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Journal of Chromatography B, 852 (2007) 40–48
JOURNAL OF CHROMATOGRAPHY B
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Isolation and characterization of antioxidant and antibacterial compound from mango ginger (*Curcuma amada* Roxb.) rhizome

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Received 1 July 2006; accepted 28 December 2006
Available online 16 January 2007

Abstract

The chloroform extract of mango ginger (*Curcuma amada* Roxb.) rhizome was subjected to antioxidant activity-guided purification by repeated silica gel column chromatography to obtain a pure antioxidant compound. The structure was deduced by analyzing UV, IR, liquid chromatography–mass spectrometry (LC–MS) and two-dimensional heteronuclear multiple quantum coherence transfer spectroscopy (2D-HMQCT) NMR spectral data, and named it as "Amadannulen", a novel compound. It exhibited DPPH radical scavenging activity, super oxide radical scavenging activity, lipid peroxidation inhibitory activity and metal chelating activity. Amadannulen also showed antibacterial activity against both Gram-positive and Gram-negative bacteria tested. It also exhibited bactericidal activity against *M. luteus, B. cereus* and *B. subtilis*.

Keywords: *Curcuma amada*; Mango ginger; Rhizome; Antioxidant activity; Antibacterial activity; Amadannulen

1. Introduction

Fruits, vegetables and spices are known to contain health-promoting components such as vitamins, minerals, antioxidants and prebiotics [1]. There is a renewed interest in bioactive compounds from fruits, vegetables and spices. In particular, spices are used in foods, because they impart desirable flavours and may fulfill more than one function to which they are added [2]. Extensive research is being conducted on traditional medicines, vis-à-vis in different plant species and their therapeutic principles all over the world. Experimental evidence suggests that free radicals and reactive oxygen species (ROS) have been implicated in more than 100 diseases, including malaria, acquired immunodeficiency syndrome, heart disease, stroke, arteriosclerosis, diabetes, cancer and gastric ulcer [3–5]. Antioxidants can protect the human body from free radicals and ROS effects and retard the progress of many chronic diseases as well as lipid oxidative rancidity in foods [6–8]. However, BHA and BHT, the most commonly used antioxidants at present, are suspected of being responsible for liver damage and carcinogenesis [9,10]. Therefore, the exploration and utilization of more effective antioxidants and antibacterial compounds from natural sources are desired. As plants produce a lot of antioxidants to control the oxidative stress, they represent a natural source of antioxidant and antibacterial activity, which can be observed in fruits, vegetables, roots, leaves and seeds [11]. When compared with plethora of synthetic substances, natural products offer the prospects of discovering a greater number of compounds, with sterically more complex structures [12,13].

The genus *Curcuma* (family Zingiberaceae) comprises of over 80 species of rhizomatous herbs. The genus is originated in the Indo-Malayan region and widely distributed in the tropics of Asia to Africa and Australia [14]. Mango ginger (*Curcuma amada* Roxb.) is a unique spice, which morphologically resembles the ginger (*Zingiber officinale*) but, it imparts mango (*Mangifera indica*) flavour. Mango flavor is mainly attributed to car-3-ene and *cis*-ocimene [15,16]. Mango ginger rhizome is used for therapeutic purpose and in the manufacture of pickles, as a source of raw mango flavour. Ayurveda, the oldest system of medicine in India, has highlighted the importance of this rhizome as an appetizer, alexteric, antipyretic, aphrodisiac and a laxative. It is also used in biliousness, itching, skin diseases, bronchitis, asthma, hiccups and inflammation due to injuries [17–19].

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doi:10.1016/j.jchromb.2006.12.036

According to the Unani system of medicine, it is a diuretic, maturant, emollient, expectorant, antipyretic and appetizer. It is also useful against inflammation of mouth, ear, and gleet, ulcers on the male sex organs, scabies, lumbago and stomatitis [18–20]. Mango ginger extract has been reported to inhibit trypsin enzyme [21]. It has been reported to induce hypertriglyceridemic activity in Triton-induced hyperlipidemic rats [22,23]. Ethyl alcohol extract of mango ginger comprises of chemical compounds with hydroxyl, ester, carbonyl and olefin functional groups. They were found to be responsible for anti-inflammatory activity of the extract in acute and chronic administration in albino rats [24]. In spite of medicinal properties and therapeutic uses of mango ginger, there are no reports on bioactive molecules. The present study proposed to investigate the antioxidant potential of mango ginger rhizome and isolation and characterization of antioxidant compound.

2. Material and methods

2.1. Plant material

Fresh and healthy mango ginger (*C. amada* Roxb.) rhizomes were procured from the local market, Mysore, India, during December 2004. The rhizomes were washed, sliced and dried in a hot air oven at 50 °C for 72 h and powdered to 60 meshes in an apex grinder (Apex Constructions, London).

