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Research Article
High Altitude Medicinal Plants Against Multidrug Resistant Microorganisms: Screening and Chemical Profiling of the Most Active *Nepeta longibracteata*

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Abstract

Background and Objective: Development of resistance amongst microbes towards commonly used antibiotics is of major concern in recent years. Considering the growing attention for search of new antimicrobial agents, seven relatively unexplored high altitude plants were screened for antimicrobial property against multidrug resistant clinical isolates. **Methodology:** Solvent extraction followed by fractionation afforded 28 components. Antibacterial activity was evaluated against Gram-positive *Staphylococcus aureus* and three Gram-negative viz., *Serratia* sp., *Acinetobacter* sp. and *E. coli* clinical isolates. Antifungal activity was evaluated against *Aspergillus niger*, *Fusarium moniliforme* and *Candida albicans*. All experiments were carried out in triplicates and expressed as Mean±SD. **Results:** The *n*-hexane fraction of *Nepeta longibracteata* (NlH) and *Rhodiola imbricata* (RiH) exhibited most significant antibacterial activity against *E. coli* and *S. aureus* (MIC 125 μg mL^{-1}), while antifungal activity was exhibited only by NlH with MIC 250 μg mL^{-1}. Phytochemical profiling of NlH by GC-MS showed 14 compounds where *n*-hexadecanoic acid and methyl hexadecanoate constituted 67.7% of the total constituents. Scanning Electron Microscopy (SEM) was performed with NlH at MIC to study the morphological changes on *E. coli* cell membrane. This study revealed shortening and swelling of cells and multiple blisters formation on cell surface compared to untreated control. **Conclusion:** The results of the present study have identified NlH as the most potent fraction against multidrug resistant microorganisms. The membrane permeabilizing activity of NlH was also established through SEM analysis. These results may be exploited in future to increase the efficacy of conventional antibiotics by enhancing their cellular uptake. Such a strategy holds immense potential in the current scenario of continuous emergence of antibiotics resistant microorganisms.

Key words: *Nepeta longibracteata*, multidrug resistant, clinical isolates, GC-MS, scanning electron microscopy

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The emergence of resistance to multiple antimicrobial agents has become a major threat to public health. Hence, fresh efforts towards new drug identification and development are greatly needed. Plants have long been used in traditional Indian medicine for numerous therapeutic benefits and low toxicity. The high altitude of the Himalayan cold desert represents a valuable habitat of natural resources. The extreme climatic condition manifested by intense mutagenic UV-radiation, physiological drought, desiccation and strong winds, makes the survival of plants really difficult. As a consequence of this atmospheric stressor, the plants produce unique metabolites which play a preventive role in intrinsic mechanism of sustenance[1]. However, therapeutic potential of these plants have little been revealed despite its wide use in traditional medicine. In the present study, seven plants of Ladakh and Kashmir valley were selected to examine their antibacterial potential against a number of clinical isolates including, ampicillin resistant *Escherichia coli* and *Staphylococcus aureus*; ciprofloxacin resistant *Serratia* species and imipenem resistant *Acinetobacter* species. The antifungal activity was evaluated against *Aspergillus niger*, *Fusarium moniliforme* and *Candida albicans*. The literature search related to the biological activity of the plants revealed that, *Perovskia abrotanoides* possesses anti-inflammatory, antimicrobial, antifungal and cytotoxic activity[2] while, *Echinacea purpurea* is used for common cold and flu[3]. The plant extracts of *Rhodiola imbricata* is used for high-altitude sickness, sexual dysfunction and sleep disorder[4] and *Lepidium latifolium* (perennial pepper weed) is one of the preferred phytofoods among locals. A wide range of phytochemicals have been reported including echinacoside and quercetin from *E. purpurea*[5]; abrotandiol, abrotanone, cirsimaritin and hesperidin from *P. abrotanoides*[6]; salidrosides from *R. imbricata*[7], sinigrin from *L. latifolium* and negletein and gardenin from *Actinocarya tibetica*[8]. However, very little scientific information was available for *Nepeta longibracteata*[9] and *Dendrobium brunonianum*[10].

