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Original scientific paper

Constituents and antimicrobial activity of the essential oils of six Himalayan *Nepeta* species

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Abstract: The essential oils from six Himalayan *Nepeta* species, *viz. Nepeta leucophylla* Benth., *Nepeta discolor* Royle ex Benth., *Nepeta govaniana* Benth., *Nepeta clarkei* Hook. f., *Nepeta elliptica* Royle ex Benth. and *Nepeta erecta* Benth., were tested for their *in vitro* antimicrobial activity against six pathogenic bacterial and two fungal strains. The results showed that *Pseudomonas aeruginosa* was the most sensitive strain tested to the essential oils of *Nepeta* species. The essential oils of *N. elliptica* and *N. erecta* exhibited the highest activity against *P. aeruginosa*, followed by the essential oils of *N. leucophylla* and *N. clarkei*. The essential oils from *N. elliptica* and *N. erecta* were also found to be very effective against *Serratia marcescens*; while the essential oil from *N. leucophylla* displayed significant activity against *Proteus vulgaris* and *Staphylococcus aureus*. Other bacterial strains displayed variable degree of susceptibility against one or more of the tested essential oils. The essential oil from *N. leucophylla* also showed the highest antifungal activity against both tested fungal strains, *viz. Candida albicans* and *Trichophyton rubrum*, followed by the essential oils from *N. clarkei*, *N. govaniana* and *N. erecta*. Iridodial derivatives, *viz.* iridodial *β*-monoenol acetate (25.4 %), dihydroiridodial diacetate (18.2 %) and iridodial dienol diacetate (7.8 %) were identified as the major constituents of *N. leucophylla*, while the essential oils from *N. elliptica* and *N. erecta* were dominated by (7*R*)-*trans*,*trans* nepetalactone (83.4 %) and isoiridomyrmecin (66.7 %), respectively. The essential oil of *N. discolor* was characterized by 1,8-cineole (25.5 %) and *β*-caryophyllene (18.6 %), while *N. clarkei* was dominated by *β*-sesquiphellandrene (22.0 %) and germacrene D (13.0 %). Isoiridomyrmecin (35.2 %) and pregeijerene (20.7 %) were identified as the major constituents of *N. govaniana*. In general the *Nepeta* species containing constituents with an iridoid or lactone skeleton were found to have the

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greater antagonistic activity against most of the microbial strains as compared to those containing regular terpene constituents.

Keywords: *Nepeta*; essential oils; antimicrobial activity; iridoids; isoiridomyrmecin; nepetalactone.

INTRODUCTION

Nepeta genus (Lamiaceae) is comprised of about 250 species, widely distributed in temperate Europe, Asia, North America, North Africa and in the Mediterranean region.[1,2] Besides being used as antispasmodic, diuretic, febrifuge, diaphoretic, antimicrobial and antiseptic agents, *Nepeta* species are used as laxatives in the treatment of dysentery, for tooth troubles and for kidney and liver diseases.[3,4] Various biologically active iridoids/monoterpene nepetalactones were reported in *Nepeta* species possessing diverse biological activities, *viz*. feline attractant, canine attractant, insect repellant and arthropod defense.[5–8] Some of them act as pheromones and catnip response factors and are also known as ginseng of the cat.[9] The essential oil composition and antimicrobial activity of various *Nepeta* species were previously reported.[10–23] In earlier communications, some Himalayan *Nepeta* species were screened for their terpenoid constituents.[18–23] Iridodial β-monoenol acetate isolated from essential oil of *N. leucophylla* Benth. and actinidine isolated from essential oil of *N. clarkei* Hook. f. were shown to have significant antibacterial and antifungal activities.[24,25] The present communication reports the antibacterial and antifungal activities of the essential oils from six *Nepeta* species from Himalayan region of Uttarakhand, India.

