Antimicrobial, antioxidant, cytotoxicity and platelet aggregation inhibitory activity of a novel molecule isolated and characterized from mango ginger (*Curcuma amada* Roxb.) rhizome

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Mango ginger (*Curcuma amada* Roxb.) rhizome is used in the manufacture of pickles and other food preparations due to its unique raw mango flavour. The chloroform extract of mango ginger rhizome was subjected to antibacterial activity-guided purification by repeated silica gel column chromatography to obtain a pure compound. The structure of the isolated compound was deduced by analysing UV, IR, LC-MS and 2D-HMQCT NMR spectral data, and named it as amadaldehyde, a novel compound. It exhibited a wide range of antibacterial activity with potential bactericidal activity against several bacteria. The purified compound also exhibited antioxidant activity, cytotoxicity and platelet aggregation inhibitory activities.

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1. Introduction

Antimicrobials from natural sources such as plants have been used for food safety since antiquity (Alzoreky and Nakahara 2003). Concerns over the rise of antimicrobial resistance have sparked an interest in investigating the use of natural products to improve health and growth (Cowan 1999). Plants have an almost limitless ability to synthesize phytochemicals, which serve as plant defence mechanisms against predation by microorganisms, insects and herbivores. Many compounds are responsible for plant flavour and some of these herbs and spices used by humans to season food also yield useful medicinal compounds.

Spices are used in food primarily because they impart desirable flavours but they may fulfil more than one function to the food to which they are added (Nasar-Abbas and Kadir Halkman 2004). Mango ginger (*Curcuma amada* Roxb.) is a unique perennial herb and its rhizomes have a morphological resemblance to ginger (*Zingiber officinale*) but impart a mango (*Mangifera indica*) flavour. Many species belonging to this genus have considerable value as medicines, dyes and spices. The main use of mango ginger is in the manufacture of pickles, as a source of raw mango flavour for foods and for therapeutic purposes. Ayurveda, the oldest system of medicine in India, has given importance to the rhizome as an appetizer, alexteric, antipyretic, aphrodisiac and laxative.

Keywords. Amadaldehyde; antibacterial activity; antioxidant; *Curcuma amada*; cytotoxicity; mango ginger; MBC; MIC

Abbreviations used: ADP, adenosine diphosphate; BHA, butylated hydroxy anisole; DMEM, Dulbecco modified Eagle medium; DMEM-PR, Dulbecco modified Eagle medium without phenol red; DMRT, Duncan multiple range test; DMSO, dimethyl sulphoxide; DPPH, 1,1-diphenyl-2-picryl-hydrazyl; EDTA, ethylene diamine tetra-acetic acid; ESI, electrospray ionization; Fr., fraction; HPLC, high performance liquid chromatography; IR, infra red; LC-MS, liquid chromatography-mass spectrometry; MBC, minimum bactericidal concentration; MIC, minimum inhibitory concentration; MTT, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide; NADH, nicotinamide adenine dinucleotide; NBCS, newborn calf serum; NBT, nitroblue tetrazolium; NMR, nuclear magnetic resonance; OD, optical density; PMS, phenazine methosulphate; PPP, platelet-poor plasma; PRP, platelet-rich plasma; SRB, sulphorhodamine B; TBA, thiobarbituric acid; TCA, trichloroacetic acid; TLC, thin-layer chromatography; UV, ultraviolet

It is also used in biliousness, itching, skin diseases, bronchitis, asthma, hiccough and inflammation due to injuries (CSIR 1950; Kirtikar and Basu 1984; Warrier *et al.* 1994). According to the Unani system of medicine, it is a diuretic, maturant, emollient, expectorant, antipyretic and appetizer. It is useful against inflammation of the mouth, ear and gleet, ulcers on the male genitalia, scabies, lumbago and stomatitis (Kirtikar and Basu 1984; Hussain *et al.* 1992; Warrier *et al.* 1994).

In spite of its numerous medicinal properties and high food value for its exotic flavour, there are no reports on bioactive molecules from mango ginger. Recently, two bioactive molecules – difurocumenonol and amadannulen – were isolated and characterized from mango ginger by Policegoudra *et al.* (2007a, b). These compounds exhibited potential multifunctional bioactivities such as antibacterial activity, platelet aggregation inhibitory activity, cytotoxicity and antioxidant activities such as 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging activity, lipid peroxidation inhibitory activity, metal chelating activity and superoxide radical scavenging activity. This plant represents a major source of valuable drugs, pigments and phytochemicals, which are important for the food, cosmetic and pharmaceutical industries. Therefore, the objective of the present study was to isolate and characterize the bioactive compounds from mango ginger rhizomes.

