SUPPLEMENTARY MATERIAL

Chemical Composition and Antibacterial Activity of Essential Oil of Nepeta graciliflora Benth. (Lamiaceae)

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Abstract

The chemical composition of the essential oil obtained from aerial parts of \textit{N. graciliflora} was analyzed, for the first time by, GC-FID and GC-MS. A total of twenty-seven compounds were identified, constituting over 91.44\% of oil composition. The oil was strongly characterized by sesquiterpenes (86.72\%), with $\beta$-sesquiphellandrene (28.75\%), caryophyllene oxide (12.15\%), $\alpha$-bisabolol (8.97\%), $\alpha$-bergamotene (8.51\%), $\beta$-bisabolene (6.33\%) and $\beta$-caryophyllene (5.34\%) as the main constituents. The \textit{in vitro} activity of the essential oil was determined against four microorganisms in comparison with chloramphenicol by the agar-well diffusion and broth dilution method. The oil exhibited good activity against all tested organisms.

\textbf{Keywords:} \textit{Nepeta graciliflora}, essential oil, chemical composition, antibacterial activity.

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**Experimental**

**Plant material**

The aerial parts of *N. graciliflora* at full flowering stage were collected (August, 2012) from Mandi district of Himachal Pradesh, India. The taxonomic identification of plant material was confirmed by Dr. Brij Lal, Senior Taxonomist, CSIR- IHBT, Palampur. A voucher (specimen number PLP-16523) has been deposited at the Herbarium of Biodiversity division, CSIR- IHBT, Palampur (H.P.) India.

**Isolation of the essential oil**

The fresh aerial parts (2 kg) of *N. graciliflora* were subjected to hydro distillation in a Clevenger-type apparatus for 6 hours. After distillation the oil was collected, dried with anhydrous Na$_2$SO$_4$, and kept in a vial at a temperature of -4°C for further analysis.

**GC and GC-MS analysis**

GC analysis of the essential oil was carried out on a Shimadzu GC-2010 gas chromatograph fitted with FID detector and a DB-5 fused silica capillary column (30 m × 0.25 mm i.d.; 0.25 µm film thickness). The oven temperature was programmed from 40°C (4 min hold) to 220°C (15 min hold) at the rate of 4°C min$^{-1}$ using N$_2$ as carrier gas with a flow rate of 1.2 mL min$^{-1}$. The injector and detector temperatures were set at 250°C; sample injection volume, 2µL; split ratio was 1: 50. Gas chromatography–mass spectrometry (70eV) was performed on a GC–MS (QP2010 Shimadzu, Tokyo, Japan) equipped with AOC-5000 Auto injector and DB-5 (SGE International, Ringwood, Australia) fused silica capillary column (30 m × 0.25 mm i.d.; 0.25 µm film thickness) under the same conditions as those of GC analysis using Helium as carrier gas with a flow rate of 1.2 mL min$^{-1}$. Mass spectrometer source temperature, 200°C; interface temperature, 250°C; injector temperature, 250°C. Sample injection volume, 2µL; split ratio, 1:10 and mass scan, 40-800 amu.

**Identification of constituents**

The retention indices were calculated for all volatile constituents using a homologous series of *n*-alkanes. Further identification was performed by matching their mass spectra with those stored in the computer library such as Wiley, New York mass spectral (MS) library, National Institute of Standards and Technology (NIST) (Stein, 1990) and their retention indices (RI) were compared with values available in the literature (Adams, 1989).

**Antibacterial Activities**
**Test microorganisms**

The sesquiterpenes (86.72%) rich essential oil was evaluated for its antibacterial activities against four microorganisms viz., *Staphylococcus aureus* (MTCC 3160), *Bacillus cereus* (MTCC 430), *Klebsiella pneumoniae* (MTCC 7162) and *Pseudomonas aeruginosa* (MTCC 424) by agar diffusion and micro dilution method. The organisms were obtained from the Microbial Type Culture Collection, Institute of Microbial Technology (IMTECH), Chandigarh, India.

**Determination of inhibitory effect**

Antibacterial activity of essential oil of *N. graciliflora* was investigated by Agar-well diffusion method (Oke *et al*., 2009). The essential oil was dissolved in dimethyl sulfoxide (DMSO) to prepare test samples of different concentrations (4, 8, 12 and 16 μL/well). 20 ml of sterilized nutrient agar was inoculated with 100 μl of bacterial suspension (10⁸ CFU mL⁻¹) and then poured on to each sterilized perti plates. The agar plates were left to solidify at room temperature. Wells of 6mm was bored into the agar plates and test solution was inserted in each well. DMSO was used as negative control. The perti plates were then incubated at 37°C for 24 hrs for bacterial growth and antibacterial activities were determined by measuring zone of inhibition (mm). The diameters of zone of inhibition produced by test samples were compared with commercially available antibiotics, 20μL cloramphenicol (10mg/mL). The assay was performed in triplicates.

