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INVESTIGATION OF THE ANTIMICROBIAL, ANTIOXIDANT AND ANTI-INFLAMMATORY ACTIVITY OF COMPOUND ISOLATED FROM *MURRAYA KOENIGII*

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ABSTRACT: In the present investigation, the compound responsible for antioxidant, antimicrobial and anti-inflammatory properties in methanolic extract of leaves of *Murraya koenigii L.* was determined by Perkin- Elmer GC Claurus 500 system and Gas Chromatograph interfaced to a Mass Spectrometer GC/MS technique. GC-MS analysis of methanol extract of the leaves of the plant revealed the existence of 1-Methyl-pyrrolidine-2-carboxylic acid (69.00%), Ethyl α-d glucopyranoside (13.36%), Isolongifolene, Isolongifolene (3.68%), c-Himachalene (2.88%), 1,2-Ethanediol, monoacetate (2.79%), 1,2-Benzenedicarboxylic acid, di-isooctyl ester (2.55%). The pure compounds were separated using a Shimadzo LC 2010 HPLC system (Kyoto, Japan), equipped with a Shimadzo LC 2010 UV-VIS detector with a thermostated flow cell and a selectable two wavelengths of 190 - 370 nm or 371–600nm. These were further screened for their antimicrobial, antioxidant and anti-inflammatory properties. All the compounds possessed some or the other activity. It was found that the compound 9, 12 octadecadienoic acid having the retention time 18.81 and the peak area 0.60 % had potent antioxidant, antimicrobial and anti-inflammatory properties. The compound showed potent antimicrobial activity against *Bacillus subtilis*, *E.coli*, *Proteus vulgaris*, *Salmonella typhimurium*, *Staphylococcus aureus*, *Candida albicans*, *Saccharomyces cerevisae*, *Aspergillus niger* and *Penicillium* notatum at MIC value from 0.05-0.56 µg/ml. The compound showed less activity against *Pseudomonas aeruginosa* in comparison to other pathogens. The compound possessed to have strong antioxidant activity with IC_{50} value of 45.65 µg/ml as measured by DPPH assay. The compound possessed 85 % reduction in paw edema at a dose of 150 µg/ml in reference to standard anti-inflammatory drug, aspirin which showed 68.62 % reduction. The compound was further assayed for cellular toxicity to fresh sheep erythrocytes and found to have no cellular toxicity.
Key words: *Murraya koenigii*, methanol extract, compound 9, 12 octadecadienoic acid.

INTRODUCTION

In developing countries and particularly in India low income people such as farmers, people of small isolated villages and native communities use folk medicine for the treatment of common infections (Fabricant, 2001). These plants are ingested as decoctions, teas and juice preparations to treat respiratory infection. They are also made into a poultice and applied directly on the infected wounds or burns. Traditional healers claim that some medicinal plants are more efficient to treat infectious diseases than synthetic antibiotics. It is necessary to evaluate, in a scientific base, the potential use of folk medicine for the treatment of infectious diseases produced by common pathogens. Medicinal plants might represent an alternative treatment in non-severe cases of infectious diseases (Gonzalez, 1980). The country like India is blessed with the natural medicines which are plant derived and these plant medicines are cheap, no side effects and are found to be effective in various resistant pathogenic microorganisms. These are also the good source of antioxidant and also cure various disorders associated with inflammation. Latest and previous studies have concluded the beneficial aspects of plant derived drugs as good source of antibiotics, antioxidants and anti-inflammatory agents (Mathur, et al., 2010; 2011).

MATERIALS AND METHODS

All the chemicals and reagents used were from C.D.H and Ranchem. Glass wares used were from Borosil. The media and broth used for microbial culture were from Hi-Media Pvt. Limited, Bombay, India.

Plant material

The authenticated sample of *Murraya koenigii* was collected from local gardens of Dehradun (U.K), India and was further confirmed in Botanical Survey of India (BSI), Dehradun. Voucher specimens have been deposited in BSI, Dehradun, India.