2. Materials and methods

2.1 *Plant material*

Fresh and healthy mango ginger (*Curcuma amada* Roxb.) rhizomes were harvested from Sulthan Bathery, Kerala, India. The plant was identified by Professor Shivamurthy, Head, Department of Botany, University of Mysore, Mysore, India. 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 extraction and column chromatography were of analytical grade from Merck Limited, Mumbai, India. High performance liquid chromatography (HPLC) grade methanol was from Ranbaxy Fine Chemicals Limited, Mumbai, India. Silica gel (60–120 mesh) used for column chromatography was from Qualigens Fine Chemicals, Mumbai, India; silica gel (100–200 mesh) used for column chromatography was from Loba Chemie Pvt. Ltd. Mumbai, India. Silica gel used for thin-layer chromatography (TLC) was from Glaxo Laboratories, Mumbai, India. Nutrient agar and nutrient broth were from HiMedia Laboratories Limited, Mumbai, India. Folin–Ciocalteu phenol reagent, potassium ferricyanide, 3-(2-pyridyl)-5, 6-bis (4-phenyl-sulphonic acid)-1, 2, 4-triazine (ferrozine) and ferric chloride were purchased from M/s Sisco Research Laboratories, Mumbai, India. Gallic acid, DPPH, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT), sulphorhodamine B (SRB) dye, Trizma-base collagen, nicotinamide adenine dinucleotide (NADH), BHA and trichloroacetic acid (TCA), newborn calf serum (NBCS) were purchased from Sigma (Sigma-Aldrich GmbH, Sternheim, Germany). Nitroblue 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, USA). Dulbecco modified Eagle medium (DMEM) was purchased from HiMedia Lab Ltd, Mumbai, India. All other reagents were of analytical grade and the other chemicals used in this study were of the highest purity. The software used for the chemical structure drawing was ChemDraw Ultra from Cambridge Soft Corporation, MA, USA.

2.3 *Bacterial strains and inoculum preparation*

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.* These bacterial strains were isolated from clinical samples and obtained from the Department of Microbiology, Mysore Medical College, Mysore, India. Their cultural characteristics and morphological features were reconfirmed and also subjected to standard biochemical tests (Krieg and Holt 1984; Peter *et al.* 1986) before the experiments. The test organisms were maintained on nutrient agar slants.

2.4 *Isolation of antimicrobial 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) at an atmospheric pressure for 48 h by shaking at 100 rpm/min speed. The extracts were filtered and concentrated by using a rotary evaporator (Buchi Rotavapor R-124). The concentrated extracts were freeze-dried and stored in a refrigerator. The promising antioxidant activity of the chloroform extract prompted us to isolate and purify the antioxidant compound from it. The yield of each extract was noted. All the extracts were screened for antibacterial activity by the agar well-diffusion method. Since the chloroform extract showed

high antibacterial activity, it was selected for isolation and purification of the bioactive compound.

2.4.2 *Fractionation of the chloroform extract:* Activated silica gel (60–120 mesh) was packed onto a glass column (450 × 40 mm) using *n*-hexane solvent. The chloroform extract was loaded on top of the silica gel and eluted step-wise: 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 the rotary evaporator.

2.4.3 *Thin-layer chromatography (TLC):* An aliquot of all the concentrated fractions was loaded on the activated silica gel TLC plates (20 × 20 cm). The plates were developed using hexane:chloroform (25:75), chloroform: ethyl acetate (95:5), ethyl acetate:methanol (90:10) solvents, and chloroform:ethyl acetate:acetone (24:75:1). Spots were located by exposing the plate to iodine fumes. Fractions having the same number of spots with similar R_f values on the TLC plate were pooled into five fractions. All the five pooled fractions were tested for antibacterial activity as described below.

2.4.4 *Further purification of the bioactive fraction:* Since fraction two (Fr. 2) obtained from the first step of column chromatography (figure 1) showed high antibacterial activity, it was selected for further purification. Bioactive Fr. 2 was further purified using a silica gel (60–120 mesh) column (450 × 20 mm). The column was eluted step-wise with 100 ml of hexane, 200 ml of hexane:chloroform (95: 5, 75:25, 50:50 v/v), 300 ml of hexane:ethyl acetate (95: 5, 75:25, 0:100 v/v) and 100 ml of methanol (0:100 v/v). About 33 fractions measuring 100 ml each were collected and concentrated on a rotary evaporator. An aliquot of all the fractions was loaded on the TLC plate, and fractions having similar R_f values were pooled into eight sub-fractions (Fr. 2.1–Fr. 2.8). These eight fractions were tested for antibacterial activity.

Sub-fraction four (Fr. 2.4) obtained from second-step chromatography showed high antibacterial activity, and was hence selected for further purification. About 1.2 g of bioactive sub-fraction four (Fr. 2.4) was further purified on a silica gel (100–200 mesh) column (600 × 15 mm). The column was eluted step-wise with 200 ml of hexane: chloroform (75:25 v/v) and 200 ml of hexane:acetone (95: 05 v/v). About 21 fractions measuring 25 ml each were collected and concentrated. Fractions having similar R_f values on the TLC plate were pooled into five sub-fractions (Fr. 2.4.1–Fr. 2.4.5). Among these, fraction number five (Fr. 2.4.5) obtained from third-step chromatography showed a