EXPERIMENTAL

Plant materials

The fresh flowering aerial parts of *Nepeta leucophylla*, *N. discolor*, *N. govaniana*, *N. clarkei*, *N. elliptica* and *N. erecta* were collected from different locations of the Himalayan region of Uttarakhand, India (Table I). The plant herbaria were identified by the Botanical Survey of India, Dehradun and voucher specimens have been deposited in the Phytochemistry Laboratory, Chemistry Department, Kumaun University, Nainital.

TABLE I. Sites for collection of the *Nepeta* species and the essential oil yields

Plants	Collection site[a]	Oil yield[b], % (v/w)
Nepeta leucophylla	Nainital, 2400 m	0.68
Nepeta discolor	Malari, Chamoli, 2800 m	0.90
Nepeta govaniana	Bhundiar, Chamoli, 2600 m	0.85
Nepeta clarkei	Malari, Chamoli, 2800 m	0.70
Nepeta elliptica	Clips, Nainital 2700 m	0.92
Nepeta erecta	Hemkund, 3250 m	0.76

[a]Locations in Uttarakhand (Himalayan region, India); [b]oil yields were determined on fresh weight basis, average of three extraction (*SD* = 0.04)

Isolation of essential oil and major isolates

The fresh flowering aerial parts (2 kg) of each plant were subjected to steam distillation using an electric copper still, fitted with spiral glass condensers. The distillates were saturated with NaCl and extracted with *n*-hexane and dichloromethane. The organic phase was dried over anhydrous Na_2SO_4 and the solvent was distilled off in a rotary vacuum evaporator at 30 °C. The essential oils were fractionated by column chromatography (CC) on silica gel (230––400 mesh, Merck, 600×25 cm column) packed with hexane, and eluted with hexane followed by a gradient elution with Et_2O/hexane (5–20 %). The isolated compounds (**1**–**12**) were further purified using a Waters HPLC fitted with a μ-Porosil column (250 mm×7.8 mm) and an RI detector at an attenuation of 32X, using 5–15 % Et_2O in hexane at a flow rate of 2.0 mL/min and a pressure of 3000 psi.

GC and GC–MS analysis

The oils were analyzed using a Nucon 5765 gas chromatograph equipped with an Rtx-5 non-polar fused silica capillary column (30 m×0.32 mm, film thickness: 0.25 μm). The oven temperature (60–210 °C) was programmed at 3 °C/min and N_2 was used as the carrier gas at 4 kg/cm^2. The injector temperature was 210 °C, detector temperature 210 °C and the injection volume 0.5 μL, using a 10 % solution of the oil in *n*-hexane. GC–MS was conducted on a ThermoQuest Trace GC 2000 fitted with an Rtx-5 non-polar fused silica capillary column (30 m×0.25 mm, film thickness: 0.25 μm) and interfaced with a Finnigan MAT Polaris Q ion trap mass spectrometer. The oven temperature (60–210 °C) was programmed at 3 °C/min and helium was used as the carrier gas at 1.0 mL/min. The injection, ion source and MS transfer line temperatures were 210, 200 and 275 °C, respectively; the injection volume was 0.10 μL and the split ratio was 1:40. The MS were taken at 70 eV with a mass range of 40–450 amu.

Identification of constituents

The identification was realized based on their linear retention index (LRI) and the retention times determined with reference to homologous series of *n*-alkanes (C_9–C_{24}, Polyscience Corp., Niles IL) and standards (Sigma) under identical experimental conditions. The identification was further supported by MS Library searches (NIST and WILEY) and by comparing MS literature data,[26,27] as well as by IR, NMR (^{1}H-and ^{13}C-NMR) data of the major isolates. The relative amounts of individual components were calculated based on the GC peak area (FID response) without using a correction factor.

