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Food Chemistry 100 (2007) 356–361
Food Chemistry
www.elsevier.com/locate/foodchem

Activity-guided isolation and identification of free radical-scavenging components from an aqueous extract of *Coleus aromaticus*

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Received 13 June 2005; received in revised form 20 September 2005; accepted 20 September 2005

Abstract

An activity-directed fractionation and purification process was used to identify the DPPH (l,l-diphenyl-2-picrylhydrazyl) free radical-scavenging components of *Coleus aromaticus* Benth. Fresh leaves of *C. aromaticus* were extracted with water and then separated into hexane, ethyl acetate, and water fractions. Among these, only the ethyl acetate phase showed strong DPPH radical-scavenging activity in vitro, when compared with water and hexane phases. The ethyl acetate fraction was then subjected to separation and purification using Sephadex LH-20 chromatography. Three compounds showing strong DPPH radical-scavenging activity were shown, by spectral methods (^{1}H NMR, ^{13}C NMR, and MS) and by comparison with literature values, to be rosmarinic acid, chlorogenic acid and caffeic acid. In addition, HPLC identification and quantification of isolated compounds were also performed. Rosmarinic acid was found as a major component and principally responsible for the radical-scavenging activity of *C. aromaticus*.

Keywords: *Coleus aromaticus*; Indian borage; Antioxidant compounds; DPPH; HPLC; Phenolic content

1. Introduction

Oxidative stress, induced by oxygen radicals, is believed to be a primary factor in various degenerative diseases, such as cancer (Muramatsu et al., 1995), atherosclerosis (Steinberg, Parthasarathy, Carew, Khoo, & Witztum, 1989), gastric ulcer (Das, Bandyopadhyay, Bhattacharjee, & Banerjee, 1997) and other conditions (Oliver, Ahn, Moerman, Goldstein, & Stadtmaan, 1987; Smith et al., 1996). Many antioxidant compounds, naturally occurring, from plant sources, have been identified as free radical- or active oxygen-scavengers (Duh, 1998; Zheng & Wang, 2001). Recently, interest has increased considerably in finding naturally occurring antioxidants in food or medicinal flora to replace synthetic antioxidants, which are being restricted, due to their adverse side effects, such as carcinogenicity (Ito, Fukushima, Hasegawa, Shibata, & Ogiso, 1983). Natural antioxidants can protect the human body from free radicals and retard the progress of many chronic diseases as well as retard lipid oxidative rancidity in food, cosmetics and pharmaceutical materials (Kinsella, Frankel, German, & Kanner, 1993; Lai, Chou, & Chao, 2001). In the search of plants as a source of natural antioxidants, some medicinal plants and fruits have been extensively studied for their antioxidant activity and radical-scavenging in the past few decades (Singh, Murthy, & Jayaprakasha, 2002; Velioglu, Mazza, Gao, & Oomah, 1998). Among the various natural antioxidants, phenolic compounds are reported to be active, quenching oxygen-derived free radicals by donating hydrogen atom or an electron to the free radical (Wanasundara & Shahidi, 1996; Yuting, Rongliang, Zhongjian, & Young, 1990). Also, phenolic compounds of plant materials have been shown to neutralize free radicals in various in vitro model systems (Ruch, Cheng, & Klaunig, 1989; Zhang et al., 1996).

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doi:10.1016/j.foodchem.2005.09.051

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 the cases of chronic cough and asthma (CSIR, 1992). It is considered to be antispasmodic, and a stimulant and stomachic and is used for the treatment of headache, fever, epilepsy and dyspepsia (Khory & Katrak, 1999; Morton, 1992). In our previous studies, aqueous extract prepared from the fresh leaves of *C. aromaticus* showed potent antioxidant activities in different in vitro models (Kumaran & Karunakaran, 2005). Hence, the present work investigates the possible antioxidative compounds present in the aqueous extract of fresh leaves of *C. aromaticus*. In this study, an activity-directed fractionation and purification process was used to isolate DPPH radical-scavenging components of *C. aromaticus*. In addition, high performance liquid chromatography (HPLC) identification and quantification of isolated compounds were also performed to find out the major active compounds.