2.2. Chemicals

All the chemicals used for column chromatography were from Merck Limited, Mumbai, India. HPLC grade methanol was purchased from Ranbaxy Fine Chemicals Limited, Mumbai, India. Silica gel (60–120 mesh) was purchased from Qualigens Fine Chemicals, Mumbai, India; silica gel (100–200) was purchased from Loba Chemie Pvt. Ltd., Mumbai, India. Silica gel was purchased from Glaxo Laboratories, Mumbai, India. 1,1-Diphenyl-2-picryl-hydrazyl (DPPH), nicotinamide adenine dinucleotide (NADH), BHA and trichloroacetic acid (TCA) were purchased from Sigma (Sigma–Aldrich GmbH, Sternheim, Germany). Potassium ferricyanide, 3-(2-pyridyl)-5,6-bis(4-phenyl-sulfonic acid)-1,2,4-triazine (ferrozine) and ferric chloride were purchased from M/s Sisco Research Laboratories, Mumbai, India, nitro blue tetrazolium (NBT), phenazine methosulphate (PMS), thiobarbituric acid (TBA), and ethylene diamine tetra-acetic acid (EDTA) were purchased from M/s Sigma Chemicals Co. (St. Louis, MO). Nutrient agar and nutrient broth were from HiMedia Laboratories Limited, Mumbai, India. All other reagents were of analytical grade and other chemicals used in this study were of highest purity.

2.3. Bacterial strains and inoculum preparation

The antibacterial activity was tested against *Pseudomonas aeruginosa*, *Escherichia coli*, *Salmonella typhi*, *Klebsiella pneumoniae*, *Enterobacter aerogenes*, *Proteus mirabilis*, *Yersinia enterocolitica*, *Micrococcus luteus*, *Staphylococcus aureus*, *Enterococcus fecalis*, *Bacillus subtilis*, *Bacillus cereus* and *Listeria monocytogenes*. The above bacterial strains isolated from clinical samples were obtained from the Department of Microbiology, Mysore Medical College, Mysore, India. Their cultural characteristics and morphological features were confirmed and also subjected to standard biochemical tests before experimentation [25,26]. The test organisms were maintained on nutrient agar slants.

2.4. Isolation of bioactive compound from chloroform extract

2.4.1. Preparation of extracts

About 100 g of dry mango ginger powder was defatted using *n*-hexane, followed by chloroform at room temperature (27 °C) and at atmospheric pressure, for 48 h by shaking at 100 rpm/min speed. The extracts were filtered and concentrated by using rotary evaporator (Buchi Rotavapor R-124). The concentrated extracts were freeze-dried and stored in refrigerator. Promising antioxidant activity of chloroform extract prompted us for the isolation and purification of antioxidant compound from it.

2.4.2. Fractionation of the chloroform extract

Activated silica gel (60–120 mesh) was packed on to a glass column (450 mm × 40 mm) using *n*-hexane solvent and 15 g of crude chloroform extract was loaded on the top of silica gel. The column was eluted step wise with 500 ml of hexane, 2000 ml of hexane:chloroform (75:25 to 0:100, v/v), 2000 ml of chloroform:ethyl acetate (75:25 to 0:100, v/v), 2000 ml of ethyl acetate:acetone (75:25 to 0:100, v/v) and 1500 ml of acetone:methanol (75:25 to 0:100, v/v). About 82 fractions measuring 100 ml each were collected and concentrated by using rotary evaporator.

2.4.3. Thin layer chromatography (TLC)

An aliquot of all the concentrated fractions were loaded on the activated silica gel TLC plates (20 cm × 20 cm). The plates were developed using hexane:chloroform (80:20), chloroform:ethyl acetate (90:10) and ethyl acetate:methanol (90:10) solvents. The spots were located by exposing the plate to iodine fumes. Fractions having same number of spots with similar R_f values on TLC plate were pooled. The pooled fractions were numbered (Fr.1–Fr.5). All the five-pooled fractions were tested for the antioxidant activity.

2.4.4. Further purification of bioactive fraction

Since fraction five (Fr.5) obtained from first step column chromatography (Fig. 1) showed high antioxidant activity, it was selected for further purification. About 2.1 g of bioactive fraction five (Fr.5) was further purified using silica gel (60–120 mesh) column (450 mm × 20 mm). The column was eluted step wise with 100 ml of hexane, 200 ml of hexane:chloroform (90:10 to 0:100, v/v), 500 ml of chloroform:ethyl acetate (90:10 to 0:100, v/v), 1000 ml of ethyl acetate:acetone (90:10 to 0:100, v/v) and 1000 ml of acetone:methanol (90:10 to 0:100, v/v). Twenty-eight

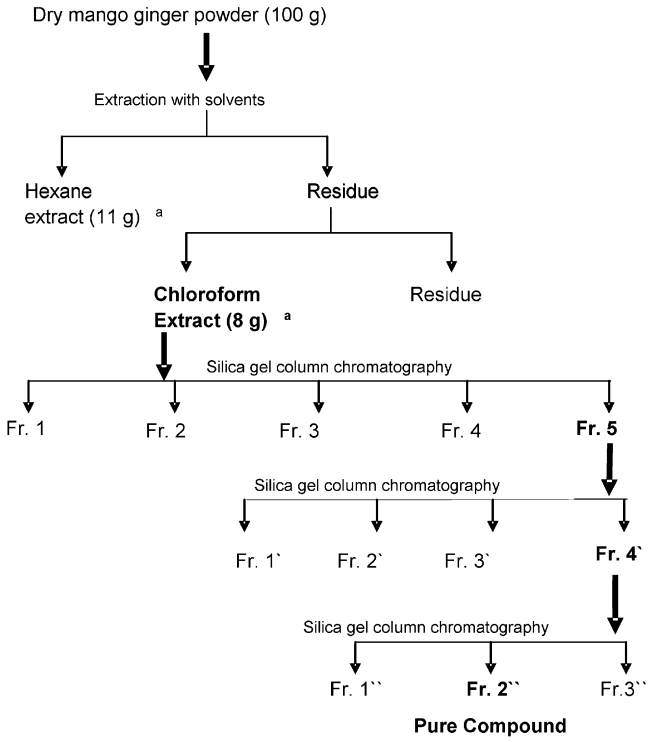

Fig. 1. Schematic representation of extraction and isolation of antioxidant compound from chloroform extract of mango ginger by column chromatography. [a]Yields of hexane and chloroform extract (g/100 g dry powder).