Preparation of plant extracts

The method (Alade and Irobi, 1993) was adopted for preparation of plant extracts with little modifications. Briefly four 20 g portions of the powdered plant material were soaked separately in 100 ml of methanol for 72 h. The mixture was stirred after every 24 h using a sterile glass rod. At the end of extraction, each extract was passed through Whatmann filter paper no1 (Whatmann, England). The filtrate obtained were concentrated in vacuo using rotary evaporator at 30^{o}C.

Phytochemical screening of the extract

The portion of the dry extract was subjected to the Phytochemical screening using the method adopted by Trease and Evans (1983) and Harbourne (1983). Phytochemical screening was performed to test for alkaloids, saponin, tannins, flavanoids, steroids, sugars and cardiac glycosides.

Test for alkaloids

The 0.5 g of the plant extract was dissolved in 5 ml of 1% HCl and was kept in water bath for about 2 minutes. 1ml of the filtrate was treated with Dragendroff's reagent Turbidity or precipitation was taken as indicator for the presence of alkaloids.

Test for Tannins

About 0.5 g of the sample was dissolved in 10 ml of boiling water and was filtered. Few ml of 6% $fecl_3$ was added to the filtrate. Deep green colour appeared confirmed the presence of Tannins (Trease and Evans, 1989).

Test for Flavanoids

About 0.2 gm of the extract was dissolved in methanol and heated for some time. A chip of mg metal was introduced followed by the addition of few drops of conc. HCl. Appearance of red or orange color was Indicator of the flavanoids.

Test for Saponin

About 0.5 g of the plant extract was stirred with water in the test tube. Frothing persists on warming was taken as a evidence for the presence of saponin.

Test for Steroids

Salkowaski method was adopted for the detection of steroids. About 0.5 g of extract was dissolved in 3 ml of chloroform & filtered. To the filtrate, conc. $H_2 SO_4$ was added to form a lower layer. Reddish brown color was taken as positive for the presence of steroids ring.

Test for Cardiac glycoside

About 0.5 g of the extract was dissolved in 2ml of glacial acetic acid containing 1 drop of 1% $Fecl_3$. This was under laid with conc. $H_2 SO_4$. A brown ring obtained at the interphase indicates the presence of deoxy sugar. A violet ring appeared below the ring while in the acetic acid layer a greenish ring appeared just above ring and gradually spread throughout this layer.

Test for reducing Sugars

1ml each of Fehling's solutions, I and II was added to 2 ml of the aqueous solution of the extract. The mixture was heated in a boiling water bath for about 2-5 minutes. The production of a brick red precipitate indicated the presence of reducing sugars.

Structure elucidation of isolated compounds by combination of different techniques

Gas Chromatography–Mass Spectrometry (GC/MS) analysis

GC/MS analysis of some of the potent volatile constituents present in the extract(s) was performed at Roorkee Research and Analytical Laboratory Pvt. Ltd., Roorkee (Uttarakhand), India using a Perkin Elmer GC Claurus 500 system and Gas Chromatograph interfaced to a Mass Spectrometer (GC/MS) equipped with Elite-1 fused silica capillary column (30 m × 0.25 mm) composed of 100% Dimethyl poly siloxane) from Perkin Co., Germany. For GC/MS detection, an electron ionization system with ionization energy of 70 eV was used. Helium gas (99.999%) was used as the carrier gas at a constant flow rate of 1 ml/min and an injection volume of 2 µl was employed (split ratio of 10:1). Injector temperature 250°C; Ion-source temperature 280°C. The oven temperature was programmed from 110°C (isothermal for 2 minutes), with an increase of 10°C/min, to 200°C, then 5°C/min to 280°C, ending with a 9 minutes isothermal at 280°C. Mass spectra were taken at 70 eV; a scan interval of 0.5 seconds and fragments from 45 to 450 Da. Total GC running time was 36 minutes. The relative percentage amount of each component was calculated by comparing its average peak area to the total areas. Software adopted to handle mass spectra and chromatograms was a Turbo Mass Ver5.2.0