Test microorganisms

The *in vitro* antibacterial activities of the essential oils were evaluated against a total of six bacteria, which includes five gram negative bacteria *viz*. *Pseudomonas aeruginosa* (MTCC 424), *Escherichia coli* (MTCC 443), *Pasteurella multocida* (MTCC 1148), *Proteus vulgaris* (MTCC 1771) and *Serratia marcescens* (MTCC 8708) with one gram positive bacterium *Staphylococcus aureus* (MTCC 737). The antifungal activity of the oils was performed against *Candida albicans* (MTCC 183) and *Trichophyton rubrum* (MTCC 296). The test strains were purchased from the Institute of Microbial Technology (IMTECH), Chandigarh, India. Microbial technology culture collection (MTCC) numbers represent the standard strain numbers assigned to these microorganisms. The cultures of bacteria and fungi were maintained on their appropriate agar slants at 4 °C throughout and used as stock cultures.

Determination of zone of inhibition

The antimicrobial activity of the essential oils was investigated by the disc diffusion method using 24–48 h grown strains reseeded on nutrient broth (bacterial strains) and potato

dextrose agar (PDA, fungal strains).[28] The cultures were adjusted to 5×10^6 CFU/mL with sterile water. 100 μL of the suspensions were spread over nutrient agar and PDA plates to obtain uniform microbial growth. Filter paper discs (6.0 mm in diameter) were impregnated with 20 μL of the oils and then placed onto the agar plates which had previously been inoculated with the test microorganism. The petri dishes were kept at 4 °C for 2 h. The plates were incubated at 37 (24 h) and at 30 °C (48 h) for bacterial and fungal strains, respectively. The diameter of the inhibition zones (mean values) were measured in millimeter and considered as the zone of inhibition (ZOI). All experiments were performed in triplicate.

Determination of the minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) values were determined using a modified agar-well diffusion method.[28] In the agar-well diffusion technique, two-fold serial dilutions of the essential oils were prepared by diluting oil with hexane to achieve a decreasing concentration range from 50 to 0.19 μL/mL (for the fungi) and 50 to 0.15 μL/mL (for the bacteria), using 100 μl of a suspension containing 5×10^6 CFU/ml of bacteria spread on nutrient agar plates, whereas the fungal strains were reseeded on PDA. The wells were filled with 20 μl of essential oil solutions in the inoculated nutrient/PDA agar plates. The bacterial cultures were incubated at 37 °C for 24 h, while fungal cultures were incubated at 30 °C for 48 h. The least concentration of each essential oil showing a clear zone of inhibition was taken as the *MIC*. Hexane was used as the negative control. Streptomycin and clotrimazole were used as positive controls for bacteria and fungi, respectively.

RESULTS AND DISCUSSION

The oil yield from different *Nepeta* species varied from 0.70 to 0.92 % calculated on the basis of the fresh weight of the plant material (Table I). The marker constituents identified in the essential oils of *Nepeta* species are listed in Table II. Iridodial derivatives, *viz*. iridodial *β*-monoenol acetate (**1**), dihydroiridodial diacetate (**2**) and iridodial dienol diacetate (**3**) were the major constituents of the essential oil of *N. leucophylla*. The essential oil of *N. discolor* was characterized by 1,8-cineole (**4**), *β*-caryophyllene (**5**) and *p*-cymene (**6**). Isoiridomyrmecin (**7**) and pregeijerene (**8**) were identified as the major constituents of *N. govaniana*. The essential oil from *N. clarkei* was dominated by *β*-sesquiphellandrene (**9**), germacrene D (**10**), *α*-guaiene (**11**) and diastereomeric iridodial esters. On the contrary, the essential oil from *N. elliptica* and *N. erecta* were dominated by a single major constituent, *viz*. (7*R*)-*trans*,*trans*-nepetalactone (**12**) and isoiridomyrmecin (**7**), respectively. The structures of marker compounds/ /major constituents are given in Fig. 1.

The essential oils were screened for possible antagonistic activity against six bacterial and two fungal strains. The results of *in vitro* test (Table III) showed that almost all the tested essential oils showed moderate to high activities against one or more of the tested pathogens in the petri plate assay, based on the zone of inhibition and *MIC* values. Some of the essential oils showed significant antibacterial and antifungal activities when the zone of inhibition values were compared to those of the standards, *viz*. streptomycin and clotrimazole. The mean zones of inhibition for the bacterial strains were in the range 6.0 to 28.4 mm, with the *MIC*

values ranging from 0.15 to 30.34 μL/mL. The inhibition zones for the fungal strains varied from 9.3 to 20.0 mm, with the *MIC* values ranging from 0.19 to 12.50 μL/mL. The positive control streptomycin (for bacteria) showed 18.6 to 27.6 mm mean inhibition zones and clotrimazole (for fungi) showed 18.2 and 19.1 mm inhibition zones. The negative control did not show an inhibitory effect on any of the tested strains.