**Determination of MIC**

Minimum inhibitory concentration of essential oil against the test microorganisms was determined by the Broth dilution method (Pattnaik *et al*., 1997). Different dilutions of the essential oil, ranging from 20 - 800 μg mL⁻¹, were prepared in Muller-Hinton broth (MHB). Exactly 0.5 MacFarland standard suspensions of the test microorganisms were inoculated in the tubes. A control test was also performed using inoculated broth only with dimethyl sulphoxide under identical conditions. The tubes were incubated at 37°C, for 24 hrs, and the lowest concentration inhibiting bacterial growth (no turbidity) was noted as MIC.

**Statistical analysis**

All tests were carried out in triplicate and the results were calculated as mean ±SD.
Table S1. Essential oil composition of *Nepeta graciliflora*

| Sr. no. | Constituents                  | LRI\textsuperscript{Lit.} | LRI\textsuperscript{Cal.} | % Composition | Identification |
|---------|-------------------------------|-----------------------------|-----------------------------|---------------|----------------|
| 1       | \(\alpha\)-pinene             | 939                         | 943                         | 0.48          | a,b            |
| 2       | Acetylcyclohexene             | 1023                        | 1031                        | 2.03          | a,b            |
| 3       | \(\gamma\)-terpinene         | 1062                        | 1062                        | 1.08          | a,b            |
| 4       | 1-octen-3-yl acetate          | 1110                        | 1102                        | 0.43          | a,b            |
|         | limonene oxide                | 1134                        | 1136                        | 0.70          | a,b            |
| 5       | \(\alpha\)-copaene            | 1376                        | 1366                        | 1.15          | a,b            |
| 6       | \(\beta\)-bourbonene         | 1384                        | 1374                        | 0.65          | a,b            |
| 7       | \(\beta\)-elemene             | 1391                        | 1381                        | 0.09          | a,b            |
| 8       | \(\beta\)-caryene             | 1418                        | 1410                        | 5.34          | a,b            |
| 9       | \(\alpha\)-bergamotene        | 1436                        | 1425                        | 8.51          | a,b            |
| 10      | (Z)-\(\beta\)-farnesene      | 1443                        | 1432                        | 2.38          | a,b            |
| 11      | (E)-\(\beta\)-farnesene      | 1458                        | 1452                        | 0.11          | a,b            |
| 12      | \(\gamma\)-gurjunene         | 1473                        | 1467                        | 1.85          | a,b            |
| 13      | germacrene D                  | 1480                        | 1473                        | 1.98          | a,b            |
| 14      | \(\beta\)-selinene            | 1485                        | 1475                        | 3.23          | a,b            |
| 15      | \(\alpha\)-zingiberene        | 1495                        | 1486                        | 0.18          | a,b            |
| 16      | \(\beta\)-bisabolene          | 1509                        | 1499                        | 6.33          | a,b            |
| 17      | \(\alpha\)-cadinene           | 1514                        | 1503                        | 0.50          | a,b            |
| 18      | \(\delta\)-cadinene           | 1524                        | 1515                        | 0.64          | a,b            |
| 19      | \(\beta\)-sesquiphellandrene | 1524                        | 1519                        | 28.75         | a,b            |
| 20      | \(\alpha\)-cadinol            | 1653                        | 1663                        | 0.29          | a,b            |
| 21      | \(\alpha\)-bisabolol          | 1683                        | 1687                        | 8.97          | a,b            |
| 22      | \(6S,7R\)-bisabolone          | 1744                        | 1740                        | 0.09          | a,b            |

Total identified: 91.44%

- **Monoterpene hydrocarbons**: 1.56%
- **Oxygenated monoterpenes**: 0.70%
- **Sesquiterpene hydrocarbons**: 61.19%
- **Oxygenated sesquiterpenes**: 25.53%
- **Others**: 2.46%

Notes: Components are listed in order of their elution from DB-5 column.  
LRI\textsuperscript{Cal.} – Linear retention index was calculated for all volatile constituents using a homologous series of *n* alkanes.  
LRI\textsuperscript{Lit.} – Retention indices taken from literature.  
\textsuperscript{a}Linear Retention Index (LRI) on DB-5 capillary column, \textsuperscript{b}MS (GC–MS).  
Compounds >2.0% are shown in bold.
Table S2. Antibacterial activity of essential oil of *N. graciliflora*.

| Microorganisms                  | Inhibition Zone Diameter (mm) | MIC (µg mL⁻¹) |
|---------------------------------|-------------------------------|---------------|
|                                 | Oil sample                    | Control (+ve) |
|                                 | 4µL/well                      | 8µL/well      | 12µL/well | 16µL/well | 20µL/well |               |
| *Staphylococcus aureus* (MTCC 3160) | 18                            | 22            | 26         | 33         | 18         | 123           |
| *Bacillus cereus* (MTCC 430)    | 19                            | 20            | 22         | 24         | 7          | 114           |
| *Klebesilla Pneumonia* (MTCC 7162) | 17                            | 21            | 23         | 25         | 15         | 212           |
| *Pseudomonas aeruginosa* (MTCC 424) | 16                            | 21            | 23         | 27         | 10         | 260           |

Notes: Control +ve Chloramphenicol, Control –ve (DMSO), NA- non-active

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