High-performance liquid chromatography (HPLC)

HPLC analysis of the pure compound was performed in Roorkee Research and Analytical Laboratory Pvt. Ltd., Roorkee (Uttarakhand), India using a Shimadzo LC- 2010 HPLC system (Kyoto, Japan), equipped with a Shimadzo LC 2010 UV-VIS detector with a thermostated flow cell and a selectable two wavelengths of 190 - 370 nm or 371–600nm. The detector signal was recorded on a Shimadzo LC2010 integrator. The column used was a C-18 block heating-type Shim-pack VP-ODS (4.6 mm interior diameter × 150 mm long) with a particle size of 5 µm. Mobile phase was designed as per the nature of the compound, containing 50 % acetonitrile along with 50 % Phosphate buffer was used at a flow rate of 3.0 ml/min, column temperature 25°C. Injection volume was 40 µl and detection was carried out at specific wavelength having maximum absorbance as calculated by UV absorption spectra at maximum wavelength.

Determination of Antibacterial and Antifungal activity

Culture Media

The media used for antibacterial test was Soyabean casein digest agar/broth and Sabouraud's dextrose agar/broth of Hi media Pvt. Bombay, India.

Inoculum

The bacteria were inoculated into soyabean casein digest agar/broth and incubated at 37^0 C for 4 h and the suspension were checked to provide approximately 10^5 CFU/ml. Similar procedure is done for fungal strains by inoculating in Sabouraud's dextrose broth for 6 h.

Microorganisms used

The test organisms (*Bacillus subtilis* ATCC 6051, *Proteus vulgaris* ATCC 6380, *Salmonella typhimurium* ATCC 23564, *Pseudomonas aeruginosa* ATCC 25619, *Escherichia coli* K-12, *Staphylococcus aureus,* were the bacterial strains obtained from institute of Microbial Technology (IMTECH) Chandigarh, India. The fungal test organisms used for study are *Candida albicans, Aspergillus niger, Saccharomyces cerevisae* and *Penicillium notatum* obtained from pure lab cultures of Roorkee Research & Analytical Laboratories, Roorkee (U.K), India.

Determination of antimicrobial activity

The agar well diffusion method (Perez, et al., 1990) was modified. Soyabean casein digest agar (SCDA) was used for bacterial cultures. The culture medium was inoculated with the microorganism separately suspended in soyabean casein digest broth. Sabouraud's dextrose agar (SDA) was used for fungal cultures. The culture medium was inoculated with the fungal strains separately suspended in Sabourauds dextrose broth. A total of 8 mm diameter wells were punched into the agar and filled with pure compound 9, 12 octadecadienoic acid (1000 ppm) dissolved in 10 % DMSO. Standard antibiotic (Chloramphenicol, concentration 1mg/ml) was simultaneously used as positive control. The bacterial plates were then incubated at 37ºC for 18 h. The antibacterial activity was evaluated by measuring the diameter of zone of inhibition observed. The same procedure was done for determining antifungal activity but in this case standard antibiotic (Fucanazole, concentration 1 mg/ml) was used as positive control and fungal plates were incubated at 37ºC for 72 h. Here also the diameter of zone of inhibition observed was measured.

Determination of MIC and MBC

The compound was then after evaluated to determine MIC and MBC values by broth dilution technique by using N-saline. The tubes were then after incubated for 48 h. The minimum dilution of the compound that kills the bacterial and fungal growth was taken as MLC (Minimum lethal count) while the minimum dilution of the compound that inhibits the growth of the organism was taken as MIC.