2. Materials and methods

2.1. General

^{1}H NMR and ^{13}C NMR spectra were obtained on a JEOL EX-270 Instrument, and mass spectra were obtained on a JEOL JMS-SX 102A Instrument. HPLC analysis was performed in a Shimadzu SPD-M6A chromatographic apparatus (Shimadzu, Kyoto, Japan), using a reversed-phase column Lichrocart 100 RP-18 (Merck) (25 × 0.4 cm, particle size, 5 μm), and isocratic elution with the mobile phase comprising water: methanol: *ortho*phosphoric acid (50:59.5:0.5, v:v:v). Sephadex LH-20 (Pharmacia Biotech AB, Uppsala, Sweden), was used as the adsorbent for open column chromatography (CC). Silica gel 60 F_{254} plates (E. Merck, Darmstadt, Germany) were used for thin-layer chromatography (TLC). 1,1-Diphenyl-2-picrylhydrazyl (DPPH) was purchased from Sigma-Aldrich (Steinheim, Germany). Gallic acid, were purchased from Merck, Mumbai, India. All solvents used for chromatography were of HPLC grade. All other chemicals were of analytical reagent grade.

2.2. Plant material

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 (Specimen No. VSCHEM 109/03).

2.3. Extraction and isolation of bioactive compounds

The fresh leaves (400 g) were washed three times with tap water and then extracted with distilled water (1:10, w/v) 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 extract (13.2 g) was dissolved in water, kept at 4 °C for 12 h, and filtered again, thus obtaining the crude extract. This crude extract was then partitioned with hexane (200 ml fractions repeatedly up to decoloration of the organic solvent), thus obtaining both the hexane fraction and the "clean" or "defatted" crude extract. The defatted crude extract was then successively partitioned with ethyl acetate (EtOAc) (as for the hexane partition), thus obtaining the EtOAc, and aqueous fractions. Among these, only the EtOAc phase showed strong antioxidant activity by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) test when compared with water and hexane phases.

The DPPH-active EtOAc extract (2.75 g) was subjected to column chromatography on Sephadex LH-20 and eluted with water and increasing amounts of methanol to 100%, affording, after TLC, 32 fractions. TLC analysis was performed on silica gel using ethyl acetate: chloroform: formic acid (5:4:1) as the mobile phase. Compounds were visualized after spraying the plates with a methanol solution of diphenylboric acid-β-ethylamino ester (Wagner, Bladt, & Zgainski, 1984). Fractions 1–5, 9–11, 17–20 and 26–32 proved to be inactive or less active and were discarded. Fractions 6–8 (0.44 g) were rechromatographed on a Sephadex LH-20 column with MeOH: H_2O (l:2) and yielded 12 mg of chlorogenic acid (1). Fractions 12–16 (1.05 g) were rechromatographed over Sephadex LH-20 and eluted with MeOH: $CHCl_3$ (1:2), affording 125 mg of rosomarinic acid (**2**). Fractions 21–25 (0.53 g), on further purification yielded 11 mg of caffeic acid (**3**).

2.4. DPPH radical-scavenging activity

The antioxidant activities of the extracts and fractions, based on the scavenging activity of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical, were determined by the method described by Braca et al. (2001). Test samples (0.1 ml) were added to 3 ml of a 0.004% MeOH solution of DPPH. 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 without sample and A_1 was the absorbance with sample).

2.5. Quantitative analysis using HPLC

The standard solutions of compounds **1–3** were prepared at 1 mg/ml in methanol. They were diluted to make five concentrations (1.0, 12.5, 25.0, 50.0, and 100 μg/ml) calibration curves. The EtOAc extract was dissolved in methanol (1 mg/ml). All solutions were filtered through a cellulose acetate membrane filter (0.45 μm) (Advantec,

Japan), and aliquots of the filtrate (10 μl) were injected on to an HPLC column using Lichrocart 100 RP-18 (Merck) (25 × 0.4 cm, particle size, 5 μm) and eluted with an isocratic solvent mixture comprising water: methanol: *ortho*phosphoric acid (50:59.5:0.5, v:v:v) with a flow rate at 1 ml/min. The UV detection was carried out at 325 nm. All of the above experiments were replicated three times each. These tested compounds in the EtOAc extract of this plant were quantified from a calibration curve.