fractions measuring 100 ml each were collected and concentrated on a rotary evaporator. An aliquot of all the fractions were loaded on the TLC plate. Fractions having same number of spots with similar R_f values were pooled and numbered (Fr.1′–Fr.4′). These four fractions were tested for the antioxidant activity.

Fraction four (Fr.4′) obtained from second step chromatography (Fig. 1) showed high antioxidant activity, hence selected for further purification. About 520 mg of bioactive fraction four (Fr.4′) was further purified on a silica gel (100–200 mesh) column (600 mm × 15 mm). The column was eluted step wise with 100 ml of hexane:chloroform (90:10 to 0:100, v/v), 100 ml of chloroform:ethyl acetate (95:05 to 0:100, v/v), 500 ml of ethyl acetate:acetone (90:05 to 0:100, v/v) and 500 ml of acetone:methanol (95:05 to 0:100, v/v). About 23 fractions measuring 50 ml each were collected and concentrated. Fractions having same number of spots with similar R_f values on TLC plate were pooled and numbered (Fr.1″–Fr.3″). Among these, fraction number two (Fr.2″) obtained from third step chromatography (Fig. 1) showed a single spot in TLC profile. This pure compound was subjected to various spectroscopic techniques for elucidation of the structure.

2.4.5. High performance liquid chromatography (HPLC)

The purified fraction was tested for its purity using HPLC, using LC-10AT liquid chromatograph (Shimadzu, Singapore) equipped with C-18 column (300 mm × 4.6 mm 5 μ Thermo Hypersil) and acetonitrile:water (60:40) as a mobile phase with a flow rate of 1 ml/min. Diode array (Shimadzu, Singapore) was used as a detector.

2.5. Identification of bioactive compound

2.5.1. UV–vis spectrophotometry

UV–vis spectrum of the isolated bioactive compound was recorded on a Shimadzu UV-160A instrument (Shimadzu, Singapore) at room temperature. About 1 mg isolated compound dissolved in 20 ml of chloroform was used to record the spectrum. The region from 200 to 800 nm was employed for scanning.

2.5.2. IR spectrometry

IR spectrum of isolated bioactive compound was recorded on a Perkin-Elmer FT-IR Spectrometer (Spectrum 2000) at room temperature. A region from 400 to 4000 cm^{-1} was used for scanning.

2.5.3. Liquid chromatography–mass spectrometry (LC–MS)

Mass spectrum of the isolated bioactive compound was recorded on instrument HP 1100 MSD series (Palo Alto, CA) by electro spray ionization (ESI) technique with a flow rate of 0.2 ml/min on C-18 column (300 mm × 4.6 mm 5 μ Thermo Hypersil). The mobile phase was acetonitrile:water (60:40) with total run time of 25 min. Diode array (Shimadzu, Singapore) was used as a detector. About 1 mg of isolated compound dissolved in 10 ml of methanol was used for recording the spectrum. The column temperature was maintained at room temperature.

2.5.4. Two-dimensional heteronuclear multiple quantum coherence transfer spectroscopy (2D-HMQCT) NMR spectra

NMR spectra were recorded on a Bruker (Rheinstetten, Germany) DRX 500 NMR instrument operating at 500 MHz for ^{1}H and 125 MHz for ^{13}C at room temperature. A region from 0 to 20 ppm for ^{1}H and 0–200 ppm for ^{13}C was employed for scanning. Signals were referred to internal standard tetramethylsilane. About 45 mg of isolated bioactive compound dissolved in $CdCl_3$ was used for recording the spectra.

2.6. Antioxidant activity

2.6.1. DPPH free radical scavenging activity

1,1-Diphenyl-2-picryl-hydrazyl radical scavenging activity was determined according to the method described earlier [27–29]. The test samples (10–100 μl) were mixed with 0.8 ml of Tris–HCl buffer (pH 7.4) to which 1 ml of DPPH (500 μM in ethanol) was added. The mixture was shaken vigorously and left to stand for 30 min. Absorbance of the resulting solution was measured at 517 nm in a UV–vis spectrophotometer (UV-160A, Shimadzu Co., Japan). The radical scavenging activity was measured as a decrease in the absorbance of DPPH. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity. Radical scavenging potential was expressed as EC_{50} value, which represents the sample concentration at which 50% of the DPPH radicals scavenged.

2.6.2. Super oxide radical scavenging activity

The super oxide scavenging ability was assessed according to the method of Nishikimi, et al. [30] with slight modifications. The reaction mixture contained NBT (0.1 mM) and NADH (0.1 mM) with or without sample to be assayed in a total volume of 1 ml of Tris–HCl buffer (0.02 M, pH 8.3). The reaction was started by adding PMS (10 μM) to the mixture, and change in the absorbance was recorded at 560 nm every 30 s for 2 min. The percent inhibition was calculated against a control without test sample. Radical scavenging potential was expressed as EC_{50} value, which represents the sample concentration at which 50% of the radicals scavenged.