Determination of cellular toxicity using sheep erythrocytes

The method (Ursula and Xian-guo, 1994) was employed to study cellular toxicity. Briefly 10 fold serial dilution of the compound were made in phosphate buffered saline. A total volume of 0.8ml for each dilution was placed in an ependroff tube. A negative control tube (containing saline only) and a positive control tube (containing tap water) were also included in the analysis. Fresh sheep erythrocytes were added to each tube, to give a final volume of 1 ml. Solutions were incubated at 37ºC for 30 minutes and all tubes were centrifuged for 5 minutes and then observed for hemolysis.

Determination of *in vitro* antioxidant activity

Determination of Antioxidant Activity by DPPH Radical Scavenging Method

The compound solution for the DPPH test (Fargare, 1995) was prepared by re-dissolving 0.2 g of the pure compound in 10 ml methanol. The concentration of DPPH solution was 0.025 g in 1000 ml of methanol. Two ml of the DPPH solution was mixed with 10 µl of compound solution and transferred to a cuvette. The reaction solution was monitored at 515 nm, after an incubation period of 30 minutes at room temperature, using a UV-Visible Systronics spectrophotometer. The inhibition percentage of the absorbance of DPPH solution was calculated using the following equation:

Inhibition%= (Abst=0 min -Abst=30 min)/ Abst=0 min ×100

Where Abst=0 min was the absorbance of DPPH at zero time and Abst=30 min was the absorbance of DPPH after 30 minutes of incubation. Ascorbic acid (0.5 mM) was dissolved in methanol and used as a standard to convert the inhibition capability of plant extract solution to the Ascorbic acid equivalent. IC_{50} is the concentration of the sample required to scavenge 50% of DPPH free radicals.

Determination of *in vivo* anti-inflammatory activity

Animals

Male albino rats (180–200 g) were used taking into account international principles and local regulations concerning the care and use of laboratory animals (Olfert, et al., 1993). The animals had free access to a standard commercial diet and water *ad libitum* and were kept in rooms maintained at 22 ± 1ºC with a 12-h light/dark cycle. The institutional animal ethical committee has approved the protocol of the study.

Carrageenan-induced edema in rats

3 Groups of five animals each were used. Paw swelling was induced by injection of 0.1 ml 1% sterile carrageenan in saline into the right hind paw. The compound solublized in 10 % DMSO was subjected at a dose of 1000 ppm were administered orally 60 minutes before carrageenan injection. Aspirin (10 mg/kg) was used as reference drug. Control group received the vehicle only (10 ml/kg). The inflammation was quantified by measuring the volume displaced by the paw, using a plethysmometer at time 0, 1, 2, 3, and 4 h after carrageenan injection.

Statistical analysis

The results were expressed as mean ± S.D. Statistical significance was determined by analysis of variance and subsequently followed by Turkey's tests. P values less than 0.05 were considered as indicative of significance. The analysis was performed using INSTAT statistical software.

RESULTS AND DISCUSSION

Phytochemical screening

Different conventional methods were followed to determine qualitatively the presence of phytochemical constituents present in the potent extract. The methanol extract possessed all the active constituents except flavanoids. The results are indicated in Table 1.

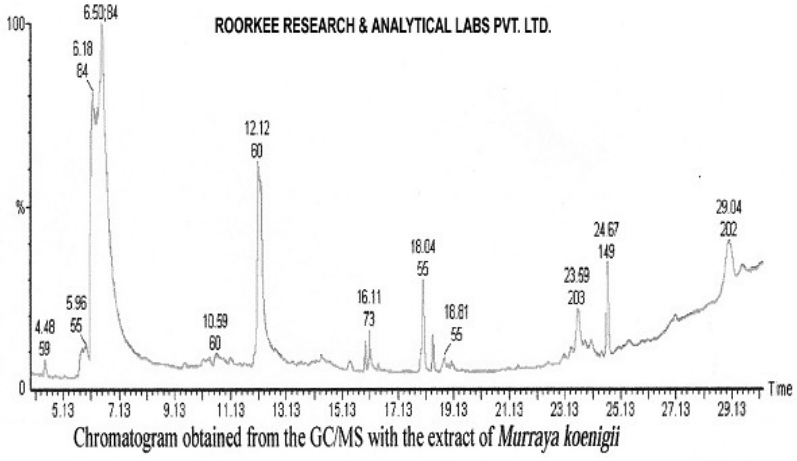
Table 1: Phytochemical Investigation of active constituents in potent extract of the plant

