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Antioxidant and free radical scavenging activity of an aqueous extract of *Coleus aromaticus*

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Abstract

The leaves of *Coleus aromaticus* Benth., (Lamiaceae), commonly called Indian Borage, are often eaten raw with bread and butter. The chopped leaves are also used as a substitute for sage (*Salvia officinalis* Linn.) in stuffing. In the present study, the antioxidant potency of freeze-dried aqueous extract of *C. aromaticus* was investigated, employing various established in vitro systems, such as the β-carotene-linoleate model system, 1,1-diphenyl-2-picrylhydrazyl (DPPH)/superoxide/nitric oxide radical scavenging, reducing power, and iron ion chelating activity. Freeze-dried aqueous extract of *C. aromaticus* (CAE) showed notable inhibitory activity in the β-carotene-linoleate model system. Furthermore, CAE exhibited a moderate concentration-dependent inhibition of the DPPH radical. The multiple antioxidant activity of CAE was evident as it showed significant reducing power, superoxide scavenging ability, nitric oxide-scavenging activity and also ferrous ion chelating potency. The data obtained in the in vitro models clearly establish the antioxidant potency of freeze-dried extract of *C. aromaticus*.

Keywords: Antioxidant activity; *Coleus aromaticus*; Free radicals; Scavenging effect

1. Introduction

The role of free radicals in many disease conditions has been well established. Several biochemical reactions in our body generate reactive oxygen species and these are capable of damaging crucial bio-molecules. If they are not effectively scavenged by cellular constituents, they lead to disease conditions (Halliwell & Gutteridge, 1990; Halliwell, Gutteridge, & Cross, 1992). The harmful action of the free radicals can, however, be blocked by antioxidant substances, which scavenge the free radicals and detoxify the organism. Current research into free radicals has confirmed that foods rich in antioxidants play an essential role in the prevention of cardiovascular diseases and cancers (Gerber et al., 2002; Kris-Etherton et al., 2002; Serafini, Bellocco, Wolk, & Ekstrom, 2002) and neurodegenerative diseases, including Parkinson's and Alzheimer's diseases (Di Matteo & Esposito, 2003), as well as inflammation and problems caused by cell and cutaneous aging (Ames, Shigrenaga, & Hagen, 1993). In recent years, one of the areas, which has attracted a great deal of attention, is antioxidants in the control of degenerative diseases in which oxidative damage has been implicated. Several plant extracts and different classes of phytochemicals have been shown to have antioxidant activity (Al Saikhan, Howard, & Miller, 1995; Bergman, Varshavsky, Gottlieb, & Grossman, 2001; Cao, Sofic, & Prior, 1996; Oomah & Mazza, 1994; Wang, Cao, & Prior, 1996; Yen & Duh, 1995). The search for newer natural antioxidants, especially of plant origin, has ever since increased.

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Coleus aromaticus Benth., (Lamiaceae), commonly called Indian Borage, is a medicinal plant and several medicinal properties are attributed to this plant in the Indian system of medicine. The leaves of the green type of country borage are often eaten raw with bread and butter. The chopped leaves are also used as a substitute for sage (*Salvia officinalis* Linn.) in stuffing. *C. aromaticus* is used for seasoning meat dishes and in food products (Uphof, 1959) while a decoction of its leaves is administered in cases of chronic cough and asthma (CSIR, 1992). It is considered to be an antispasmodic, stimulant and stomachic and is used for the treatment of headache, fever, epilepsy and dyspepsia (Khory & Katrak, 1999; Morton, 1992). As far as our literature survey could ascertain, antioxidant activities of this plant have not previously been published. Hence, the present work investigates the possible antioxidative effects of freeze-dried powder obtained from aqueous extract of fresh leaves of *C. aromaticus*. In this study, we examined the antioxidant activity of CAE, employing various in vitro assay systems, such as the β-carotene-linoleate model system, DPPH/superoxide/nitric oxide radical scavenging, reducing power and iron ion chelation, in order to understand the usefulness of this plant as a foodstuff as well as in medicine.