2.6.3. Lipid peroxidation inhibitory activity

Lipid peroxidation inhibitory activity was determined according to the method described earlier [31]. In brief, egg lecithin (3 mg/ml phosphate buffer, pH 7.4) was sonicated in dr. Hielscher Gmb_H, UP 50H ultraschallprozessor (DrHielscher GmbH, Teltow, Berlin, Germany). The test samples (100 μl) were added to 1ml of liposome mixture, control was without test sample. Lipid peroxidation was induced by adding 10 μl $FeCl_3$ (400 mM) and 10 μl L-ascorbic acid (400 mM). After incubation for 1 h at 37 °C, the reaction was stopped by adding 2 ml of 0.25N HCl containing 15% TCA and 0.375% TBA and the reaction mixture was boiled for 15 min then cooled, centrifuged and absorbance of the supernatant was measured at 532 nm. Inhibitory activity was expressed as EC_{50} value, which is sample concentration inhibited 50% of lipid peroxidation.

2.6.4. Metal chelating activity

The chelation of ferrous ions by the test sample was estimated by the method described earlier [32,33]. Briefly, the test samples at different concentrations were 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) and the mixture was vigorously shaken and left standing at room temperature for 10 min. After the mixture had reached equilibrium, the absorbance of the mixture was read at 562 nm against a blank. EDTA was used as positive control. Results were expressed as EC_{50} value, which represents the sample concentration at which 50% of metal chelation occurred.

2.6.5. Total reducing power

The reducing power was quantified by the method described earlier by Yen and Chen [34] with minor modifications. Reaction mixture, containing test samples at different concentrations (10–100 μl) in phosphate buffer (0.2 M, pH 6.6), was incubated with potassium ferricyanide (1%, w/v) at 50 °C for 20 min. The reaction was terminated by the addition of TCA solution (10%, w/v) and the mixture was centrifuged at 3000 rpm for 20 min. The supernatant was mixed with distilled water and ferric chloride (0.1%, w/v) solution and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power.

2.7. Antibacterial activity

2.7.1. Minimum inhibitory concentration (MIC)

The minimum inhibitory concentration was determined according to the method described by Jones et al. [35]. Different concentrations (20–300 ppm) of isolated compound and 100 μl of the bacterial suspension (10^5 CFU/ml) was placed aseptically in10 ml of nutrient broth separately and incubated for 24 h at 37 °C. The growth was observed both visually and by measuring O.D. at 600 nm at regular intervals followed by pour plating. The plating was carried out by transferring bacterial suspension (10^5 CFU/ml) to sterile Petri plate and mixed with molten Nutrient agar medium (HiMedia Laboratories Limited, Mumbai, India) and allowed to solidify. The lowest concentration of test sample showing no visible growth was recorded as the minimum inhibitory concentration. Triplicate sets of tubes were maintained for each concentration of test sample. Amoxicillin (100 μg/ml) was used as a positive control.

2.7.2. Determination of minimum bactericidal concentration (MBC)

Minimum bactericidal concentration was determined according to the method of Smith-Palmer et al. [36]. Test tubes containing nutrient broth with different concentrations of isolated compound were inoculated with 100 μl of the bacterial suspension (10^5 CFU/ml). Inoculated tubes were incubated for 24 h at 37 °C and growth was observed both visually and by measuring O.D. at 600 nm. About 100 μl from the tubes not showing growth were plated on Nutrient agar as described above. Triplicate sets of tubes were maintained for each concentration of test sample. Minimum bactericidal concentration is the concentration at which bacteria failed to grow in nutrient broth and nutrient agar inoculated with 100 μl suspension.

2.8. Statistical analysis

The experiments were carried out in triplicates. Significant differences ($P < 0.05$) were determined by Duncan's Multiple Range Test (DMRT).

3. Results and discussion

3.1. Isolation of bioactive compound

Extraction of 100 g of mango ginger powder using solvents hexane and chloroform yielded 11 and 8 g of extract, respectively (Fig. 1). Chloroform extract exhibited promising lipid peroxidation inhibitory activity and moderate metal chelating activity (Table 1). Hence, it was selected for the isolation of bioactive compound responsible for the antioxidant activity. Silica gel column chromatography of crude chloroform extract yielded five fractions (Fr.1–Fr.5). Among these, fifth fraction (Fr.5) showed 68% DPPH scavenging activity, remaining fractions viz. Fr.1, Fr.2, Fr.3 and Fr.4 showed 18, 22, 25 and 36% DPPH scavenging activity (at 2 mg/ml), respectively (Fig. 2). Further purification of the fifth fraction (Fr.5) yielded four fractions (Fr.1′–Fr.4′), wherein fourth fraction (Fr.4′) exhibited high DPPH scavenging