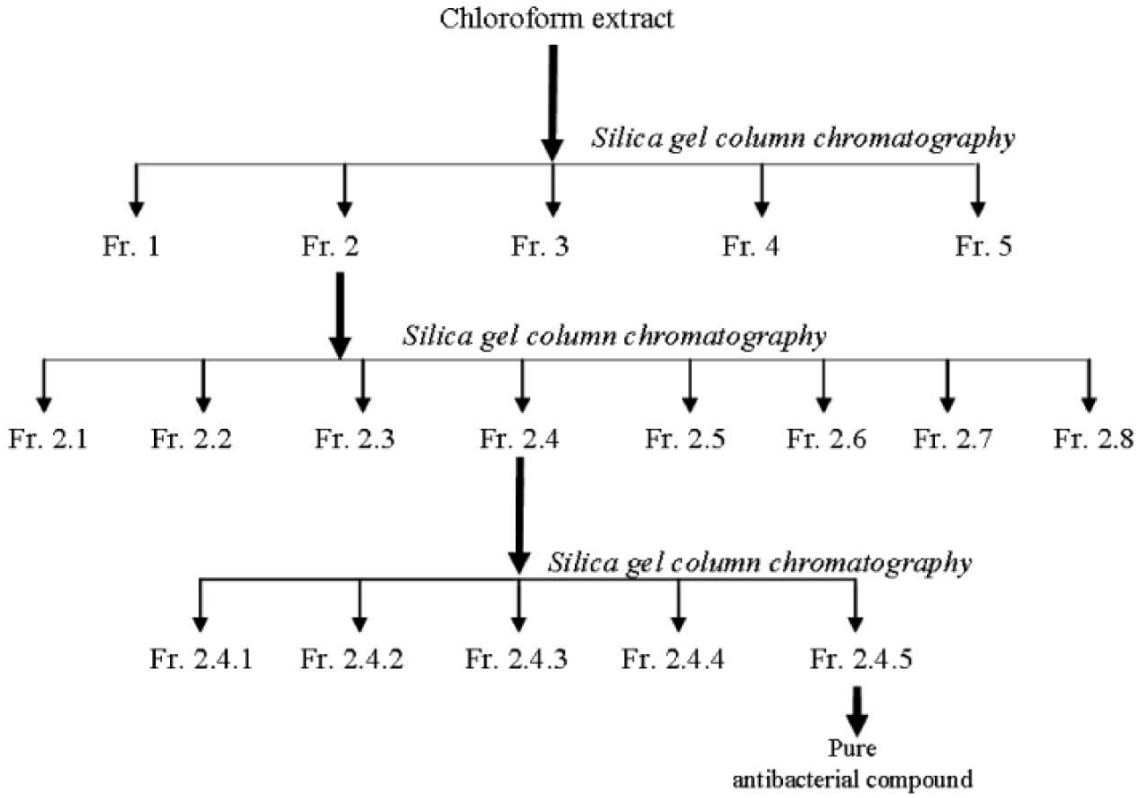

Figure 1. Flow chart for isolation of the antibacterial compound.

single spot in the 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 isolated compound (Fr. 2.4.5) was filtered and freeze-dried before the sample was used for HPLC analysis using an LC-10AT liquid chromatograph (Shimadzu, Singapore) equipped with a C-18 column (300 × 4.6 mm 5 μ, Thermo Hypersil). The gradient programme used for the mobile phase was as follows; 0 min, methanol:water (25:75 v/v); 5 min, methanol:water (40:60 v/v); 10 min, methanol:water (50:50 v/v); 20 min, methanol:water (70:30 v/v); 40 min, methanol:water (100:0 v/v), with a flow rate of 1 ml min^{-1}. UV detection was carried out with a diode array detector (Shimadzu, Singapore).

2.5 *Characterization of the antimicrobial molecule*

2.5.1 *Ultraviolet (UV) spectrophotometry:* The UV-visible spectrum of the isolated compound was recorded on a Shimadzu UV-160A instrument (Shimadzu, Singapore) at room temperature. About 1 mg of isolated compound dissolved in 20 ml of chloroform was used to record the spectrum (from 200 to 800 nm).

2.5.2 *Infra red (IR) spectrometry:* The IR spectrum of the isolated compound was recorded on a Perkin-Elmer FT-IR spectrometer (Spectrum 2000) at room temperature. About 1 mg of isolated compound dissolved in 10 ml of chloroform was used to record the spectrum (frequencies between 4000 and 400 cm^{-1}).

2.5.3 *Liquid chromatography-mass spectrometry (LC-MS):* The mass spectrum of the isolated compound was recorded on an HP 1100 MSD series instrument (Palo Alto, CA) by the electrospray ionization (ESI) technique with a flow rate of 0.2 ml min^{-1} on a C-18 column and a total run time of 40 min. Diode array was used as a detector. About 1 mg of isolated compound dissolved in 5 ml of methanol was used to record the spectrum.

2.5.4 *Nuclear magnetic resonance (NMR) spectra:* NMR spectra were recorded on a Bruker DRX 700 NMR instrument (Rheinstetten, Germany) operating at 500 MHz for ^{1}H and 125 MHz for ^{13}C at room temperature. Regions from 0 to 12 ppm for ^{1}H and 0–200 ppm for ^{13}C were employed. Signals were referred to the internal standard tetramethylsilane. About 20 mg of isolated compound dissolved in 0.75 ml of $CDCl_3$ was used for recording the spectra.

