which has a long wavelength absorption spectrum without the involvement of any intermediary radical. The antioxidant activity of the extract by this assay implies that action may be by either inhibiting or scavenging the ABTS radicals since both inhibition and scavenging properties of antioxidants towards this radical have been reported in earlier studies (Rice Evans and Miller, 1997).

Nitric oxide, which is produced in mammalian cells, involved in the regulation of various physiological processes, is a free radical containing an odd number of electrons and can form a covalent link with other molecules by sharing a pair of electrons (Hussain and Ray, 2002, Ialenti *et al.*, 1993). In the present study the *C. fenestratum* extract scavenged the free radicals produced *in vitro* by using sodium nitroprusside. This may be due to the antioxidant principles in the extract, which compete with oxygen to react with nitric oxide (Marcocci *et al.*, 1994).

Superoxide is a highly reactive molecule that can react with many substrates, produced in various metabolic processes, including phagocytosis. It can cause oxidation or reduction of solutes depending on their reduction potential. Both aerobic and anaerobic organisms possess super oxide dismutase enzymes, which catalyze the breakdown of super oxide radical (Cross and Jones, 1991). In our study, alkaline DMSO used for superoxide generation indicates that *C. fenestratum* is a potent superoxide scavenger.

Lipid peroxidation has been implicated in the pathogenesis of a number of diseases and clinical conditions. Malondialdehyde and other aldehydes have been identified as products of lipid peroxidation that react with thiobarbituric acid to give a pink coloured species. The aldehyde products are responsible for DNA damage, generation of cancer and aging related diseases (Wiseman *et al.*, 1996). The decrease in the concentration of malondialdehyde level with increase in the concentration of *C. fenestratum* extract indicated the antioxidant role of the extract.

C. fenestratum extract exhibits its antioxidant action in several ways; removal of oxygen, scavenging of reactive oxygen species and nitrogen species or their precursors, inhibiting reactive oxygen species and reactive nitrogen species, binding metal ions needed for catalysis of reactive oxygen generation and up regulation of endogenous antioxidant defenses. Antioxidant potential of *C. fenestratum* observed in the study may be due to the presence of phenolic compounds and other constituents responsible for the potent antioxidant activity.

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