Table 1
Antioxidant activity of chloroform extract and bioactive compound

Antioxidant activity	EC_{50} value* ($\mu g\ ml^{-1}$)		
	Chloroform extract	Amadannulen	BHA
DPPH radical scavenging activity	N.D.[n]	178 ± 2.8^{b}	5 ± 0.8^{a}
Super oxide radical scavenging activity	N.D.[n]	132 ± 4.4^{a}	258 ± 2.4^{b}
Lipid peroxidation inhibitory activity	80 ± 1.8^{b}	65 ± 3.2^{a}	94 ± 1.6^{c}
Metal chelating activity	142 ± 3.6^{a}	216 ± 2.6^{b}	N.D.[n]

*Each value represents mean of three different observations ± S.D. Mean values with different superscript letters (a–c) differ significantly at $P < 0.05$. [n]Not detected.

activity (64%), while Fr.1′, Fr.2′ and Fr.3′ showed 22, 26 and 38%, respectively (at 2 mg/ml) (Fig. 3). The fraction four (Fr.4′) was further purified using silica gel (100–200 mesh) column chromatography and yielded three fractions (Fr.1″–Fr.3″). Among these, fraction two (Fr.2″) showed high DPPH scavenging activity (52%), while Fr.1″ and Fr.3″ exhibited 38 and 36% DPPH radical scavenging activity, respectively (at 2 mg/ml) (Fig. 4). The second fraction (Fr.2″) showed a single spot on TLC. The purity of the isolated compound was confirmed by HPLC. Amadannulen showed single peak at 240 nm at a retention time of 3.3 min in the HPLC chromatogram (Fig. 5). The schematic representation for the isolation of antioxidant compound is given in Fig. 1. The isolated compound was subjected to spectroscopic analysis to elucidate the structure.

3.2. Identification of bioactive compound

The structure of bioactive compound was elucidated after analyzing the data obtained by various spectroscopic techniques. The molecular weight was determined using LC–MS. Mass spectrum showed the parent molecular ion *m/z* at 377. Elemental analysis (VARIO EL III CHNS Elementar) revealed that the

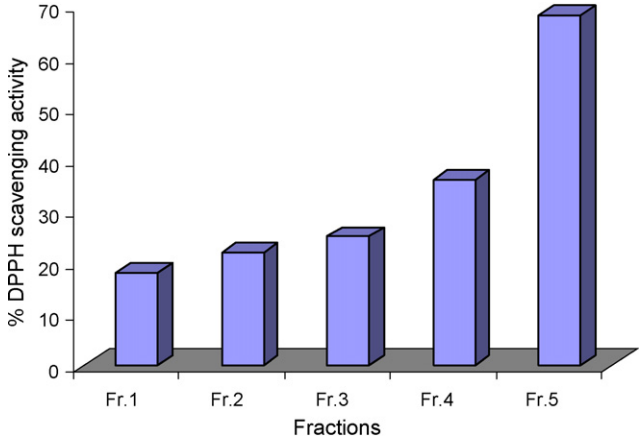

Fig. 2. DPPH radical-scavenging activity[a] of five fractions[b] (Fr.1–Fr.5) obtained from chloroform extract. [a]Each value represents mean of three different observations at a concentration of 2 mg/ml. [b]Five fractions (Fr.1–Fr.5) were obtained from the chloroform extract by silica gel column chromatography.

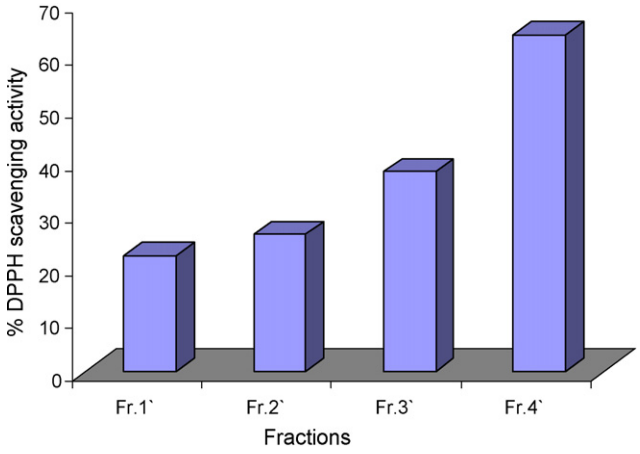

Fig. 3. DPPH radical-scavenging activity[a] of four fractions[b] (Fr.1′–Fr.4′) obtained by further purification of active fraction five (Fr.5). [a]Each value represents mean of three different observations at a concentration of 2 mg/ml. [b]Active fraction five (Fr.5) from first chromatographic step was further purified and four fractions (Fr.1′–Fr.4′) obtained were tested for DPPH scavenging activity.

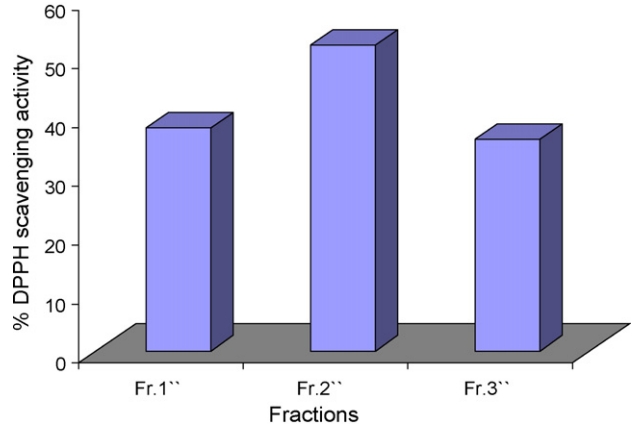

Fig. 4. DPPH radical-scavenging activity[a] of three fractions[b] (Fr.1″–Fr.3″) obtained by further purification of active fraction four (Fr.4′). [a]Each value represents mean of three different observations at a concentration of 2 mg/ml. [b]Active fraction four (Fr.4′) from second chromatographic step was further purified on silica gel column and three fractions (Fr.1″–Fr.3″) obtained were tested for DPPH scavenging activity.