2.6 *Antibacterial activity*

2.6.1 *Agar well-diffusion method: In vitro* antibacterial activity was determined by the agar well-diffusion method (Mukherjee *et al.* 1995). The overnight bacterial culture was centrifuged at 8000 rpm for 10 min at 4°C. The supernatant was discarded and bacterial cells were resuspended in saline to make a suspension of 10^5 CFU ml^{-1} and used for assay. Plating was carried out by transferring the bacterial suspension (10^5 CFU ml^{-1}) to a sterile Petri plate, mixed with molten nutrient agar medium (HiMedia Laboratories Limited, Mumbai, India) and allowed to solidify. About 75 μl of the sample (2 mg ml^{-1}) was placed in the wells and allowed to diffuse for 2 h. Plates were incubated at 37°C for 48 h and activity was determined by measuring the diameter of the inhibition zones. Solvent control and amoxicillin (Galpha Lab. Mumbai, India) were also maintained. The assay was carried out in triplicate.

2.6.2 *Minimum inhibitory concentration (MIC):* The MIC was determined according to the method described by Jones *et al.* (1985). Different concentrations (20–300 ppm) of the isolated compound (amadaldehyde) and 100 μl of the bacterial suspension (10^5 CFU ml^{-1}) were placed aseptically in 10 ml of nutrient broth separately and incubated for 24 h at 37°C. Growth was observed both visually and by measuring the optical density (OD) at 600 nm at regular intervals followed by pour plating as described above. The lowest concentration of the test sample showing no visible growth was recorded as the MIC. Triplicate sets of tubes were maintained for each concentration of test sample.

2.6.3 *Minimum bactericidal concentration (MBC):* The MBC was determined according to the method of Smith-Palmer *et al.* (1998). The test-tubes containing nutrient broth with different concentrations of isolated compound (amadaldehyde) were inoculated with 100 μl of the bacterial suspension (10^5 CFU ml^{-1}). The inoculated tubes were incubated for 24 h at 37°C and growth was observed both visually and by measuring the OD at 600 nm. About 100 μl from the tubes that did not show growth were plated on nutrient agar as described above. The MBC is the concentration at which bacteria failed to grow in nutrient broth and nutrient agar inoculated with 100 μl of suspension. Triplicate sets of tubes were maintained for each concentration of test sample.

2.7 *Antioxidant activities of mango ginger extracts*

2.7.1 *DPPH free radical scavenging activity:* DPPH radical scavenging activity was determined according to the method described earlier (Blois 1958; Moon and Terao 1998). 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-visible 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 an EC_{50} value, which represents the concentration of the sample at which 50% of the DPPH radicals were scavenged.

2.7.2 *Superoxide radical scavenging activity:* Superoxide scavenging ability was assessed according to the method of Nishikimi *et al.* (1972) with slight modifications. The reaction mixture contained NBT (0.1 mM) and NADH (0.1 mM) with or without the 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 per cent inhibition was calculated against a control without the test sample. Radical scavenging potential was expressed as an EC_{50} value, which represents the concentration of the sample at which 50% of the radicals were scavenged.

2.7.3 *Lipid peroxidation inhibitory activity:* Lipid peroxidation inhibitory activity was determined according to the method described earlier (Duh and Yen 1997). In brief, lecithin (3 mg/ml phosphate buffer, pH 7.4) was sonicated in a DrHielscher GmbH, UP 50H ultraschallprozessor (DrHielscher GmbH, Teltow, near Berlin, Germany). The test samples (100 μl) were added to 1 ml of liposome mixture, while the control was without the test sample. Lipid peroxidation was induced by adding 10 μl $FeCl_3$ (400 mM) and 10 μl ascorbic acid (400 mM). After incubation for 1 h at 37°C, the reaction was stopped by adding 2 ml of 0.25 N HCl containing 15% TCA and 0.375% TBA, and the reaction mixture was boiled for 15 min. It was then cooled, centrifuged and absorbance of the supernatant was measured at 532 nm. Inhibitory activity was expressed as an EC_{50} value, which is a sample with a concentration that inhibited 50% of lipid peroxidation.

2.7.4 *Metal chelating activity:* The chelation of ferrous ions by the test sample was estimated by the method described earlier (Decker and Welch 1990; Dinis *et al.* 1994). 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 the positive control. The results were expressed as an EC_{50} value, which represents the concentration of the sample at which 50% of metal chelation occurred.

2.8 *Cytotoxicity of mango ginger extracts*

2.8.1 *Preparation of test solutions:* For cytotoxicity studies, each extract was weighed separately, dissolved in distilled dimethyl sulphoxide (DMSO) and the volume was made up to 10 ml with DMEM, pH 7.4, supplemented with 2% inactivated NBCS (maintenance medium) to obtain a stock solution with a concentration of 1 mg/ml. This was sterilized by filtration and stored at –20°C till use. Serial twofold dilution of the extracts was prepared from the stock solution to obtain lower concentrations.

2.8.2 *Cell lines and culture medium:* Vero (normal African green monkey kidney) cell culture was procured from the National Centre for Cell Sciences (NCCS), Pune, India, and A-549 (human small cell lung carcinoma) cells from the Christian Medical College, Vellore, India. Stock cells of Vero and A-549 cell lines were cultured in DMEM medium supplemented with 10% inactivated NBCS, penicillin (100 IU/ml), streptomycin (100 μg/ml) and amphotericin B (5 μg/ml) in a humidified atmosphere of 5% CO_2 at 37°C until confluent. The cells were dissociated with TPVG solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS). The stock cultures were grown in 25 cm^2 flat bottles and all experiments were carried out in 96-well microtitre plates.