Plant	Phytochemical constituents					
	Alkaloids	Flavanoids	Tannins	Steroids	Saponin	Glycosides
Murraya koenigii	+	-	+	+ +	+	+

+, present; -, absent; ++, prominent

Gas Chromatography–Mass Spectrometry (GC/MS) analysis

The peak area of the compound 9, 12 octadecadienoic acid identified was found to be 0.60; the retention time of the compound 9, 12 octadecadienoic acid was found to be 18.81 minutes and the molecular weight was found to be 280 as recorded by GC-MS chromatogram. The results are indicated in Figure 1.



Chromatogram obtained from the GC/MS with the extract of *Murraya koenigii*

Figure 1: GC-MS chromatogram of extract of *Murraya koenigii*

HPLC analysis

The HPLC chromatogram was recorded of the pure compound. The pure compound 9, 12 octadecadienoic acid showed the retention time 5.287 minutes in reference to that of standard. The results are recorded in Figure 2.

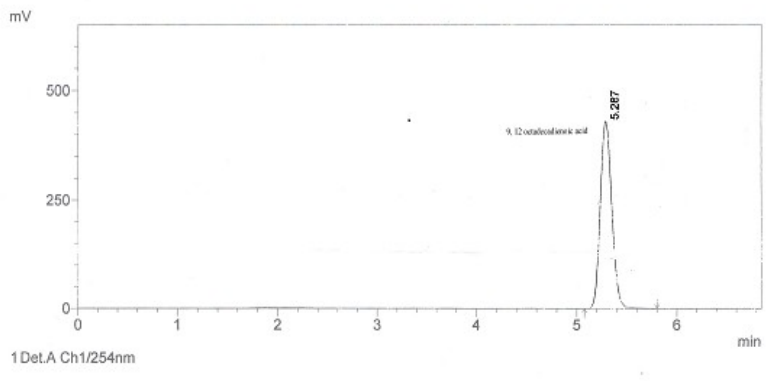


Figure 2: HPLC chromatogram of isolated compound

Determination of antimicrobial activity

The antimicrobial activity of the compound 9, 12 octadecadienoic acid isolated was determined by measuring the diameter of zone of inhibition recorded. The compound was found to be most potent antimicrobial agent. The initial screening of antimicrobial activity of compound at different concentration 1000 ppm was assayed *in vitro* by the agar diffusion method against all tested bacterial and fungal strains (Table 2 and Figure 3). The highest inhibitory effect of the compound was observed against *Bacillus subtilis* (zone of inhibition: 25.0 mm) followed by *E.coli* K-12, *Proteus vulgaris*, *Salmonella typhimurium,* (diameter of zone of inhibition: 23.0 mm). The compound showed moderate activity against *Pseudomonas aeruginosa* and *Staphylococcus aureus* (diameter of zone of inhibition: 19.0 mm) bacterial strains. The compound was found to be potent against *Candida albicans*, *Aspergillus niger* and *Penicillium notatum* (diameter of zone of inhibition: 25.0 mm) fungal strains but showed moderate activity activity against *Saccharomyces cerevisae* (diameter of zone of inhibition: 18.0 mm). In view of the results obtained by the well diffusion method, the minimal inhibitory concentration MIC of methanol extract was determined by broth micro dilution assay.