compound consists of 74.22% of carbon and 10.10% of hydrogen. The molecular formula of the compound was found to be $C_{24}H_{40}O_3$. The UV maxima (Fig. 6) observed at 242 nm indicated the presence of double bond. Identification of specific

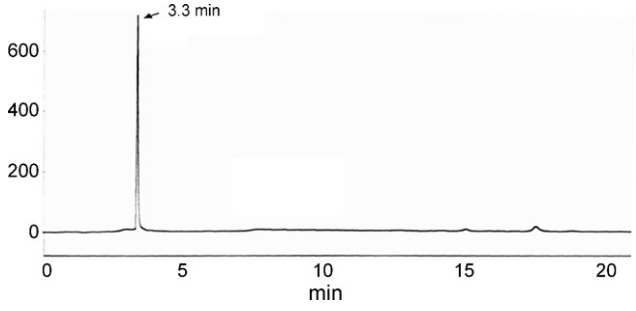

Fig. 5. Chromatogram of isolated amadannulen.

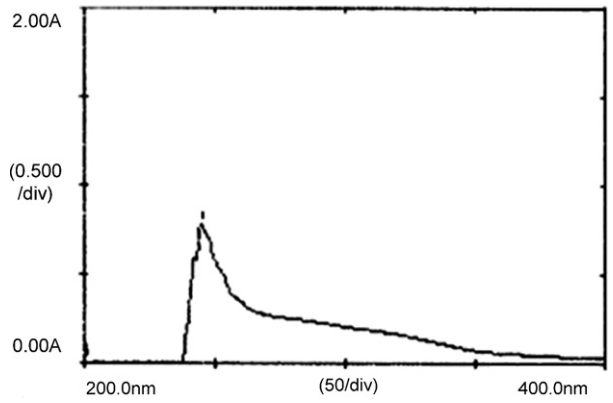

Fig. 6. UV spectrum of isolated amadannulen.

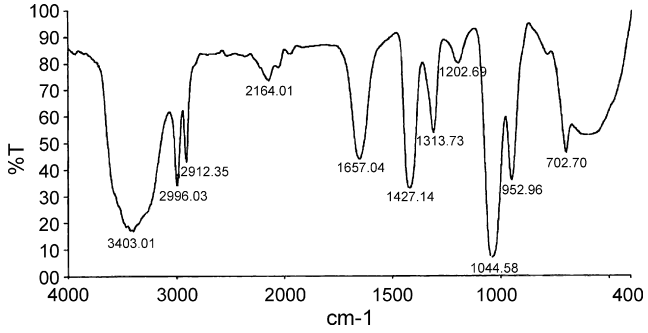

Fig. 7. FT-IR spectrum of isolated amadannulen.

functional groups was carried out using IR spectra (Fig. 7). The O–H stretching observed at 3403 cm^{-1} is the characteristic of hydrogen bonded –OH group. The stretching at 2996 and 1657 cm^{-1} can be attributed to C–H and C=O stretching vibrations, respectively. The alkyl C–H bending vibration has been observed at 1427 cm^{-1}. The C–O stretching vibration at 1202 cm^{-1} indicates the presence of ester moiety.

A two-dimensional heteronuclear multiple quantum coherence transfer NMR spectrum (Fig. 8) was recorded along with one-dimensional ^{1}H and ^{13}C NMR spectra, which gave clear indication of carbon skeleton of the compound (Table 2). No

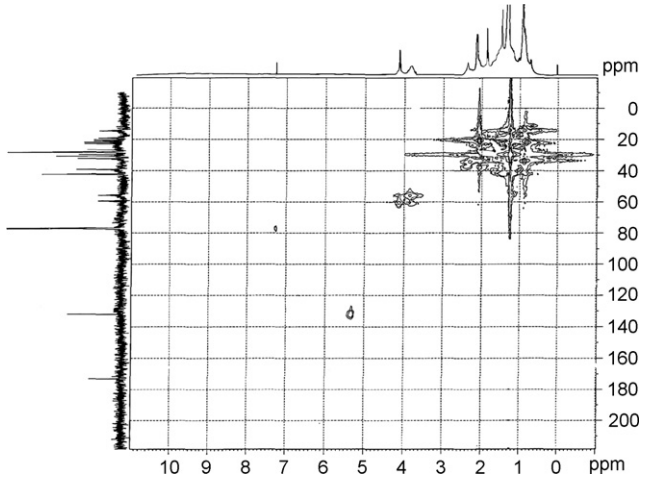

Fig. 8. 2D-HMQCT NMR spectrum of isolated amadannulen.