2.8.3 *Determination of mitochondrial synthesis by MTT assay:* MTT assay was done as described by Francis and Rita (1986). The monolayer cell culture was trypsinized and cell count adjusted to 1.0×10^5 cells/ml using DMEM containing 10% NBCS. To each well of the 96-well microtitre plate, 0.1 ml of the diluted cell suspension (approximately 10 000 cells) was added. After 24 h, when a partial monolayer had formed, the supernatant was flicked off, the monolayer washed once with medium and 100 μl of different concentrations of extract were added to the cells in the microtitre plates. The plates were then incubated at 37°C for 3 days in a 5% CO_2 atmosphere. Microscopic examination was carried out and observations were noted every 24 h. After 72 h, the extract solutions in the wells were discarded and 50 μl of MTT in DMEM-PR (Dulbecco modified Eagle medium without phenol red, 2 mg/ml) was added to each well. The plates were gently shaken and incubated for 3 h at 37°C in a 5% CO_2 atmosphere. The supernatant was removed, 50 μl of propanol added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 540 nm. The percentage growth inhibition was calculated and CTC_{50} (concentration of drug or test extract needed to inhibit cell growth by 50%) values were generated from the dose–response curves for each cell line. The pattern of all the cell lines as a group was used to rank compounds as toxic or non-toxic.

2.8.4 *Determination of total cell protein content by sulphorhodamine B (SRB) assay:* SRB assay was conducted as described by Philip *et al.* (1990). The monolayer cell culture was trypsinized and the cell count was adjusted to 1.0×10^5 cells/ml using DMEM containing 10% NBCS.

To each well of the 96-well microtitre plate, 0.1 ml of the diluted cell suspension (approximately 10 000 cells) was added. After 24 h, when a partial monolayer had formed, the supernatant was flicked off, the monolayer washed once with medium and 100 μl of different concentrations of extract were added to the cells in the microtitre plates. The plates were then incubated at 37°C for 3 days in a 5% CO_2 atmosphere, microscopic examination was carried out and observations were recorded every 24 h. After 72 h, 25 μl of 50% TCA was gently added to the wells in such a way that it formed a thin-layer over the extract to attain an overall concentration of 10%. The plates were incubated at 4°C for 1 h. They were flicked and washed five times with water to remove traces of the medium, extract and serum, and air-dried. They were stained with SRB (0.4% prepared in 1% acetic acid) for 30 min. The unbound dye was then removed by rapidly washing four times with 1% acetic acid. The plates were then air-dried. Tris base (10 mM, 100 μl) was then added to the wells to solubilize the dye. The plates were shaken vigorously for 5 min. The absorbance was measured using a microplate reader at a wavelength of 540 nm. The percentage growth inhibition was calculated using the same formula used for MTT assay. CTC_{50} values were calculated.

2.9 *Platelet aggregation inhibitory activity*

Blood samples were taken from healthy volunteers who had not taken any drugs during the two weeks prior to blood sampling. Blood was collected into buffered sodium citrate (3.8% w/v) pH 6.5 as the anticoagulant at a ratio of 9:1 v/v and used within 3 h of collection. Platelet-rich plasma (PRP) was obtained by centrifugation of the citrated blood at 1100 rpm for 20 min. The residual blood was again centrifuged at 2500 rpm for 20 min to obtain homologous platelet-poor plasma (PPP). Platelet count was adjusted to 1.6×10^7 platelets/μl of PRP. Aggregation was measured turbidimetrically at 37°C with constant stirring at 1000 rpm in a Chronolog dual-channel aggregometer. About 0.45 ml of PRP was continuously stirred at 1200 rpm at 37°C, and aggregation was induced by collagen (10 μM). The change in turbidity was recorded with reference to PPP using an omniscribe recorder for at least 5 min. The slope was calculated and it was used as the control.

Similarly, 100–500 μM of mango ginger extracts were added to PRP, incubated for 5 min after which collagen (10 μM) was added. Platelet aggregation was recorded using an omniscribe recorder for 5 min. The slope was calculated. The difference in the slope between the control and the sample treated was expressed as per cent inhibition of platelet aggregation by mango ginger extract.

2.10 *Statistical analysis*

The experiments were carried out in triplicate. Significant differences (P<0.05) were determined by the Duncan multiple range test (DMRT).

3. Results and discussion

3.1 *Preparation of extracts*

Mango ginger rhizome powder was extracted with hexane followed by chloroform. Extraction of 100 g of mango ginger powder yielded 11 g and 8 g of extract, respectively.

