Analysis and antimicrobial activity of essential oil of the leaves of *Cymbopogon flexuosus* (Nees ex Steud.) W. Watson

Vijender Singh, Mohammed Ali and Shahnaz Sultana

Abstract
*Cymbopogon flexuosus* (Nees ex Steud.) W. Watson (Poaceae), known as Lemongrass, yields an essential oil which is used to treat headache, abdominal and muscle pains, stress, anxiety, irritability, insomnia, drowsiness and hair loss. Hydro-distillation of the fresh leaves (1.5 kg) gave a pale yellow essential oil (0.5%). The GLC and GC-MS analysis of the oil indicated that it was composed of large amount of monoterpenes (93.4%). Out of the thirteen monoterpenes, there were five alcohols (54.4%), four aldehydes (31.7%), one ester (0.7%) and three hydrocarbons (6.7%). The predominant monoterpenes characterized were geraniol (51.7%), geranial (29.0%), α-pinene (4.8%), citronellal (1.3%), citronellol (1.2%), myrcene (1.1%) and trans-verbenol (1.0%). Among four identified sesquiterpenes (6.5%), spathulenol (2.9%) and β-selinene (2.0%) were the main constituents. The significant antimicrobial activities were observed with the essential oil and ethanolic extract of the leaves against *Staphylococcus aureus*, *Escherichia coli*, *Candida albicans* and *Aspergillus niger*.

Keywords: *Cymbopogon flexuosus*, leaves, essential oil, geraniol, geranial, antimicrobial activity

1. Introduction
*Cymbopogon flexuosus* (Nees ex Steud.) W. Watson; syn. *C. travancorensis* Bor; *Andropogon flexuosus* Nees ex Steud.; *A. ampliflorus* Steud. (Poaceae), known as Cochinchin grass, Lemongrass or Malabar grass, is a perennial grass native to India, Sri Lanka, Burma and Thailand. It was introduced in India about a century back and is now commercially cultivated along western Ghats of Maharashtra and Kerala, in Karnataka, Tamil Nadu, foot-hills of Arunachal Pradesh and Sikkim. Apart from India, lemongrass is also cultivated on large scale in Australia, Thailand, Europe, Mexico, Dominica, Haiti, Madagascar, Indonesia and China [1]. Lemongrass is a tall, perennial sedge with dense fascicles of leaves from a short rhizome; culm stout, erect, up to 1.8 meter high; leaves long, glaucous, green, linear tapering upwards and along the margins; ligule very short; sheaths terete, those of the barren shoots widened and tightly clasping at the base, others narrow and separating, with flowering inflorescence in a long spike.

Lemongrass is used for treating digestive tract spasms, stomach ache, hypertension, convulsions, pain, cough, rheumatism, period cramps, ringworm, athlete's foot, toothache, migraine, fever, common cold, vomiting, exhaustion, to kill germs and as a mild astringent. The plant and its essential oil are applied to the skin to relieve headache and abdominal and muscle pains. The oil is inhaled in aromatherapy to alleviate muscle ache. It works as an air freshener and deodorizer, especially when blended with other essential oils like geranium or bergamot. It repels insects such as mosquitoes and ants due to its high citral and geraniol contents. Its aroma helps to relieve stress, anxiety, irritability and insomnia and to prevent drowsiness and hair loss. Lemon grass oil has a strong lemon-like odour due to high percentage of citral and is used for scenting of soaps, deodorants, shampoos, lotions, tonics, detergents and insect repellent preparations. Citral is utilized in perfumery, cosmetics, beverages and is a starting material for manufacture of ionones, which produce vitamin A. The citral rich oil has germicidal, medicinal and flavouring properties. Lemongrass oil is analgesic, antiseptic, carminative, astringent, febrifuge, fungicidal, bactericidal and antidepressant [10].