TABLE II. Marker constituents of the essential oils from Himalayan *Nepeta* species

Plant	Marker constituents[a]	Content, % (based on FID response)
Nepeta leucophylla	Iridodial *β*-monoenol acetate (**1**)	25.4
	Dihydroiridodial diacetate (**2**)	18.2
	Iridodial dienol diacetate (**3**)	7.8
Nepeta discolor	1,8-Cineole (**4**)	25.5
	β-Caryophyllene (**5**)	18.6
	p-Cymene (**6**)	9.8
Nepeta govaniana	Isoiridomyrmecin (**7**)	35.2
	Pregeijerene (**8**)	20.7
Nepeta clarkei	Iridodial *β*-monoenol acetate diastereomers	25.3
	β-Sesquiphellandrene (**9**)	22.0
	Germacrene D (**10**)	13.0
	α-Guaiene (**11**)	10.0
Nepeta elliptica	(7*R*)-*trans*,*trans*-Nepetalactone (**12**)	83.4
Nepeta erecta	Isoiridomyrmecin (**7**)	66.7

[a]Mode of identification: linear retention index (LRI, based on a homologous series of *n*-alkanes; C_9–C_{24}), co-injection with standards (Sigma), MS (GC–MS)

The data indicated that *P. aeruginosa* was the tested strain most sensitive to the essential oils of the *Nepeta* species, with the strongest inhibition zones varying from 18.2 to 28.4 mm. The essential oils of *N. elliptica* and *N. erecta* were found to exhibit the largest zones of inhibition (28.4 mm, *MIC* = 0.31 μL/mL; 28.0 mm, *MIC* = 0.62 μL/mL) against *P. aeruginosa* followed by *N. leucophylla* (27.4 mm, *MIC* = 0.42 μL/mL) and *N. clarkei* (22.0 mm, *MIC* = 0.15 μL/mL).

P. aeruginosa is known to have a high level of intrinsic resistance to virtually all known antimicrobials and antibiotics due to a very restrictive outer membrane barrier, which is highly resistant even to synthetic drugs. Furthermore, earlier reports on the antimicrobial activity of essential oils of *Nepeta* species stated they possessed very little or no activity against *P. aeruginosa*.[11–13,29] Thus, the results of the antibacterial activity of all the tested oils against *P. aeruginosa* evidenced in the present study are very promising. The essential oils from *N. elliptica* and *N. erecta* were also found to be very effective against *S. marcescens* (20.2 mm, *MIC* = 0.43 μL/mL; 18.3 mm, *MIC* = 1.59 μL/mL). The essential oil from *N. leucophylla* also displayed significant activity against *P. vulgaris* and *S. aureus* (21.2 mm, *MIC* = 3.21 μL/mL; 16.4 mm; *MIC* = 1.78 μL/mL). Other bac-

terial strains displayed variable degrees of susceptibility against one or more of the tested essential oils. Furthermore, most gram negative bacteria are more resistant to various antimicrobials,[30] but in the present study, the tested essential oils showed promising antimicrobial activities against most of the gram negative bacteria.