2. Materials and methods

2.1. Chemicals

Linoleic acid, β-carotene, ferrozine and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Sigma–Aldrich (Steinheim, Germany). Butylated hydroxytoluene (BHT), ascorbic acid, sulfanilamide, *N*-(1-naphthyl) ethylenediamine dihydrochloride, and ethylenediaminetetraacetic acid (EDTA) were purchased from Merck, Mumbai, India. Potassium ferricyanide, nitro blue tetrazolium (NBT), trichloroacetic acid (TCA), and ferric chloride were purchased from SD fine chemicals, India. All other reagents were of analytical grade.

2.2. Plant material and preparation of freeze-dried extract of C. aromaticus

Fresh leaves were collected from Chennai, India, during the month of October, 2003. Botanical identification was performed by Dr. D. Narashiman, Department of Botany, Madras Christian College, where the voucher specimen has been deposited. The fresh leaves were washed three times with tap water and then extracted with distilled water (1:10) for 1 h at 90 °C. The supernatant was filtered through Whatman No. 1 filter paper, and the resultant extract was lyophilized in vacuo. The lyophilized powder (CAE) was stored in a dark bottle at 4 °C until used.

2.3. Antioxidant assay using a β-carotene-linoleate model system

The antioxidant activity of extract was evaluated by the β-carotene-linoleate model system (Miller, 1971). A solution of β-carotene was prepared by dissolving 2 mg of β-carotene in 10 ml of chloroform. Two millilitres of this solution were pipetted into a 100 ml round-bottom flask. After chloroform was removed under vacuum, 40 mg of purified linoleic acid, 400 mg of Tween 40 emulsifier, and 100 ml of aerated distilled water were added to the flask with vigorous shaking. Aliquots (4.8 ml) of this emulsion were transferred into different test tubes containing different concentrations of the extract (0.2 ml). As soon as the emulsion was added to each tube, the zero time absorbance was measured at 470 nm, using a spectrophotometer. The tubes were placed, at 50 °C, in a water bath and measurement of absorbance was recorded after 2 h; a blank, devoid of β-carotene, was prepared for background subtraction. The same procedure was repeated with the synthetic antioxidant, butylated hydroxytoluene (BHT), as positive control. Antioxidant activity was calculated using the following equation:

$$\text{Antioxidant activity} = (\beta\text{-carotene content after 2 h of assay}/\text{initial } \beta\text{-carotene content}) \times 100.$$

2.4. DPPH radical-scavenging activity

The antioxidant activity of the extracts, based on the scavenging activity of the stable 1,1-diphenyl-2- picrylhydrazyl (DPPH) free radical, was determined by the method described by Braca et al. (2001). Plant extract (0.1 ml) was added to 3 ml of a 0.004% MeOH solution of DPPH. Water (0.1 ml) in place of the plant extract was used as control. Absorbance at 517 nm was determined after 30 min, and the percent inhibition activity was calculated as $[(A_0 - A_1)/A_0] \times 100$, where A_0 was the absorbance of the control, and A_1 was the absorbance of the extract/standard.

2.5. Assay of superoxide radical (O_2^-)-scavenging activity

The assay was based on the capacity of the aqueous extract to inhibit formazan formation by scavenging the superoxide radicals generated in a riboflavin-light-NBT system (Beauchamp & Fridovich, 1971). The method used by Martinez, Marcelo, Marco, and Moacyr (2001) for determination of superoxide dismutase was followed after modification. The reaction mixture contained 50 mM phosphate buffer, pH 7.6, 20 μg riboflavin, 12 mM EDTA, and NBT, 0.1 mg/3 ml, added in that sequence. Reaction was started by illuminating

(fluorescent lamp) the reaction mixture with different concentrations of CAE for 90 seconds. Immediately after illumination, the absorbance was measured at 590 nm. The entire reaction assembly was enclosed in a box lined with aluminium foil. Identical tubes, with reaction mixture, were kept in the dark and served as blanks. The percentage inhibition of superoxide anion generation was calculated using the following formula:

$$\% \text{ Inhibition} = [(A_0 - A_1)/A_0] \times 100,$$

where A_0 was the absorbance of the control, and A_1 was the absorbance of the aqueous extract/standard.