3. Results and discussion

3.1. Isolation of antioxidant compound

Solvent extraction, followed by Sephadex LH-20 column chromatography, of aqueous extract of *C. aromaticus*. yielded three potential antioxidant compounds. The aqueous extract was first extracted with hexane to separate non-polar compounds, the yield of which is negligible (0.9% w/w). Then it was extracted with EtOAc which is known to extract phenolics; yield of extract was 20.8% (w/w). The remaining aqueous portion accounts for a 75.3% yield with the operational loss of 3%. Table 1 summarizes the antioxidant activity of different extracts and the isolated compounds. Antioxidant activities of EtOAc extracts were higher than those of hexane and aqueous extracts. Hence the EtOAc extract was subjected to activity guided fractionation on a Sephadex LH-20 column eluted with water and methanol mixtures. The active fractions obtained from Sephadex LH-20 column chromatography, on further purification, finally yielded antioxidant components. Fig. 1 schematically represents the extraction procedure for isolation of active compounds. All the compounds, known in the literature, were isolated for the first time from *C. aromaticus*.

3.2. Structure determinations of isolated compounds

The chemical structure confirmation of the components from the *C. aromaticus* was accomplished by comparing, mass, ^{1}H and ^{13}C NMR data obtained to those published.

Table 1
DPPH radical-scavenging activities and amount of the isolated compounds of *Coleus aromaticus*

Tested materials	EC_{50} (μg/ml ± SD)	Amounts (mg/g[a] ± SD)
Hexane extract	>500	–
Ethyl acetate extract	84.0 ± 0.35	–
Aqueous extract	348 ± 2.46	–
Chlorogenic acid	11.0 ± 0.57	1.33 ± 6.58
Rosmarinic acid	9.96 ± 0.94	44.8 ± 1.84
Caffeic acid	5.52 ± 0.35	2.42 ± 1.84
Gallic acid	1.38 ± 0.22	–

Values were determined from integration of HPLC signals and response factors calculated from standards. The results are from three separate experiments.

[a] Dry weight of the EtOAc extract of *C. aromaticus*.

Compound **1** was isolated using Sephadex LH-20 column as a white amorphous powder, MP: 208–211 °C. Its molecular weight was 354, determined by mass spectrum. Its detailed spectral data of MS, ^{1}H and ^{13}C NMR, listed in Table 2, agree well with the reported compound, chlorogenic acid (de Almeida et al., 1998).

Compound **2** was isolated and recrystallized from water as white crystals, MP: 200–201 °C; its molecular weight was 256. Detailed spectral data, listed in Table 2, agree well with the data reported (Kelley et al., 1975). So the compound was identified as rosmarinic acid.

Compound **3** was isolated and recrystallized from water as yellow crystals, MP: 220–222 °C; its molecular weight was 360. Its detailed spectral data, listed in Table 2, agree well with the data reported (Xu et al., 1994). This compound was identified as caffeic acid.

The structures of the compounds isolated from *C. aromaticus* are shown in Fig. 2.

3.3. DPPH radical-scavenging activity

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 EtOAc extract of *C. aromaticus* and the isolated components showed a concentration-dependent antiradical activity by reducing the stable radical DPPH to the yellow-coloured diphenylpicrylhydrazine derivative (Table 1). However, scavenging activity of gallic acid, a known antioxidant used as positive control, was relatively high. Rosmarinic acid, the major chemical constituent of *C. aromaticus*, showed potent DPPH free radical-scavenging activity, with an EC_{50} value of 9.96 μg/ml. The antioxidant activities of the isolated compounds decrease as follows: gallic acid > caffeic acid > rosmarinic acid chlorogenic acid.

Caffeic acid and rosmarinic acid, being common in many plants and often present in our diet, are both strong radical scavengers with caffeic acid being slightly superior to rosmarinic acid (Brand-Williams, Cuvelier, & Berset, 1995; Cuvelier, Richard, & Berset, 1992). Chemical structure–activity studies of the phenolic acids, by various methods, have consistently shown that cinnamic acid derivatives have superior antioxidant activity to the benzoic acid analogues, which may be due to the presence of the conjugated unsaturation that facilitates the delocalization of the resulting free radicals. Among the cinnamic acids, caffeic acids are much better than ferulic acids, which are in turn better than *p*-coumaric acids (Cuvelier et al., 1992; Hermann, 1993; Natella, Nardini, Di Felice, & Scaccini, 1999). The *o*-dihydroxybenzene (catechol) structure is crucial for enhanced antioxidant activities. Natural polyphenols have chain-breaking antioxidant activities and are believed to prevent many degenerative diseases, including cancer and atherosclerosis (Roginsky, 2003).