Table 2
^{1}H and ^{13}C data showing HMQC connectivities obtained from isolated compound

Carbon position	^{13}C NMR (ppm)	^{1}H NMR (ppm)
1-C	133.4	–
2-CH_2	42.7	1.85
3-CH	34.6	1.40
4-CH_2	29.1	1.35
5-CH_2	21.5	1.30
6-CH_2	24.4	1.30
7-CH_2	22.8	1.35
8-CH	29.3	1.40
9-CH_2	39.7	1.85
10-C	133.1	–
11-CH_2	42.2	2.10
12-CH	58.1	3.80
13-CH_2	42.4	2.10
14-CH_3	19.0	0.85
15-CH	31.7	1.28
16-CH_2	29.6	1.25
17-CH	27.1	1.35
18-CH_2	33.7	1.40
19-CH	39.0	2.33
20-CH_2	33.2	1.40
21-CO	175.4	–
22-CH_2	60.1	4.15
23-CH_3	14.1	0.90
24-CH_3	20.2	0.85

peaks were detected for aromatic moiety both in ^{1}H and ^{13}C NMR spectra. The –CH_3 protons showed peaks in the region 0.8–0.9 ppm. There are three –CH_3 groups and the corresponding ^{13}C peaks were detected between 14 and 20 ppm. The cyclic –CH_2 and –CH protons exhibited peaks in the range 1.2–1.7 ppm and the corresponding ^{13}C peaks have been observed between 21 and 34 ppm. The four –CH_2 protons of cyclopentyl ring showed doublet at 2.1 ppm indicating clearly both the –CH_2 groups are attached to –CH carbon. The –CH group attached to –OH moiety showed ^{1}H peak at 3.8 ppm and the corresponding ^{13}C peak at 58 ppm. The quartet at 4.15 ppm exhibited by ethyl –CH_2 group indicates that it has been attached to –CH_3 group. The corresponding ^{13}C peak has been observed at 60 ppm. Further, the ^{13}C signals at 178 and 133 ppm clearly confirms the presence of –CO and C=C groups. Based on these spectral data, the probable structure of the compound was deduced (Fig. 9) and tentatively named it as 'amadannulen'. The compound has been reported for the first time from mango ginger rhizome. Structurally similar types of bioactive compounds have been reported from *Euphorbia semiperfoliata* [37], *Mikania micrantha* [38], *Neurolaena oaxacana* [39], *Elephantopus tomentosus* [40] and gorgonian corals [41]. The conformation of the 10-membered ring in this type of compounds has been defined by the endocyclic torsion angles by earlier reports [42].

3.3. Antioxidant activities of chloroform extract and isolated compound

Various antioxidant assays like DPPH radical scavenging activity, super oxide radical scavenging activity, lipid perox-

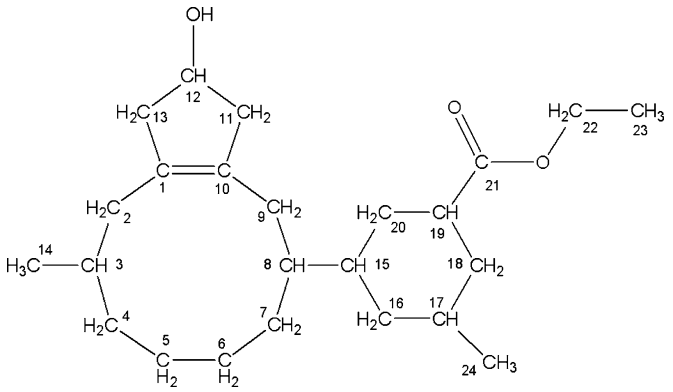

Fig. 9. Structure of amadannulen.

idation inhibitory activity, metal chelating activity and total reducing power were tested for amadannulen, chloroform extract to evaluate their antioxidant activity. BHA was used as a standard for antioxidant assay.

3.3.1. DPPH radical scavenging activity

The model of scavenging the stable DPPH radical is a widely used method to evaluate antioxidant activities in a relatively short time compared with other methods. Amadannulen showed moderate activity (EC_{50} = 178 μg/ml) compared to BHA (EC_{50} = 5 μg/ml) (Table 1). Interestingly, no activity was observed in the chloroform extract. A linear increase in free radical scavenging ability of amadannulen was observed with its increasing concentration. DPPH radical scavenging by antioxidants has been attributed to their hydrogen-donating ability of –OH and $–CH_3$ groups [43,44].

3.3.2. Super oxide scavenging activity

Amadannulen exhibited significant activity with an EC_{50} of 132 μg/ml compared to BHA (EC_{50} of 258 μg/ml). Chloroform extract did not show super oxide radical scavenging activity (Table 1). Super oxide anion plays an important role in the formation of reactive oxygen species (ROS) such as hydrogen peroxide, hydroxyl radical, and singlet oxygen, which induce oxidative damage in lipids, proteins and DNA [45–47].

3.3.3. Lipid peroxidation inhibitory activity

Lipid peroxidation causes destabilization and disintegration of the cell membrane, leading to liver injury, atherosclerosis, kidney damage, aging, and susceptibility to cancer [48]. Highest lipid peroxidation inhibitory activity was observed in amadannulen with EC_{50} of 80 μg/ml compared to chloroform extract and BHA with EC_{50} of 65 and 94 μg/ml, respectively (Table 1). Lipid peroxidation inhibitory activity was mainly attributed to the number of hydroxyl groups, solubility and hydrophobicity of the compounds [49]. Presence of methyl groups in amadannulen may be responsible for high lipid peroxidation inhibitory activity.