2.6. Assay of nitric oxide-scavenging activity

The procedure is based on the principle that, sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide which interacts with oxygen to produce nitrite ions that can be estimated using Griess reagent. Scavengers of nitric oxide compete with oxygen, leading to reduced production of nitrite ions. For the experiment, sodium nitroprusside (10 mM), in phosphate-buffered saline, was mixed with different concentrations of CAE dissolved in water and incubated at room temperature for 150 min. The same reaction mixture, without the CAE but with an equivalent amount of water, served as control. After the incubation period, 0.5 ml of Griess reagent (1% sulfanilamide, 2% H_3PO_4 and 0.1% *N*-(1-naphthyl) ethylenediamine dihydrochloride) was added. The absorbance of the chromophore formed was read at 546 nm. Curcumin was used as positive control (Sreejayan & Rao, 1997).

2.7. Reducing power determination

The reducing power of CAE was determined according to the method of Oyaizu (1986). Different amounts of extract (50–200 μg/ml) in water were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 ml, 1%). The mixture was incubated at 50 °C for 20 min. A portion (2.5 ml) of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged for 10 min. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml, 0.1%), and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power.

2.8. Metal chelating activity

The chelating of ferrous ions by the CAE was estimated by the method of Dinis, Madeira, and Almeida (1994). Briefly, the extract (50–200 μg/ml) was added to a solution of 2 mM $FeCl_2$ (0.05 ml). The reaction was initiated by the addition of 5 mM ferrozine (0.2 ml), the mixture was shaken vigorously and left standing at room temperature for 10 min. Absorbance of the solution was then measured spectrophotometrically at 562 nm. The percentage inhibition of ferrozine–Fe^{2+} complex formation was calculated as $[(A_0-A_1)/A_0] \times 100$, where A_0 was the absorbance of the control, and A_1 was the absorbance of the extract/standard.

2.9. Statistical analysis

Experimental results were means ± SD of three parallel measurements. The data were analysed by an analysis of variance ($p < 0.05$) and the means separated by Duncan's multiple range test. The EC_{50} values were calculated from linear regression analysis. Results were processed by computer programmes: Excel and Statistica software (1999).

3. Results and discussion

3.1. Antioxidant assay using a β-carotene-linoleate model system

Table 1 shows the antioxidant activity of the extract as measured by the bleaching of β-carotene. The addition of CAE and BHT at various concentrations prevented the bleaching of β-carotene to different degrees. β-Carotene in this model system undergoes rapid discoloration in the absence of an antioxidant. This is because of the coupled oxidation of β-carotene and linoleic acid, which generates free radicals. The linoleic acid free radical, formed upon the abstraction of a hydrogen atom from one of its diallylic methylene groups, attacks the highly unsaturated β-carotene molecules. As a result, β-carotene will be oxidized and broken down in part; subsequently, the system looses its chromophore and characteristic orange colour, which can be monitored spectrophotometrically. The presence of different antioxidants can hinder the extent of β-carotene bleaching by neutralising the linoleate-free radical and other free radicals formed in the system (Jayaprakasha, Singh, & Sakariah, 2001). In our present study, the CAE was found to hinder the extent of β-carotene bleaching by neutralizing the linoleate-free radical and other free

Table 1
Antioxidant activity of aqueous extract of *C. aromaticus* in β-carotene-linoleate system

Sample	Concentration (μg/ml)	Antioxidant activity (%)
Aqueous extract	125	53.2 ± 1.04
	250	83.0 ± 1.33
	500	91.3 ± 1.41
BHT	50	64.2 ± 1.81
	100	89.6 ± 1.52
	200	95.3 ± 1.33

Table 2
Antiradical activity of aqueous extract of *C. aromaticus* observed with DPPH

Sample	Concentration (μg/ml)	% Inhibition	EC_{50} (μg/ml)
Aqueous extract	60	11.3 ± 0.22	210
	120	27.0 ± 0.41	
	180	42.0 ± 1.79	
	240	58.4 ± .050	
	300	72.7 ± 0.33	
Gallic acid			1.38

radicals formed in the system. In comparison, the CAE showed an appreciable antioxidant activity of 83.0% at 250 μg/ml, while BHT, a synthetic antioxidant had 89.6% antioxidant activity at 100 μg/ml.