The objective of the present study was to explore new plant based antimicrobial agents from relatively unexplored high altitude medicinal plants of Ladakh and validate their traditional usage by local medicinal practitioners.

MATERIALS AND METHODS

Chemicals and reagents: Analytical grade solvents were used for extraction, fractionation. All microbiological media was procured from HiMedia chemicals, India. GC-MS analysis was performed on GCMS-Q2010 Ultra, Shimadzu gas chromatograph coupled with a mass selective detector. The separation was achieved using SPB-5 fused-silica capillary column (30 m×0.25 mm×0.25 μm), Supelco, Sigma-Aldrich. SEM was performed on Zeiss Evo 40 electron microscope (Germany) with magnifications ranging from 10-25 K.

Plant and microbial strains: The MDR bacterial isolates viz., *E. coli* (2461), *S. aureus* (2413), *Acinetobacter* sp. (2457) and *Serratia* sp. (2442) were obtained from Dr. Kumardeep Dutta Choudhary, Department of Medical Oncology, Rajiv Gandhi Cancer Research Institute, Delhi, India with their respective antibiotic resistance profiles (Table S1). Three fungal isolates *A. niger* (MTCC 872), *F. moniliforme* (MTCC 2088) and *C. albicans* (MTCC 227) were procured from IMTECH,

Table S1: Antibiotic resistance profiles of MDR clinical isolates obtained from Rajiv Gandhi Cancer Institute and Research Center, Delhi, India

Antibiotics	*E. coli* (2461)	*S. aureus* (2413)	*Serratia* sp. (2442)	*Acinetobacter* sp. (2457)
Amikacin	S	S	S	R
Ampicillin	R	-	R	-
Ciprofloxacin	R	S	R	R
Ceftriaxone	R	S	R	-
Chloramphenicol	R	-	R	-
Gentamicin	S	S	R	R
Imipenem	S	S	S	R
Levofloxacin	R	S	R	-
Meropenem	S	S	S	R
Nalidixic acid	R	-	-	-
Nitrofurantoin	S	-	-	-
Norfloxacin	R	-	-	-
Ofloxacin	R	S	R	-
Piperacillin	R	S	S	R
Vancomycin	-	S	-	-
Tobramycin	R	-	R	R
Amphotericin B	-	-	-	-
Ketoconazole	-	-	-	-

R: Resistant, S: Sensitive, -: Not tested

Table S2: Accession details of plants and their origin

Species	Family	Location	Altitude (m)	Voucher specimen No.	Time of collection
Nepeta longibracteata Bentham	Lamiaceae	South Polu	4500	22081	July-August
Rhodiola imbricata Edgew	Crassulaceae	Khardungla	5200	22950	July-August
Delphinium brunonianum Royle	Ranunculaceae	Khardungla	4000	21727	July-August
Lepidium latifolium L.	Brassicaceae	Leh	3500	22107	July-August
Echinacea purpurea (L.) Moench.	Asteraceae	IIIM-Jammu	300	21907	July-August
Perovskia abrotanoides Karel	Lamiaceae	Khardung-Nubra	3025	57323	July-August
Actinocarya tibetica Benth	Boraginaceae	Upshi-Leh	3600	22157	July-August

Chandigarh, India. All bacterial strains were revived in nutrient broth for antibacterial assay. Similarly fungal strains were revived and maintained on Sabouraud-Dextrose broth for antifungal assays.

The plants were collected and authenticated by Dr. S. Kitchlu, Department of Botany, Indian Institute of Integrative Medicine (IIIM) and the voucher specimen is deposited at the herbarium of IIIM, Jammu. The accession details are provided in the Table S2.