The main reported components of lemongrass oil were limonene, myrcene, citral, citronellal, citronellol, terpineol, methyl heptenone, dipentene, geraniol, geranial, geranyl acetate, nerol, neral and farnesol [1-3]. These compounds possess antifungal, antiseptic, insecticidal and counterirritant properties.
Citral is antiseptic and suppresses the growth of bacteria and fungi [4, 6]. Lemongrass oil's quality is generally determined by its citral content. The essential oil components are greatly influenced by genetic, environmental and geographical conditions [1, 7-9]. The aim of this study was to analyze the chemical composition and to evaluate antimicrobial activity of the essential oil and ethanolic extract of the leaves of *Cymbopogon flexuosus* collected from the western Uttar Pradesh state of India.

2. Material and Methods
2.1 Plant Material
The fresh leaves of *C. flexuosus* were collected from Neha nursery, Greater Noida, western Uttar Pradesh. The plant material was identified by Prof. M.P. Sharma, Department of Botany, Jamia Hamdard. A voucher specimen is preserved in the Phytochemistry Research Laboratory, Jamia Hamdard, New Delhi.

2.2 Preparation of Alcoholic Extract
A coarse powder of the dried leaves (10 g) of *C. flexuosus* was extracted with ethanol (35 ml) in a Soxhlet apparatus exhaustively. The ethanolic extract was dried under reduced pressure to get dark brown semisolid mass (1.1 g). It was stored at 4 °C prior to testing.

2.3 Isolation of Essential Oil
The chopped fresh leaves (1.5 kg) were added to deionized water (1.5 L) and subjected to hydro-distillation in a Clevenger apparatus for 4 h in accordance with the British Pharmacopoeia specification [10]. The essential oil was evaporated together with water vapour and finally collected in a condenser. The upper phase that contained the essential oil was separated from the lower one. The yield of essential oil obtained was 0.5% v/w. The pale yellow essential oil was collected in a graduated tube. It was dried over anhydrous sodium sulphate and stored at 4 °C in the dark until analysis.

2.4 GC Analysis
Analytical GC was carried out on a Varion 3300 Gas Chromatograph fitted with a silicone DB-1 capillary column (30 m x 0.25 mm i.d.), film thickness 0.25 μm, carrier gas nitrogen, flow rate 1.5 ml/min, split mode ratio was 1:25. The oven temperature was programmed from 50 °C (after 2 min) to 240 °C at 5 °C/min with column pressure of 155.1 kPa and the final temperature was held for 10 min. Injector and detector temperatures were 250 °C and 270 °C, respectively. The GC instrument was equipped with a flame ionization detector (FID). An aliquot (0.5 μL of the diluted oil) was injected into the GC. Component separation was achieved following a linear temperature programmed from 60 – 230 °C at a rate of 3 °C/min and then held at 230 °C for 9 min, with a total run time of 55.14 min. Percentage of the constituents were calculated by electronic integration of FID peak areas. A homologous series of n-alkanes were run under the same conditions for determination of retention indices.

2.5 GC-MS Analysis
GC-MS analysis of the oils was performed on a Hewlett Packard HP 6890 Gas Chromatography interfaced with Hewlett Packard 5973 mass spectrometer system equipped with a Ulbon HR-1 capillary column (30 m x 0.25 mm id, film thickness 0.25 μm). The oven temperature was programmed from 70- 240 °C at the rate of 5 °C/min. Oven temperature was programmed as 50 °C for 1 min and subsequently held isothermal for 2 min. injector port: 250 °C, detector: 280 °C, split ratio 1:50, volume injected: 1μL of the oil. The recording was performed at 70 eV, scan time was 1.5; mass range 40-750 amu. Software adopted to handle mass spectra and chromatograph was a Chem station. Helium was used as a carrier gas at a flow rate of 1 mL/min. Scanning range was 35 to 425 amu. An amount of 1.0 μL of the diluted oil in hexane was injected into the GC/MS instrument.

2.6 Identification of Components
The individual essential oil components were identified by comparing their retention indices (RI) either with those of authentic compounds available in author’s laboratory or with those stored in the spectrometer database of NBS 54 K.L, Wiley L-built libraries and with those published in the literature [12,13].