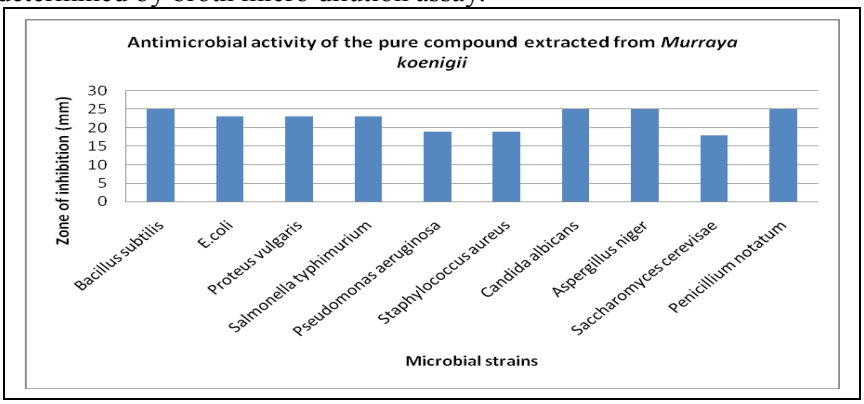


Figure 3: Antimicrobial activity of the compound against microorganisms

Table 2: Diameter of zone of inhibition (mm) of compound 9, 12 octadecadienoic acid of *Murraya koenigii*

Diameter of zone of inhibition (mm)

Microorganisms	Control			
	***Compound* 9, 12 octadecadienoic acid (1000 ppm)**	**MIC (µg/ml)**	**C**	**F**
Bacillus subtilis	25.0	0.07	25.8	----
E.coli	23.0	0.18	35.0	----
Proteus vulgaris	23.0	0.18	26.0	----
Salmonella typhimurium	23.0	0.18	27.8	----
Pseudomonas aeruginosa	19.0	0.53	25.0	----
Staphylococcus aureus	19.0	0.56	27.0	----
Candida albicans	25.0	0.05	-----	24.0
Aspergillus niger	25.0	0.05	-----	23.0
Saccharomyces cerevisae	18.0	0.17	-----	25.0
Penicillium notatum	25.0	0.05	-----	27.0

C, Chloramphenicol; F, Fucanazole (1mg ml^{-1}); -, Not tested

Determination of cellular toxicity using sheep erythrocytes

The compound showed no cellular toxicity as no hemolysis was observed.

Determination of *in vitro* antioxidant activity

Determination of Antioxidant Activity by DPPH Radical Scavenging Method

IC_{50}values of compound 9, 12 octadecadienoic acid were found to be 45.65 µg/ml in comparison to Ascorbic acid which showed IC_{50} value of 78.17 µg/ml.

Determination of *in vivo* anti-inflammatory activity

The anti-inflammatory effects of the compound 9, 12 octadecadienoic acid on carrageenan-induced edema in rat's hind paws are presented in Table 3**.** The anti-inflammatory activity of compound was found to have effect in dose-dependent manner. There was a gradual increase in edema paw volume of rats in the control group. However, in the test groups, the compound (150 µg/kg) showed a significant reduction in the edema paw volume. There was no reduction in inflammation found in case of rats treated with 10 % DMSO extracts. The results showed that the compound causes significant reduction in inflammation i.e. 85 % (150 µg/kg) compared to standard anti-inflammatory drug aspirin i.e. 68.62% (25 mg/kg).

Table 3: Anti-inflammatory activities of compound extracted from *Murraya koenigii*

Paw volume (ml) ± SD

Experiment	Control (10% DMSO)	Aspirin (25mg/kg orally)	Compound (150 µg/kg)
1h after treatment	0.25±0.003	0.21± 0.003	0.23± 0.003
2h after treatment	0.25±0.003	0.18± 0.003	0.20± 0.003
4h after treatment	0.25±0.003	0.16± 0.002	0.10± 0.002

±, S.D, Standard Deviation

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