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 *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 β -sesquiphellandrene (28.75%), caryophyllene oxide (12.15%), α -bisabolol (8.97%), α -bergamotene (8.51%), β -bisabolene (6.33%) and β -caryophyllene (5.34%) as the main constituents. The *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.

Keywords: *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- IHB, Palampur. A voucher (specimen number PLP-16523) has been deposited at the Herbarium of Biodiversity division, CSIR- IHB, 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 Na₂SO₄, 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 N₂ 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, 2 µL; 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, 2 µL; 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 $\mu\text{L}/\text{well}$). 20 ml of sterilized nutrient agar was inoculated with 100 μl of bacterial suspension (10^8 CFU mL^{-1}) and then poured on to each sterilized petri 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 petri 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, 20 μL chloramphenicol (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 $\mu\text{g mL}^{-1}$, 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 $\pm$ SD.

Table S1. Essential oil composition of *Nepeta graciliflora*

Sr. no.	Constituents	LRI ^{Lit.}	LRI ^{Cal.}	% Composition	Identification
1	α -pinene	939	943	0.48	a,b
2	Acetylcyclohexene	1023	1031	2.03	a,b
3	γ -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
6	α -copaene	1376	1366	1.15	a,b
7	β -bourbonene	1384	1374	0.65	a,b
8	β -elemene	1391	1381	0.09	a,b
9	β -caryophyllene	1418	1410	5.34	a,b
10	α -bergamotene	1436	1425	8.51	a,b
11	(Z)- β -farnesene	1443	1432	2.38	a,b
12	(E)- β -farnesene	1458	1452	0.11	a,b
13	γ -gurjunene	1473	1467	1.85	a,b
14	germacrene D	1480	1473	1.98	a,b
15	β -selinene	1485	1475	3.23	a,b
16	α -zingiberene	1495	1486	0.18	a,b
17	β -bisabolene	1509	1499	6.33	a,b
18	Sesquicineole	1514	1503	0.50	a,b
19	δ -cadinene	1524	1515	0.64	a,b
20	β -sesquiphellandrene	1524	1519	28.75	a,b
21	(E)- γ -bisabolene	1532	1535	1.61	a,b
22	caryophyllene oxide	1581	1577	12.15	a,b
23	humulene epoxide-II	1606	1613	1.02	a,b
24	α -eudesmol	1652	1661	0.90	a,b
25	α -cadinol	1653	1663	0.29	a,b
26	α -bisabolol	1683	1687	8.97	a,b,
27	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^{Cal.} – Linear retention index was calculated for all volatile constituents using a homologous series of *n* alkanes.

LRI^{Lit.} - Retention indices taken from literature.

^aLinear Retention Index (LRI) on DB-5 capillary column, ^bMS (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 ($\mu\text{g mL}^{-1}$)
	Oil sample				Control (+ve)	
	4 μL / well	8 μL / well	12 μL / well	16 μL / well	20 μL / well	
<i>Staphylococcus aureus</i> (MTCC 3160)	18	22	26	33	18	123
<i>Bacillus cereus</i> (MTCC 430)	19	20	22	24	7	114
<i>Klebesilla Pneumonia</i> (MTCC 7162)	17	21	23	25	15	212
<i>Pseudomonas aeruginosa</i> (MTCC 424)	16	21	23	27	10	260

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

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