2.7 Antimicrobial Activity
The antimicrobial activities of essential oil and dried alcoholic extract of *C. flexuosus* fresh leaves were performed in the Department of Microbiology, R V Northland Institute, Greater Noida. The identification of microbial strains was based on morphological, cultural and biochemical tests. *Staphylococcus aureus*, *Escherichia coli*, *Candida albicans* and *Aspergillus niger* were used to evaluate antimicrobial activity of the essential oil. Identification of each culture was done by conventional methods. Pure chloramphenicol and ketoconazole (Ranbaxy Ltd., Gurgaon) 50 mcg/ml were used as standards for comparison of antimicrobial activities.

2.8 Media
Dehydrated nutrient agar media was prepared in distilled deionized water. The media (g/100 ml) was composed of peptone (5.1 g), sodium chloride (5.0 g), beef extract (1.5 g), yeast extract (1.5 g) and agar (1.5 g).

2.9 Preparation of Media
All media were prepared in distilled water by dissolving agar (15 g), peptone (5 g), beef extract (1.5 g), dextrose (1 g), sodium chloride (3.5 g), dipotassium-hydrogen phosphate (3.68 g) and potassium dihydrogen phosphate (1.32 g), and pH of the media was adjusted between 6.95-7.05. Dehydrated nutrient agar medium (28 g) was accurately weighed and suspended in 1000 ml of distilled water in a conical flask. It was heated on a water bath to dissolve the medium completely. The conical flask containing the nutrient agar medium was plugged with the help of a non-absorbent cotton plug. Direct heating was avoided as it may lead to charring of the medium components and render it useless for the purpose.

2.10 Sterilization of Media
The conical flask containing the nutrient agar medium was plugged with the help of a non-absorbent cotton plug. The mouth of the conical flask and the cotton bung were properly covered with an aluminium foil. The medium was then sterilized at 121 °C by autoclaving at 15 lbs/in² pressure for 30 minutes.

2.11 Preparation of Standard Solution
For the preparation of standard solution, pure chloramphenicol and ketoconazole were dissolved in dimethyl sulphoxide for comparison of antibacterial and antifungal activities, respectively. The concentration of both standard drug solutions was 10 mg./ ml.
2.12 Preparation of Test Solutions
The essential oil (0.1% v/v, 0.5% v/v, 1% v/v) and dried alcoholic extract (5.0% w/w) were dissolved in dimethyl sulfoxide (DMSO) for anti-microbial activity.

2.13 Preparation of Organisms
The test organisms were maintained on slants of medium and transferred to a fresh slant once a week. The slants were incubated at 37°C for 24 hours. Using 3 ml of saline solution, the organisms were washed from the agar slant on to a large agar surface (medium) and incubated for 24 hours at 37±2 °C. The growth from the nutrient surface was washed using 50 ml of distilled water. A dilution factor was determined which gave 25% light transmission at 530 nm. The amount of suspension to be added to each 100 ml agar or nutrient broth was determined by use of test plates or test broth. The test organisms were stored under refrigeration. The anonomic bacteria were cultured as mentioned in the literature.[14]

2.14 Temperature Control
Thermostatic control is required in several stages of a microbial assay when culturing a micro-organism and preparing its inoculums and during inoculation in a plate assay.

2.15 Cup and Plate Method
The antibacterial activities of the ethanolic extract and leaf essential oil were studied against four microorganisms, viz., Staphylococcus aureus, Escherichia coli, Candida albicans and Aspergillus niger. The dried ethanolic extract was dissolved in ethanol. Pure chloramphenicol and ketoconazole, 50 c mg/ml each, were used as standard for comparison of antimicrobial activity. The antimicrobial activities were screened by the agar well diffusion method. Nutrient agar plates were swabbed with the respective broth culture of the organisms and kept for 15 minutes in laminar chamber for absorption to take place. Wells were made in agar plates using a sterile cork borer and 10 µl of different concentrations of extracts were added to different wells. Excess amounts of the suspensions were drained and discarded properly with the help of a filter paper strip. Wells of 6 mm in diameter and 2 cm apart were pricked in the culture media using sterile cork borer. Equal volumes of the test samples and standards were administered to fullness in each well. Micropipette was used to deliver the solutions into the holes. The plates were then left for standing for 1 h for proper diffusion of the drug solutions. The plates were incubated at 37±2 °C for bacteria and 25 ± 2 °C for fungus for 24 hours. The diameters of the zone of inhibitions were measured accurately in millimeter. The tests were performed in triplicate. The observations are tabulated in Table – 2.