Extraction and fractionation of plants: Air dried whole plant (100 g) material was crushed and macerated in methanol-water (9:1; 100 mL) for overnight. Next day, the mixture was sonicated in an ultrasonic bath at 35°C for 30 min, twice and filtered. The filtrate was collected and the residue was again extracted with minimum volume of water. The combined organic extract was concentrated under reduced pressure, below 50°C while, the aqueous extract was concentrated in a lyophilizer. The concentrated organic fraction was resuspended in 75 mL of water and extracted with *n*-hexane, dichloromethane and ethyl acetate successively as described previously by Mishra *et al.*[11]. The organic and aqueous fractions were concentrated appropriately and subjected to preliminary phytochemical screening.

Preliminary phytochemical analysis: Phytochemical analysis for the presence of alkaloids, flavonoids, steroids, reducing sugars, cardiac glycosides, terpenoids, anthraquinones, tannins, phlobatannins and saponins were conducted with each fraction by using standard protocol[12].

Evaluation of antimicrobial activity: Antibacterial and antifungal activities of plant fractions were determined by agar well diffusion method as described by Rojas *et al.*[13]. Briefly, the nutrient agar plates were inoculated with standardized inoculum at a concentration of 10^7 CFU mL^{-1} of the test organism. Each well was treated with 50 µL of the sample, where, tetracycline and ketoconazole were used as positive control for antibacterial and antifungal activity respectively. The plates were incubated at 37°C for 24 h. The antimicrobial activity was expressed as the mean diameter of inhibition zones (mm) with standard deviation produced by the tested fractions. Minimum Inhibitory Concentrations (MIC) values were determined for the most potent extract by tube dilution method. A series of dilutions of each extract ranging from 5-0.1 mg mL^{-1} were done in Muller Hinton broth and were inoculated with 0.1 mL of suspension of the test organism. The tubes were incubated at 37°C for 24 h and checked for turbidity. Minimum inhibitory concentration was determined as highest dilution of the extract that showed no visible growth.

Cell cytotoxicity assay: The cell cytotoxicity was performed by MTT assay as described by Kakad and Dhembare[14]. In brief, fibroblast cells obtained from chick embryo were cultured in DMEM medium supplemented with Fetal Bovine Serum (FBS) and gentamicin. The cells suspension (2 mL) was treated with sample solution at MIC concentration and twice the concentration of MIC. The microtiter plate was incubated aseptically in CO_2 incubator for 24 h at 37°C. After incubation, cells were disaggregated using trypsin (0.25%) and cell viability (%) was calculated.

DPPH free radical scavenging assay: The DPPH (1,1-diphenyl-2-picrylhydrazyl) free radical scavenging assay was performed according to Yesil-Celiktas *et al.*[15]. An aliquot of the sample was mixed with 1 mM DPPH solution followed by incubation of 30 min in dark. The absorbance of each sample was read at 517 nm. Ascorbic acid and quercetin were used as positive control. The antioxidant activity was expressed in terms of concentration required to inhibit 50% methanolic DPPH radical formation (IC_{50} µg mL^{-1}).

Sample preparation for GC-MS analysis: The most potent antimicrobial fraction, *n*-hexane fraction of *N. longibracteata* (NlH) was accurately weighed to 5 mg and dissolved in acetonitrile. The sample was centrifuged at

3000 rpm for 15 min. The supernatant was concentrated to dryness in a rotary evaporator at 50°C under reduced pressure. The residue was reconstituted with methanol as required. An aliquot of 1.0 µL was injected for the GC-MS experiment.

GC-MS analysis: The GC-MS analysis were repeated three times on GCMS-Q2010 Ultra, Shimadzu gas chromatograph coupled with a mass selective detector. The injector and interface were operated at 260 and 270°C, respectively. The oven temperature was raised from 80-280°C at a heating rate of 5°C for 3 min and then isothermally held for 17 min. Helium at 1.0 mL min^{-1} was used as a carrier gas. One microliter of the solution of the sample in methanol (1:100) was injected in pulsed split mode (first 3 min at 1.5 mL min^{-1} and the rest time period at 1.0 mL min^{-1}, split ratio 1:10). Mass selective detector operated at the ionization energy of 70 eV, in 40-650 amu range with a scanning speed of 0.33 sec. Retention Indices (RI) were determined in relation to a homologous series of n-alkanes (C_7-C_{33}) under the same conditions. Peak identification was accomplished by comparison of their mass spectra with those stored on GC-MS databases (NIST 11 and Wiley 8).