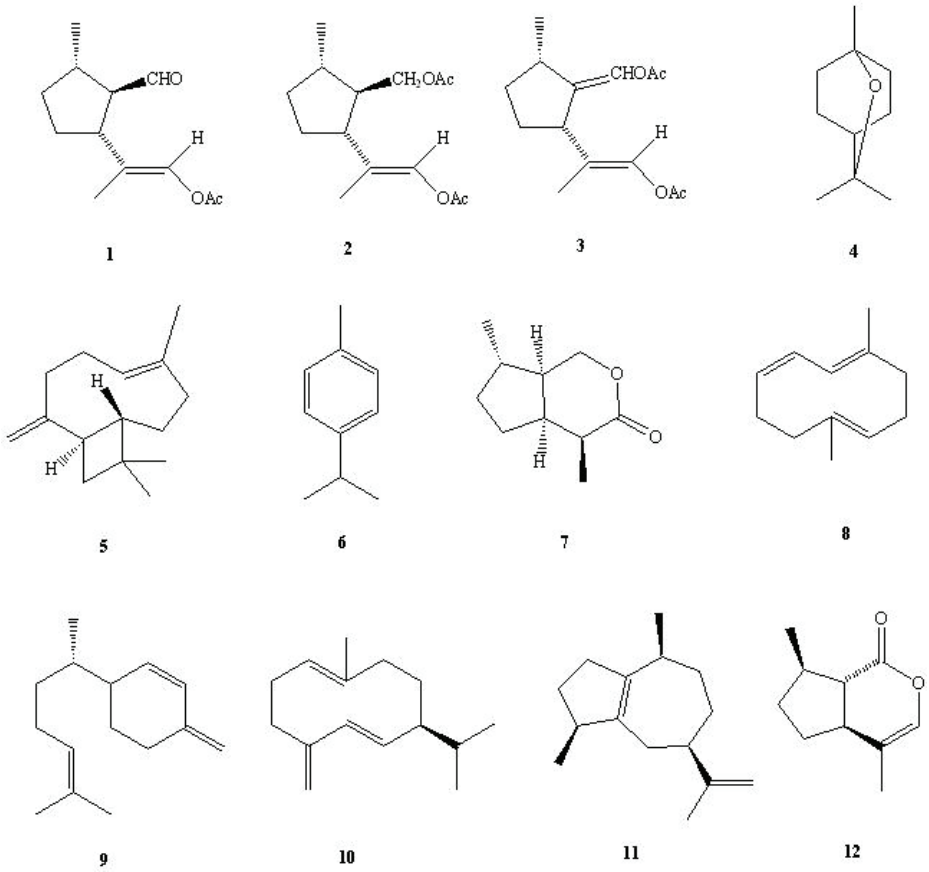

Fig. 1. Structures of major/marker constituets of Himalayan *Nepta* species.

The essential oil from *N. leucophylla* showed maximum antifungal activity against both *C. ablicans* (20.0 mm, *MIC* = 0.78 μL/mL) and *T. rubrum* (19.2 mm, *MIC* = 0.19 μL/mL). The essential oils from *N. elliptica*, *N. erecta* and *N. govaniana* also showed significant activity against both the fungal strains, whereas, *N. clarkei* and *N. discolor* showed poor activity against both the fungal strains.

The essential oils possessing chemical constituents with an iridoid or lactone skeleton were noticed to be more active antibacterial/antifungal as compared to those containing regular terpene constituents. This might be due to their higher

water solubility and diffusion coefficient through the medium and also due to their higher hydrogen bonding potential. By inhibiting the growth of almost all human and plant pathogenic and/or food spoilage bacteria and fungi tested, the essential oil from Himalayan *Nepeta* species exerted a broad antimicrobial spectrum, the iridoid/ lactone containing oils showed higher activity. Furthermore, the obtained results showing a wide spectrum of antibacterial and antifungal activities may provide support to the traditional applications of these plants.