3. Results
Hydro-distillation of the leaves of C. flexuosus yielded 0.5% pale yellowish essential oils. The composition of the oil is tabulated in Table 1. The constituents are listed in order of their elution on the ULbon HR-1 column. A total 16 components were identified. The significant antimicrobial activities were observed with the essential oil and ethanolic extract of the leaves against S. aureus, E. coli, C. albicans and A. niger. The dried ethanolic extract was dissolved in ethanol. The zone of inhibitions of the microorganisms were compared with standard samples of chloramphenicol and ketoconazole (Table 2).

4. Discussion
The components of the oil, the percentage of each constituent and their RI values are summarised in Table -1. The constituents are arranged in order to GLC and GC-MS elution on silicon DB-1 and ULbon HR-1 fused silica columns, respectively. Analysis of the oil by GC-MS resulted in the identification of eighteen components. The oil was composed of large amount of monoterpenes (93.4%). Out of the thirteen monoterpenes, there were five alcohols (54.4%), four aldehydes (31.7%), one ester (0.7%) and three hydrocarbons (6.7%). The predominant monoterpenene characterized was geraniol (51.7%) followed by geranial (29.0%), α-pinene (4.8%), citronellol (1.3%), citronellol (1.2%), myrcene (1.1%) and trans-verbenol (1.0%). Among four identified sesquiterpenes (6.5%), there were two hydrocarbons (2.2%) and two alcohols (3.7%) in which spathulenol (2.9%) was the predominant sesquiterpene followed by β-selinene (2.0%), (E,E)-farnesol (0.8%) and β-caryophyllene (0.2%). There was one benzoic ester derivative occurring in trace amount (0.1%). Antimicrobial activities of dried alcoholic extract and different concentrations of the essential oil of C. flexuosus fresh leaves from Greater Noida, U.P. were summarized in Table 2. The maximum antibacterial activity was shown with 1% v/v of the volatile oil on S. aureus (15.3 mm) followed by E. coli (12.8 mm), and maximum antifungal activity was shown by 1% v/v of the volatile oil against C. albicans (12.5 mm) and A. niger (10.2 mm).

5. Conclusion
The leaf essential oil of C. flexuosus was composed prominently of monoterpenes (93.4%) including geraniol (51.7%), geranial (29.0%), α-pinene (4.8%), citronellol (1.3%), citronellol (1.2%), myrcene (1.1%) and trans-verbenol (1.0%). The main sesquiterpenes (6.5%) were spathulenol (2.9%) and β-selinene (2.0%). The leaf essential oil and ethanolic extract exhibited antimicrobial activity against Staphylococcus aureus (15.3 mm), Escherichia coli (12.8 mm), Candida albicans (12.5 mm) and Aspergillus niger (10.2 mm).