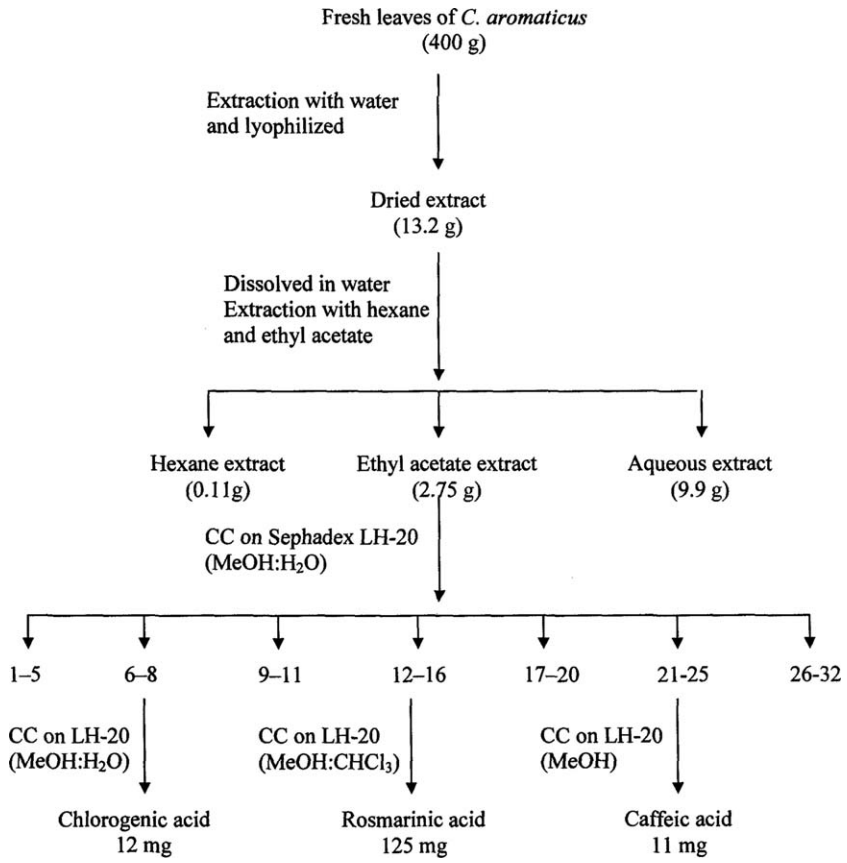

Fig. 1. Extraction scheme for the isolation of antioxidant compounds from *Coleus aromaticus*.

Table 2
Spectral data of the compounds isolated from *Coleus aromaticus*

Name of compounds	^{1}H NMR(270 MHz) TMS as int. standard	^{13}C NMR(68 MHz) TMS as int. standard	FAB MS (*m*/*z*)
(**1**) Chlorogenic acid	1.62–2.12 (m) 3.54–4.03 (m) 5.03–5.19 (m) 6.28 (d) 6.78 (d) 6.99 (dd) 7.06 (d) 7.57 (d)	36.56, 37.27, 68.34, 70.65, 70.94, 73.63, 114.33,114.86, 115.75, 121.31, 125.64, 144.94, 145.57, 165.82, 175.15,	355 $[M + H]^+$, 377 $[M + Na]^+$
(**2**) Rosmarinic acid	3.10 (2q) 5.24 (dd) 6.32 (d) 6.68 (dd) 6.77 (d) 6.87 (d) 6.88 (d) 7.05 (dd) 7.18 (d) 7.58 (d)	37.40, 73.73 114.77,115.16, 115.95,116.33, 117.28,120.39, 121.63, 122.78 127.37, 129.09, 144.71, 145.63, 146.21, 146.64, 148.90, 166.93, 171.36	361 $[M + H]^+$, 383 $[M + Na]^+$
(**3**) Caffeic acid	6.77 (d) 6.96 (dd) 7.05 (d) 6.28 (d) 7.42 (d)	114.64,115.24, 115.83,121.20, 125.80, 144.60, 145.63, 148.17, 168.00	181 $[M + H]^+$, 203 $[M + Na]^+$