TABLE III. Antibacterial and antifungal activities of essential oils of six Himalayan *Nepeta* species

Plant	Zone of inhibition[a], mm (*MIC* / μL mL^{-1})							
	Bacteria						Fungi	
	E. coli	*P. aeruginosa*	*S. aureus*	*P. multocida*	*P. vulgaris*	*S. marcescens*	*C. albicans*	*T. rubrum*
N. leucophylla	9.0 (15.62)	27.4 (0.42)	20.0 (6.24)	na[b]	21.2 (3.21)	16.4 (1.78)	20.0 (0.78)	19.2 (0.19)
N. discolor	10.4 (7.54)	18.2 (1.42)	na	na	9.4 (7.2)	na	12.0 (3.21)	9.3 (7.30)
N. govaniana	13.0 (10.24)	20.1 (0.37)	7.3 (3.90)	16.0 (11.34)	na	na	16.1 (1.56)	18.1 (1.58)
N. clarkei	11.1 (9.24)	22.0 (0.15)	16.2 (11.03)	6.9 (30.34)	14.2 (1.54)	7.5 (16.52)	13.7 (3.50)	12.5 (12.50)
N. elliptica	20.8 (2.58)	28.4 (0.31)	19.4 (2.05)	10.4 (2.60)	na	20.2 (0.43)	18.0 (0.19)	19.1 (0.39)
N. erecta	22.2 (3.25)	28.0 (0.62)	18.5 (2.36)	na	18.2 (1.56)	18.8 (1.59)	18.2 (1.50)	15.4 (1.41)
Standard[c]	27.1	27.6	23.5	18.6	23.0	19.5	19.1	18.2

[a]Diameter of inhibition zones (mm) including the diameter of the disc (6.0 mm); values are the mean of three replicates; oil concentration 20 μL/disc; [b]not active; [c]standard: streptomycin (10 μg/disc for bacterial strains) and clotrimazole (20 μg/disc for fungal strains)

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И З В О Д

САСТАВ И АНТИМИКРОБНА АКТИВНОСТ ЕТАРСКИХ УЉА ИЗОЛОВАНИХ ИЗ ШЕСТ ВРСТА БИЉКЕ *Nepeta* СА ХИМАЛАЈА

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Испитана је антимикробна активност спрам шест врста патогених бактерија и две врсте гљива етарских уља изолованих из шест врста биљке *Nepeta*, која расте на Хималајима: *Ne-*

peta leucophylla Benth., *Nepeta discolor* Royle ex Benth., *Nepeta govaniana* Benth., *Nepeta clarkei* Hook. f., *Nepeta elliptica* Royle ex Benth. и *Nepeta erecta* Benth. Резултати су показали да је *Pseudomonas aeruginosa* најосетљивији сој на етарска уља врста *Nepeta*. Етарска уља *N. elliptica* и *N. erecta* су имала највећу активност спрам *P. aeruginosa*, а затим етарска уља *N. leucophylla* и *N. clarkei*. Етарска уља *N. elliptica* и *N. erecta* су била веома активна спрам *Serratia marcescens*, док је уље *N. leucophylla* испољило значајну активност спрам *Proteus vulgaris* и *Staphylococcus aureus*. Остали бактеријски сојеви су испољили различит степен осетљивости на тестирана етарска уља. Етарско уље *N. leucophylla* је имало највећу антифунгалну активност, спрам обе врсте тестираних гљива: *Candida albicans* и *Trichophyton rubrum*, док су уља *N. clarkei*, *N. govaniana* и *N. erecta* испољила мању активност. Деривати иридодиала, иридодиал-β-моноенол-ацетат (25,4 %), дихидроиридодиал диацетат (18,2 %) и иридодиал-диенол-диацетат (7,8 %) су главни састојци уља *N. leucophylla*, док су у уљима *N. elliptica* и *N. erecta* доминантни (7R)-*trans*,*trans*-непеталактон (83,4 %) и изоиридомирмецин (66,7 %). У етарском уљу *N. discolor* је највише 1,8-цинеола (25,5 %) и β-кариофилена (18,6 %), док је у уљу *N. clarkei* највише β-сесквифеландрена (22,0 %) и гермакрена Д (13,0 %). Изоиридомирмецин (35,2 %) и прегеијерен (20,7 %) су главни састојци *N. govaniana*. Уопштено, *Nepeta* врсте које садрже једињења са иридоидним или лактонским скелетом имају већу антагонистичку активност спрам микробних сојева у поређењу са врстама која садрже стандардне терпенске састојке.

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