Table 1. Antibacterial activity of eight sub-fractions obtained from second-step column chromatography of the second fraction

Bacteria	Inhibition zone (mm)* exhibited by eight sub-fractions$^{\Psi}$ obtained from second-step chromatography							
	Fr. 2.1	Fr. 2. 2	Fr. 2.3	Fr. 2.4	Fr. 2.5	Fr. 2.6	Fr. 2.7	Fr. 2.8
P. aeruginosa	–	–	–	–	–	–	–	–
E. coli	–	–	–	–	–	–	–	–
S. typhi	–	–	–	–	–	13 ± 0 ^{a}	–	–
K. pneumoniae	–	–	–	16 ± 0 ^{a}	–	–	–	–
E. aerogenes	–	–	–	–	–	–	13 ± 0 ^{a}	–
P. mirabilis	–	–	–	13 ± 0 ^{a}	–	14 ± 1 ^{b}	–	13 ± 0^{a}
Y. enterocolitica	–	–	–	–	–	–	–	–
M. luteus	12 ± 0 ^{a}	11 ± 1 ^{a}	18 ± 0 ^{c}	20 ± 0 ^{d}	15 ± 0 ^{b}	–	–	–
S. aureus	11 ± 1 ^{a}	12 ± 0 ^{a}	–	–	–	14 ± 0 ^{b}	–	–
E. fecalis	–	–	13 ± 0 ^{a}	16 ± 0 ^{b}	13 ± 0 ^{a}	–	14 ± 0 ^{a}	14 ± 0^{a}
B. cereus	13 ± 0 ^{a}	13 ± 0 ^{a}	16 ± 1^{c}	18 ± 1^{d}	15 ± 1^{b}	17 ± 0^{c}	13 ± 1^{a}	
B. subtilis	13 ± 0 ^{a}	14 ± 0 ^{b}	16 ± 0 ^{c}	19 ± 1 ^{d}	14 ± 0 ^{b}	6 ± 0 ^{c}	14 ± 0 ^{b}	12 ± 0^{a}
L. monocytogenes	–	–	–	15 ± 0 ^{a}	–	–	–	

*Each value represents the mean of three different observations. Mean values with different superscripts ($^{a, b, c}$ and ^{d}) differ significantly at P<0.05.

$^{\Psi}$Eight sub-fractions (Fr. 2.1– Fr. 2.8) were obtained by second-step chromatography of the 'active fraction' (Fr. 2).

Table 2. Antibacterial activity of five sub-fractions obtained from third-step column chromatography

Bacteria	Inhibition zone (mm)* exhibited by five sub-fractions$^{\Psi}$ (Fr. 2.4.1–Fr. 2.4.5) obtained from third-step chromatography				
	Fr. 2.4.1	Fr. 2.4.2	Fr. 2.4.3	Fr. 2.4.4	Fr. 2.4.5
P. aeruginosa	17 ± 0^{a}	–	–	–	18 ± 0^{a}
E. coli	–	–	–	–	–
S. typhi	–	–	–	–	19 ± 0^{a}
K. pneumoniae	–	17 ± 0^{a}	–	16 ± 0^{a}	20 ± 0^{b}
E. aerogenes	16 ± 0^{a}	–	–	–	20 ± 0^{b}
P. mirabilis	–	–	–	13 ± 0^{a}	–
Y. enterocolitica	18 ± 0^{a}	–	–	–	17 ± 0^{a}
M. luteus	15 ± 0^{b}	12 ± 1^{a}	17 ± 0^{c}	20 ± 0^{d}	21 ± 0^{d}
S. aureus	13 ± 1^{a}	14 ± 0^{a}	–	–	15 ± 0^{b}
E. fecalis	–	–	14 ± 0^{a}	16 ± 0^{b}	14 ± 0^{a}
B. cereus	16 ± 0^{a}	15 ± 0^{a}	15 ± 1^{a}	18 ± 1^{b}	16 ± 1^{a}
B. subtilis	14 ± 0^{a}	17 ± 0^{b}	15 ± 0^{a}	19 ± 1^{c}	17 ± 0^{b}
L. monocytogenes	–	–	–	15 ± 0^{a}	20 ± 0^{b}

*Each value represents the mean of three different observations. Mean values with different superscripts ($^{a, b, c}$ and ^{d}) differ significantly at $P<0.05$.

$^{\Psi}$Five sub-fractions (Fr. 2.4.1– Fr. 2.4.5) were obtained by second-step chromatography of the 'active fraction' (Fr. 2.4).

3.2 *Isolation of antibacterial compound*

The chloroform extract was highly antibacterial against clinical isolates such as *B. cereus*, *S. pneumoniae*, *M. luteus, S. aureus, S. typhi, L. monocytogenes* and *E. fecalis* (Policegoudra *et al.* 2007a). Compared with other solvent extracts, the chloroform extract exhibited the highest antibacterial activity for a wide range of clinical isolates. It also exhibited promising lipid peroxidation inhibitory activity and moderate metal chelating activity (Policegoudra *et al.* 2007b). Thus, high antibacterial activity along with antioxidant properties of the chloroform extract prompted us to attempt isolation, purification and characterization of an antibacterial compound from this extract. Two antibacterial and antioxidant compounds were successfully isolated and characterized from the chloroform extract by Policegoudra *et al.* (2007a, b). In continuation of the above work, it was felt that isolation and characterization of potential bioactive compounds from the chloroform extract would be worthwhile.

Bioactivity-guided fractionation of the chloroform extract by repeated silica gel column chromatography yielded a pure antibacterial compound. The schematic representation of isolation of the antimicrobial compound is shown in figure 1.

3.2.1 *High performance liquid chromatography:* The purity of the isolated antibacterial compound was tested using HPLC. The isolated compound showed a single peak at 242 nm at a retention time of 3.3 min in the chromatogram.