3.2. DPPH radical-scavenging activity

The CAE showed a concentration-dependent antiradical activity by inhibiting DPPH radical with an EC_{50} value of 210 μg/ml (Table 2). DPPH is usually used as a substrate to evaluate antioxidative activity of antioxidants (Oyaizu, 1986). The method is based on the reduction of methanolic DPPH solution in the presence of a hydrogen donating antioxidant, due to the formation of the non-radical form DPPH-H by the reaction. The extract was able to reduce the stable radical DPPH to the yellow-coloured diphenylpicrylhydrazine. It has been found that cysteine, glutathione, ascorbic acid, tocopherol, polyhydroxy aromatic compounds (e.g., hydroquinone, pyrogallol, gallic acid), and aromatic amines (e.g., *p*-phenylene diamine, *p*-aminophenol), reduce and decolorise 1,1-diphenyl-2-picrylhydrazyl by their hydrogen donating ability (Blois, 1958). It appears that the CAE possesses hydrogen donating capabilities and acts as an antioxidant. The scavenging effect increased with increasing concentration of the extract. However, scavenging activity of gallic acid, a known antioxidant, used as positive control, was relatively more pronounced than that of CAE.

3.3. Assay of superoxide radical (O_2^-)-scavenging activity

Table 3 shows the superoxide radical (O_2^-)-scavenging activity of the extract, as measured by the riboflavin-NBT-light system in vitro. Superoxide radical is known to be very harmful to cellular components as a precursor of more reactive oxygen species (Halliwell & Gutteridge, 1985). Photochemical reduction of flavins generates O_2^-, which reduces NBT, resulting in the formation of blue formazan (Beauchamp & Fridovich, 1971). The extract was found to be a moderate scavenger of superoxide radical generated in riboflavin-NBT-light system in vitro. The extract inhibited the formation of the blue formazan and the % inhibition was proportional to the concentration with an EC_{50} value of 73.9 μg/ml. These results indicated that the tested extract had a notable effect on scavenging of superoxide when compared with ascorbic acid, which was used as positive control.

Table 3
Superoxide anion-scavenging activity of aqueous extract of *C. aromaticus* observed with the riboflavin-light-NBT system

Sample	Concentration (μg/ml)	% Inhibition	EC_{50} (μg/ml)
Aqueous extract	25	13.3 ± 1.89	73.9
	50	35.4 ± 1.14	
	75	52.5 ± 1.30	
	100	66.5 ± 1.05	
Ascorbic acid			17.4

Table 4
In vitro NO-scavenging activity of aqueous extract of *C. aromaticus*

Sample	Concentration (μg/ml)	% Inhibition	EC_{50} (μg/ml)
Aqueous extract	25	14.4 ± 1.08	173
	50	20.2 ± 0.79	
	100	35.1 ± 0.77	
	200	55.6 ± 1.02	
Curcumin			25.4

3.4. Assay of nitric oxide-scavenging activity

The extract also showed a moderate nitric oxide-scavenging activity between 25 and 200 μg/ml in a dose-dependent manner (EC_{50} = 173 μg/ml) (Table 4). In addition to reactive oxygen species, nitric oxide is also implicated in inflammation, cancer and other pathological conditions (Moncada, Palmer, & Higgs, 1991). The plant/plant products may have the property to counteract the effect of NO formation and in turn may be of considerable interest in preventing the ill effects of excessive NO generation in the human body. Further, the scavenging activity may also help to arrest the chain of reactions initiated by excess generation of NO that are detrimental to human health. The extract showed a moderate nitric oxide-scavenging activity. The % inhibition was increased with increasing concentration of the extract. Curcumin, a natural antioxidant was used as a positive control for comparison (Sreejayan & Rao, 1997).

3.5. Reducing power determination

Fig. 1 shows the reductive capabilities of sample extract compared to BHT. For the measurements of the reductive ability, we investigated the Fe^{3+}–Fe^{2+} transformation in the presence of CAE, using the method of Oyaizu (1986). Earlier authors (Pin-Der-Duh, 1998; Pin-Der-Duh, Pin-Chan-Du, & Gow-Chin Yen, 1999; Tanaka, Kuie, Nagashima, & Taguchi, 1988) have