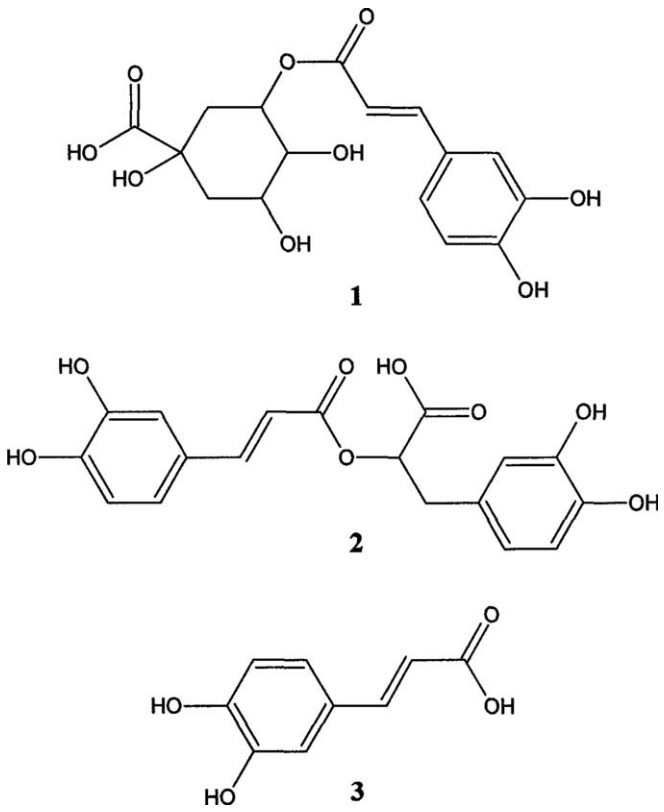

Fig. 2. Compounds isolated from *Coleus aromaticus* leaves.

3.4. HPLC assay for measuring the compounds in C. aromaticus

In order to determine the variations in components with free radical-scavenging activities, an HPLC method was developed to quantify chlorogenic acid, rosmarinic acid and caffeic acid in EtOAc extract of *C. aromaticus*. The HPLC chromatogram of the tested compound is shown in Fig. 3. The retention times of chlorogenic acid, caffeic acid and romarinic acid were at 3.4, 4.7 and 7.2 min, respectively. Amount of tested compounds in the extract are shown in Table 1. The content of rosmarinic acid was about 18-fold higher than that of caffeic acid and 33-fold higher than that of chlorogenic acid, indicating that rosmarinic acid is the major phenolic component with free radical-scavenging activity in *C. aromaticus*. The caffeic acid possesses higher radical-scavenging activity than romarinic acid, but is present in lower amount in the extract. Therefore the antioxidant activity is mainly due the presence of romarinic acid.

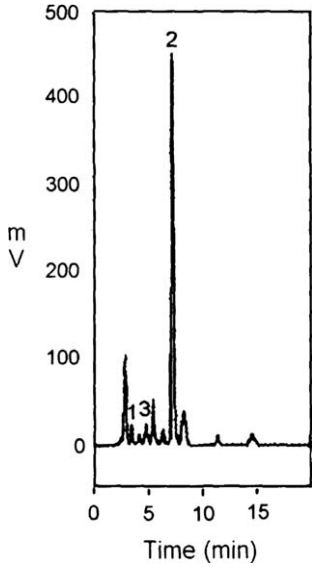

Fig. 3. HPLC chromatogram of the ethyl acetate extract of *Coleus aromaticus* leaves. Chlorogenic acid (**1**), rosmarinic acid (**2**), and caffeic acid (**3**) were detected at 325 nm.

4. Conclusion

Activity-guided isolation of radical-scavenging antioxidant compounds from *C. aromaticus* resulted in the identification of three compounds, namely, rosmarinic acid, chlorogenic acid and caffeic acid. The antioxidant activity assessments of isolated compounds, by measuring their capacity to scavenge the DPPH; showed that they were potent antioxidants. Being the major polyphenol in *C. aromaticus*, the rosmarinic acid was more likely to be responsible for most of the observed antioxidant activity of the *C. aromaticus*. The potency of these compounds could provide a chemical basis for some of the health benefits claimed for *C. aromaticus* in folk medicine and warrant further studies to assess their potential as effective natural remedies.

Acknowledgement

The authors are thankful to the CSIR, New Delhi for financial assistance.

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