3.3 *Characterization of isolated antimicrobial molecule*

The structure of the isolated bioactive compound was deduced by analysing UV, IR, MS and NMR spectral data. The compound exhibited UV λ maxima at 242 nm corresponding to π-π* transition, indicating the presence of double and triple bonds. The IR spectra showed alkyl stretching at 2964 cm^{-1} and carbonyl stretching at 1720 cm^{-1}, indicating the presence of C-H and C=O groups, respectively. The transmittance peaks at 2360 and 2336 cm^{-1} indicated the presence of C≡C groups. One-dimensional ^{1}H and ^{13}C NMR (table 3) spectra gave a clear indication of the structure of the compound. The spectrum indicated the presence of two methyl groups at δ 0.76 and 0.85 ppm. One methyl signal was assigned to the methyl group located at the terminal end. The compound showed the presence of many CH_2 groups in the region of δ 0.6–2.5 ppm. The signal at δ 9.40 (s) ppm indicated the presence of an aldehyde proton. The signals at δ 4.41, 4.88 and 6.7 ppm indicated the presence of an allylic olefinic group (-CH=CH-CH=CH-). The signals at δ 0.85 and 3.39 showed the presence of an ethoxy group (-HC-O-CH_2CH_3). The mass spectrum showed the M^+ ion at m/z 930 and two other ions at m/z values 686 and 187, indicating cleavage at the allylic olefininc and ethoxy groups, respectively. The corresponding ^{13}C signals were also observed in the carbon NMR spectrum. The signal at δ 193.6 is assigned to carbon (C-1) of the aldehyde group. The signals at δ 128.5 and 130.6 were assigned to carbons of the allylic olefinic group at C-17 to 20. The signals at δ 13.7 and 57.8 were assigned to the methyl carbon and C-O-C in the ethoxy group. Several methylene carbons were observed in the region of δ 14.0–42.0. In addition to these four, methine carbon signals (C-7 to C-10) were observed at δ 55.1, 56.1 and 67.9 ppm, indicating two C≡C groups. The terminal methyl carbon was observed at δ 10.6 ppm. All these spectral characters indicated that the molecule was aliphatic in nature and the structure of the compound

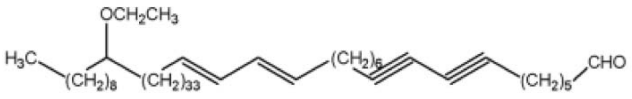

Figure 2. Structure of amadaldehyde.

Table 3. Spectral data of the isolated compound

Carbon / Proton number	Amadaldehyde	
Nucleus	^{1}H (δ)	^{13}C (δ)
1	9.40 (s)	193.6
2–6	0.6–2.5	14.0–42.0
7, 10	–	55.1, 56.1
8, 9	–	67.9
11–16	0.6-2.5	14.0–42.0
17, 20	6.73 (dd)	130.6
18,19	4.41(dt), 4.88(dt)	128.5
21–53	0.6–2.5	14.0–42.0
54	3.39 (m)	57.8
55–62	0.6–2.5	14.0–42.0
63	0.76 (d)	10.6
$O\mathbf{CH_2}CH_3$	3.39 (q,9.5)	57.8
$OCH_2\mathbf{CH_3}$	0.85 (d)	13.7

was deduced and tentatively named as amadaldehyde (figure 2).

3.4 *Antibacterial activity*

Amadaldehyde and various fractions of the chloroform extract exhibited antibacterial activity against both Gram-positive and Gram-negative bacteria. The isolated compound was more effective against *B. cereus*, *E. fecalis, S. pneumoniae, S. aureus* and *M. luteus.* It also showed activity against Gram-negative bacteria such as *P. mirabilis, S. typhi* and *E. aerogenes.* The first and second column fractions showed moderate activity against *B. cereus, S. pneumoniae, E. aerogenes* and *P. mirabilis* (tables 1 and 2). The most striking increase in activity was observed against *B. cereus*.

3.4.1 *Minimum inhibitory concentration (MIC):* The chloroform extract was very active against *B. cereus* with an MIC of 60 ppm. It also inhibited the growth of *M. luteus* and *S. aureus* at 80 ppm. *S. typhi, E. fecalis* and *L. monocytogenes* were completely inhibited at 180, 140 and 100 ppm, respectively (Policegoudra *et al.* 2007a). The MIC for amadaldehyde ranged from 100 to 180 ppm (table 4). Amadaldehyde inhibited both Gram-positive and Gram-negative bacteria. The chloroform extract was more effective than the amadaldehyde (Policegoudra *et al.* 2007a). This may be due the synergistic effect of other bioactive compounds present in the chloroform extract.

Table 4. Antibacterial activity of amadaldehyde

Bacteria	Amadaldehyde	
	MIC (ppm)*	MBC (ppm)*
P. aeruginosa	–	–
E. coli	180	–
S. typhi	160	160
K. pneumoniae	140	180
E. aerogenes	–	–
P. mirabilis	120	140
Y. enterocolitica	–	–
M. luteus	120	150
S. aureus	140	140
E. fecalis	140	–
B. cereus	100	100
B. subtilis	100	120
L. monocytogenes	–	–

*Each value represents the mean of three different observations.
– No inhibition
MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration

3.4.2 *Minimum bactericidal concentration (MBC):* MBC values reveal the concentration at which the organisms are completely killed. Amadaldehyde showed bactericidal effect against *B. cereus, B. subtilis, M. luteus, S. aureus, K. pneumoniae, P. mirabilis* and *S. typhi* at concentrations of 100 ppm, 120 ppm, 150 ppm, 140 ppm, 180 ppm, 140 ppm and 160 ppm, respectively (table 4). The other fractions obtained from the third chromatographic step also showed bactericidal activity. The mode of action of several terpenoids has been studied, but the actual structure–activity relationships of the terpenoids are not well understood. It is known that carbonylation of terpenoids increases their bacteriostatic activity but not necessarily their bactericidal activity (Naigre *et al.* 1996).