Sample preparation for SEM: Scanning electron microscopy (SEM) was performed as described by Tang *et al.*[16] on *E. coli* cells treated with 125 µg mL^{-1} of NIH for 4 h. Both control and treated cells were prepared for morphological observation. The cells were collected by centrifugation and washed thrice with sodium phosphate buffer. The samples were then fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.3, at 4°C overnight and post fixed in 1% osmium tetroxide in the phosphate buffer for 1 h at room temperature. This was followed by three times washing in phosphate buffer for 10 min and subsequently dehydration by a series of ethanol concentrations (30, 50, 70, 90 and 95%), for 15 min each. The samples were finally subjected to 100% ethanol and CO_2 to achieve the critical point. An aliquot of 20 µL of bacterial pellets were applied on poly-L-lysine slide and subjected to gold coating and observed under ZeissEvo 40 (Germany) scanning electron microscope.

Statistical analysis: All experiments were carried out in triplicates and statistical analysis was carried out using Graph pad Prism version 4.00 for Windows, GraphPad Software, San Diego California USA. Data are presented as Mean±SD.

RESULTS

Qualitative phytochemical analysis of the plant fractions were conducted for alkaloids, flavonoids, steroids, reducing sugar, cardiac glycosides, terpenoids, anthraquinones, tannins, phlobatannins and saponins, which helped us to understand the chemical composition of the active fraction. The results are presented in Table 1.

Flavonoids, steroids, alkaloids and tannins were detected by NaOH-HCl test, Salkowski reaction, Dragendorff reagent and ferric chloride test, respectively.

Additional tests were carried out to check the presence of reducing sugar, cardiac glycosides, phlobatannins, anthraquinones, saponins and terpenoids[12].

The antibacterial screening identified *n*-hexane fractions of *N. longibracteata* (NlH) as most active against *E. coli* and *S. aureus* with MIC 125 µg mL^{-1} (Table 2, 3). The NlH also exhibited substantial antifungal activity against *A. niger* and *F. moniliforme* with MIC 250 µg mL^{-1}.

The phytochemical profile of NlH, conducted by GC-MS showed the presence of 22 compounds, 14 compounds constituted 93.8% of the total constituents. The constituents were identified by comparing their Retention Indices (RI) with those stored on GC-MS databases, where the RI were determined in relation to a homologous series of n-alkanes (C7-C33) under the same operating conditions. The relative concentrations of the components according to their elution order on a SPB-5 column are presented in Table 4. Mostly, long chain fatty acids and esters viz., *n*-hexadecanoic acid (50.32%), hexadecanoic acid methyl ester (17.4%), pentadecanoic acid (8.75%), 12-methyl tetradecanoic acid methyl ester (6.1%) and tridecanoic acid methyl ester (2.5%) constituted the active fraction. The compounds could be classified in five categories including, fatty acids (87.7%) saturated ketone (4.2%), aliphatic alcohols (2.0%), unknown compounds (5.6%) and miscellaneous types (0.5%). It was very difficult to place minor constituents separately in a pie-chart hence, were clubbed together as miscellaneous types (Fig. 1).

To understand the effect of NlH on cell morphology, *E. coli* cells treated with MIC of NlH were studied on SEM and the cell morphology was compared with untreated cells under same condition. The result showed that cell membrane of untreated *E. coli* cells were intact even after incubation whereas, the treated cells were swollen up and showed blisters of various shapes thus confirming damage to the structural integrity of the cells (Fig. 2).