3.3.4. Metal chelating activity

Amadannulen showed metal chelating activity with EC_{50} of 216 μg/ml compared to chloroform extract with EC_{50} of

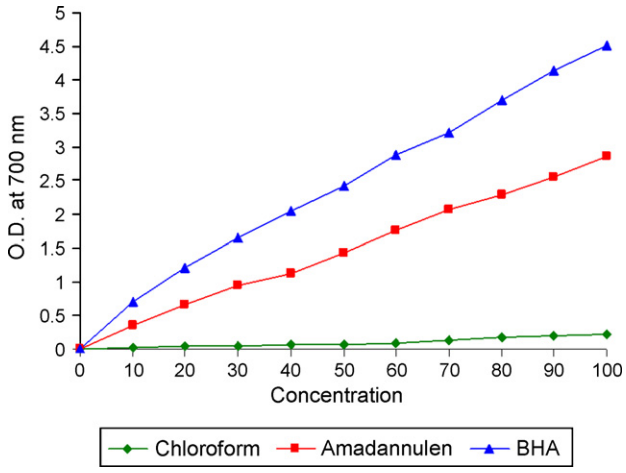

Fig. 10. Total reducing power of amadannulen[a], chloroform extract[b] and BHA[c]. [a,c]Concentration of test sample was 2 mg/ml. [b]Concentration of test sample was 1 mg/ml.

142 μg/ml. It was reported that the structures containing two or more of the following functional groups: –OH, –SH, –COOH, $–PO_3H_2$, C=O, $–NR_2$, –S– and –O– in a favorable structure-function configuration is responsible for metal chelating activity [50,51]. Amadannulen, which is having both –OH and C=O functional groups can contribute for the metal chelating activity. Ferrous ions can stimulate lipid peroxidation by Fenton reaction, and also accelerates peroxidation by decomposing lipid hydro peroxides into peroxyl and alkoxyl [3,52]. Since they are the most effective proxidant in the food system [53], high chelating abilities of amadannulen and chloroform extract would be beneficial.

3.3.5. Total reducing power

The reducing capacity of a compound from Fe^{3+}/ferricyanide complex to the ferrous form may serve as a significant indicator of its antioxidant capacity [54]. Fig. 10 shows the reductive capability of amadannulen and chloroform extract compared with BHA. A linear increase in reducing power with increasing concentration of amadannulen was observed. Chloroform extract showed negligible reducing power compared to BHA.

3.4. Antibacterial activity

3.4.1. Minimum inhibitory concentration

Amadannulen exhibited antibacterial activity against both Gram-positive and Gram-negative bacteria. It was more effective against wide spectrum of bacteria viz. *P. aeruginosa*, *S. typhi*, *K. pneumoniae*, *E. aerogenes*, *Y. enterocolitica*, *M. luteus*, *S. aureus*, *E. fecalis*, *B. cereus*, *B. subtilis* and *L. monocytogenes*. The most striking increase in activity was observed against *B. cereus*, *B. subtilis* and *M. luteus* with MIC of 70, 60 and 100 ppm, respectively. It also inhibited the growth of five Gram-negative bacteria viz. *P. aeruginosa*, *S. typhi*, *K. pneumoniae*, *E. aerogenes*, and *Y. enterocolitica* (Table 3). Amadannulen showed more activity than the chloroform extract (data not shown).

Table 3
Antibacterial activity of amadannulen

Bacteria	Amadannulen	
	MIC (ppm)*	MBC (ppm)*
P. aeruginosa	180	–
E. coli	–	–
S. typhi	140	–
K. pneumoniae	190	–
E. aerogenes	220	–
P. mirabilis	–	–
Y. enterocolitica	90	–
M. luteus	100	180
S. aureus	80	–
E. fecalis	170	–
B. cereus	70	120
B. subtilis	60	130
L. monocytogenes	80	–

*Each value represents mean of three different observations. (–) No inhibition.

3.5. Determination of bactericidal effect

Amadannulen was found to be bactericidal against *M. luteus*, *B. cereus* and *B. subtilis* (Table 3) with minimum bactericidal concentration of 180, 120 and 130 ppm, respectively. It did not show bactericidal activity against other bacterial strains tested even at higher concentrations. Amadannulen was bacteriostatic for most of the strains. Investigations are in progress to test the mode and site of action of the amadannulen.

4. Conclusion

Amadannulen was isolated by sequential extraction of mango ginger rhizome followed by bioactivity-guided fractionation with silica gel column chromatography and characterized by different spectral analysis. Amadannulen is a novel compound not reported previously from any other source. It exhibited a multisystem antioxidant activities and also antimicrobial activities. The antioxidant activity of amadannulen includes reductive ability, metal chelator, hydrogen donating ability and scavengers of super oxide radicals. Bactericidal activity of amadannulen against the bacteria tested is of high significance. Antioxidant and antibacterial activity shown by amadannulen need to be exploited.

Acknowledgements

We are thankful to Dr. V. Prakash, Director, CFTRI, Mysore, for his keen interest in the work and encouragement. Mr. R.S. Policegoudra thanks the CSIR, New Delhi, India, for awarding the Senior Research Fellowship. We also thank Sophisticated Instruments Facility, IISc, Bangalore, for the NMR analysis and Molecular Biophysics Unit, IISc, Bangalore, for the LC–MS analysis.

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