Investigations have shown that the site of action of cyclic hydrocarbons (including terpene hydrocarbons) is at the cell membrane (Sikkema *et al.* 1995). Terpenoid compounds were shown to permeabilize the membranes, making them swell. This inhibits respiratory enzymes, which leads to a partial dissipation of the pH gradient and electrical potential, which are crucial to the energy system in a cell (Sikkema *et al.* 1992; Sikkema *et al.* 1994).

3.5 *Antioxidant activity*

3.5.1 *DPPH radical scavenging activity:* Amadaldehyde showed DPPH radical scavenging activity with an IC_{50} of 170 μg (table 5). The antioxidant activity of this compound may be attributed to the presence of -OH and C=O groups, as reported in structurally similar types of compounds (Chen and Ho 1995; Nikolaos *et al.* 2003).

Table 5. Antioxidant activity of amadaldehyde

Antioxidant activity	EC_{50} value (μg)	
	Amadaldehyde	BHA
DPPH radical scavenging activity	170 ± 3^{b}	5 ± 0^{a}
Superoxide radical scavenging activity	120 ± 3^{a}	258 ± 2^{b}
Lipid peroxidation inhibitory activity	92 ± 2^{a}	94 ± 1^{a}
Metal chelating activity	158 ± 2^{a}	–

* Each value represents the mean of three different observations ± SD.
Mean values with different superscripts (^{a} and ^{b}) differ significantly at $P<0.05$.

3.5.2 *Superoxide radical scavenging activity:* Amadaldehyde showed superoxide radical scavenging activity with an IC_{50} of 120 μg (table 5). The activity was higher than that of BHA (IC_{50}=258 ± 2 μg). Superoxide radicals are generated during the normal physiological process mainly in the mitochondria. Although superoxide anion is by itself a weak oxidant, it gives rise to powerful and dangerous hydroxyl radicals as well as singlet oxygen, both of which contribute to oxidative stress (Dahl and Richardson 1978; Meyer and Isaksen 1995). Therefore, superoxide radical scavenging by antioxidants has physiological implications.

3.5.3 *Lipid peroxidation inhibitory activity:* Lipid peroxidation is a free radical-mediated propagation of oxidative insult to polyunsaturated fatty acids involving several types of free radicals. Its termination occurs in the biological system through enzymatic means or by radical scavenging activity by antioxidants (Heim *et al.* 2002). Amadaldehyde showed lipid peroxidation inhibitory activity with an IC_{50} of 92 μg (table 5). Hydroxyl radical and perferryl ion are highly reactive, and act as the actual initiating type for cellular lipid peroxidation (Fridovich 1989).

3.5.4 *Metal chelating activity:* Amadaldehyde showed metal chelating activity with an IC_{50} of 158 μg (table 5). Iron is known to generate free radicals through the Fenton and Haber–Weiss reactions (Halliwell and Gutteridge 1990). Metal ion chelating activity of an antioxidant molecule prevents oxyradical generation and consequent oxidative damage. Metal ion chelating capacity plays a significant role in the antioxidant mechanism since it reduces the concentration of the catalysing transition metal in lipid peroxidation (Duh *et al.* 1999). Hence, amadaldehyde can play a very important role as a natural antioxidant.

3.6 *Platelet aggregation inhibitory activity*

Amadaldehyde showed potential platelet aggregation inhibitory activity with an IC_{50} of 113 μg in the presence of ADP as an agonist. Platelets readily aggregate in response to a variety of endogenous substances and they can initiate thrombus formation, leading to ischaemic diseases. In addition, the interactions between platelets and blood vessel walls are important in the development of thrombosis and cardiovascular diseases (Dinerman and Mehta 1990; Hirsh 1987). Therefore, inhibition of platelet function represents a promising approach for the prevention of thrombosis.

3.7 *Cytotoxicity*

Amadaldehyde exhibited cytotoxicity against the A-549 cell line when compared with Vero cell lines with a CTC_{50} of 102 μg and 118 μg, respectively, in MTT assay. Similar results were obtained in the SRB assay, in which amadaldehyde exhibited cytotoxicity with a CTC_{50} of 110 μg and 120 μg, respectively.

4. Conclusion

Antibacterial activity-guided fractionation of a chloroform extract of mango ginger yielded a pure compound. The structure of the isolated antibacterial compound was elucidated with spectroscopic techniques and named as amadaldehyde, a novel compound. It showed bactericidal activity against a wide range of bacteria. It also showed potential antioxidant activity, cytotoxicity and platelet aggregation inhibitory activity. The bioactive properties of the compound have great potential and are worth exploring for pharmaceutical purposes.

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