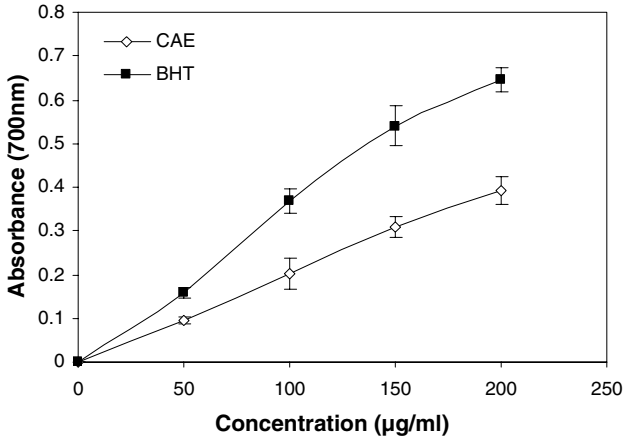

Fig. 1. Reducing power of CAE and BHT (50–200 µg/ml); CAE, freeze-dried aqueous extract of *C. aromaticus*; BHT-butylated hydroxytoluene.

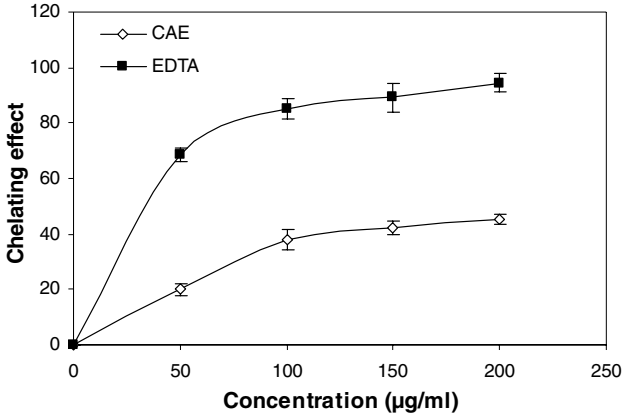

Fig. 2. Chelating effect of CAE and EDTA (50–200 µg/ml) on Fe^{2+} ion. CAE, freeze-dried aqueous extract of *C. aromaticus*; EDTA-ethylenediaminetetraacetic acid.

observed a direct correlation between antioxidant activities and reducing power of certain plant extracts. The reducing properties are generally associated with the presence of reductones (Pin-Der-Duh, 1998), which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom (Gordon, 1990). Reductones are also reported to react with certain precursors of peroxide, thus preventing peroxide formation. Our data on the reducing power of CAE suggest that it is likely to contribute significantly towards the observed antioxidant effect. However, the antioxidant activity of antioxidants have been attributed to various mechanisms, among which are prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity and radical-scavenging (Diplock, 1997). Like the antioxidant activity, the reducing power of CAE increased with increasing amount of sample. However, the reducing power of BHT was relatively more pronounced than that of CAE.

3.6. Metal chelating activity

The chelating of ferrous ions by the extract was estimated by the method of (Dinis et al., 1994). Ferrozine can quantitatively form complexes with Fe^{2+}. In the presence of other chelating agents, the complex formation is disrupted with the result that the red colour of the complexes decreases. Measurement of the rate of colour reduction therefore allows estimation of the chelating activity of the coexisting chelator (Yamaguchi, Ariga, Yoshimira, & Nakazawa, 2000). In this assay, both extract and EDTA, interfered with the formation of ferrous and ferrozine complex, suggesting that it has chelating activity and captures ferrous ion before ferrozine. The absorbance of Fe^{2+}–ferrozine complex was decreased dose-dependently; otherwise the activity was increased on increasing concentration from 50 to 200 µg/ml. Metal chelating capacity was significant since the extract reduced the concentration of the catalysing transition metal in lipid peroxidation (Duh & Tu, 1999). It was reported that chelating agents, which form σ-bonds with a metal, are effective as secondary antioxidants because they reduce the redox potential, thereby stabilising the oxidized form of the metal ion (Gordon, 1990). The data obtained from Fig. 2, reveals that CAE has an effective capacity for iron binding, suggesting that its action as an antioxidant may be related to its iron binding capacity.

4. Conclusion

The aqueous extract of *C. aromaticus* leaves exhibited different levels of antioxidant activity in all the models studied. The results from various free radical-scavenging systems revealed that the *C. aromaticus* had significant antioxidant activity and free radical-scavenging activity. The free radical-scavenging property may be one of the mechanisms by which this drug is useful as a foodstuff as well as a traditional medicine. However, further investigation of individual compounds, their in vivo antioxidant activities and in different antioxidant mechanisms is warranted.

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