6. Acknowledgments
The authors are thankful to the Head, Sophisticated Analytical Instrumentation Facility, Central Drug Research Institute (CSIR), Lucknow for recording GC-MS analysis of the essential oil.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Components</th>
<th>RI</th>
<th>% Area (GC-MS)</th>
<th>Method</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>α-Pinene</td>
<td>925</td>
<td>4.8</td>
<td>A,B</td>
</tr>
<tr>
<td>2</td>
<td>Myrcene</td>
<td>991</td>
<td>1.1</td>
<td>A,B</td>
</tr>
<tr>
<td>3</td>
<td>Limonene</td>
<td>1031</td>
<td>0.8</td>
<td>A</td>
</tr>
<tr>
<td>4</td>
<td>Citronellal</td>
<td>1153</td>
<td>1.3</td>
<td>B</td>
</tr>
<tr>
<td>5</td>
<td>trans-Verbenol</td>
<td>1162</td>
<td>1.0</td>
<td>B</td>
</tr>
<tr>
<td>6</td>
<td>Terpene-4-ol</td>
<td>1105</td>
<td>0.5</td>
<td>B</td>
</tr>
<tr>
<td>7</td>
<td>α-Terpineol</td>
<td>1109</td>
<td>0.1</td>
<td>B</td>
</tr>
<tr>
<td>8</td>
<td>Citronellol</td>
<td>1228</td>
<td>1.2</td>
<td>A,B</td>
</tr>
<tr>
<td>9</td>
<td>Geranial</td>
<td>1237</td>
<td>51.7</td>
<td>A,B</td>
</tr>
<tr>
<td>10</td>
<td>Neral</td>
<td>1240</td>
<td>0.6</td>
<td>A,B</td>
</tr>
<tr>
<td>11</td>
<td>Citral</td>
<td>1242</td>
<td>0.8</td>
<td>B</td>
</tr>
<tr>
<td>12</td>
<td>Geranial</td>
<td>1260</td>
<td>29.0</td>
<td>A,B</td>
</tr>
<tr>
<td>13</td>
<td>Geranyl acetate</td>
<td>1383</td>
<td>0.7</td>
<td>B</td>
</tr>
<tr>
<td>14</td>
<td>β-Caryophyllene</td>
<td>1405</td>
<td>0.2</td>
<td>B</td>
</tr>
<tr>
<td>15</td>
<td>2-Methyl propyl benzoate</td>
<td>1410</td>
<td>0.6</td>
<td>A,B</td>
</tr>
<tr>
<td>16</td>
<td>Spathulenol</td>
<td>1436</td>
<td>2.9</td>
<td>B</td>
</tr>
<tr>
<td>17</td>
<td>β-Selinene</td>
<td>1449</td>
<td>2.0</td>
<td>A,B</td>
</tr>
<tr>
<td>18</td>
<td>(E,E)-Farnesol</td>
<td>1722</td>
<td>0.8</td>
<td>B</td>
</tr>
</tbody>
</table>

RI= Retention Index on ULBON HR-1; A = GC-MS Analysis; B = GLC Analysis
Table 2: Anti-microbial activity of the essential oil and ethanolic extract of the leaves of *Cymbopogon flexuosus*

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Test Organism</th>
<th>Conc. of Essential Oil</th>
<th>Zone of Inhibition in mm²</th>
<th>Dried ethanolic extract</th>
<th>Standard Chloramphenicol (0.1 mg/ml)</th>
<th>Standard Ketoconazole (0.1 mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.1%v/v</td>
<td>0.5%v/v</td>
<td>1.0%v/v</td>
<td>5.0%w/v</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td><em>Staphylococcus aureus</em></td>
<td>9.2</td>
<td>12.8</td>
<td>15.3</td>
<td>10.2</td>
<td>18.2</td>
</tr>
<tr>
<td>2</td>
<td><em>Escherichia coli</em></td>
<td>7.8</td>
<td>10.2</td>
<td>12.8</td>
<td>9.8</td>
<td>14.8</td>
</tr>
<tr>
<td>3</td>
<td><em>Candida albicans</em></td>
<td>7.5</td>
<td>9.8</td>
<td>12.5</td>
<td>9.0</td>
<td>14.2</td>
</tr>
<tr>
<td>4</td>
<td><em>Aspergillus niger</em></td>
<td>6.8</td>
<td>8.2</td>
<td>10.2</td>
<td>7.5</td>
<td>13.5</td>
</tr>
</tbody>
</table>

* An average of triplicate; Chloramphenicol - against all micro-organism; Ketoconazole - against fungal strains only.

7. References