Table 1: Phytochemical screening of crude fractions and extracts of seven high altitude medicinal plants of Ladakh

Plants	Fraction	Flavonoids	Saponins	Reducing sugars	Anthraquinones	Steroids	Terpenoids	Alkaloids	Cardiac glycosides	Phlobatannins
N. longibracteata	*n*-hexane	-	++	-	-	+++	-	-	-	++
	Dichloromethane	+	-	-	-	-	-	-	++	-
	Ethyl acetate	+	++	-	-	+	-	+++	++	++
	aqueous residue	++	-	-	-	-	-	+	++	+
R. imbricata	*n*-hexane	++	-	-	-	-	++	+++	+	++
	Dichloromethane	+++	+	-	-	+++	-	-	-	-
	Ethyl acetate	++	+	-	-	+	-	-	+	+
	Aqueous residue	+	-	-	+	++	+	-	++	+++
D. brunonianum	*n*-hexane	+	-	-	+	+	-	+	+	+
	Dichloromethane	+	-	-	+	+	-	++	+	-
	Ethyl acetate	+	-	-	-	+	-	+	-	-
	Aqueous residue	++	-	-	-	+	-	++	-	-
L. latifolia	*n*-hexane	-	-	-	-	+	+	-	-	+
	Dichloromethane	-	+		-	+	++	-	-	+++
	Ethyl acetate	++	-	-	-	+	-	-	++	+
	Aqueous residue	++	-	-	-	+	-	-	-	-
E. purpurea	*n*-hexane	+	-	-	-	+	++	-	+	++
	Dichloromethane	+	+	-	-	++	++	-	++	+++
	Ethyl acetate	+	-	-	-	+	++	-	-	++
	Aqueous residue	++	-	-	+	++	+	-	-	+
P. abrotanoides	*n*-hexane	-	++	-	-	++	++	-	+	++
	Dichloromethane	+	++	-	-	+	++	-	-	++
	Ethyl acetate	+	-	-	+	+	+	-	-	++
	Aqueous residue	-	++	-	-	-	+	++	+	+
A. tibetica	*n*-hexane	+	++	-	-	++	-	-	-	-
	Dichloromethane	++	+	-	-	+	+	+	-	+
	Ethyl acetate	++	+	-	-	+	-	++	-	++
	Aqueous residue	++	+	-	-	+	-	-	-	-

+++: Highly present, ++: Moderately present, +: Present in traces, -: Absent

Table 2 : Antimicrobial activity of the plant fractions

		Antibacterial activity				Antifungal activity		
Plant	Fraction[b]	*E. coli* (2461)	*S. aureus* (2413)	*Acinetobacter* sp. (2457)	*Serratia* sp. (2442)	*A. niger* (MTCC 872)	*F. moniliforme* (MTCC2088)	*C. albicans* (MTCC 227)
N. longibracteata	*n*-hexane	20.33±0.58	21.67±0.58	13.33±1.15	17.67±0.57	18.67±0.57	13.00±1.00	12.67±1.15
	Dichloromethane	12.33±0.57	12.67±1.15	12.33±1.15	10.67±0.58	13.33±0.58	9.33±0.58	9.33±0.58
	Ethyl acetate	10.67±0.58	10.33±1.15	10.67±1.15	8.33±0.57	12.33±1.15	9.33±0.58	9.33±0.58
	Aqueous residue	10.67±0.57	10.33±0.58	10.33±0.58	9.67±0.57	12.33±1.15	9.33±0.58	9.33±0.58
R. imbricata	*n*-hexane	20.33±0.58	20.33±0.58	20.33±0.58	20.33±0.58	9.33±0.58	13.33±0.58	12.33±0.58
	Dichloromethane	12.33±0.58	12.33±0.58	18.67±0.57	18.00±1.15	9.33±0.58	9.33±0.58	8.33±0.57
	Ethyl acetate	10.67±0.57	9.33±0.58	9.33±0.58	9.33±0.58	9.33±0.58	8.33±0.57	8.33±0.57
	Aqueous residue	9.33±0.58	9.33±0.58	18.33±1.15	9.33±0.58	ND	8.33±0.57	ND
D. brunonianum	*n*-hexane	12.33±0.58	12.33±0.58	11.33±0.58	12.33±0.58	ND	ND	ND
	Dichloromethane	14.67±1.15	9.33±0.58	14.67±1.15	14.67±1.15	ND	9.33±0.58	ND
	Ethyl acetate	12.33±0.58	11.00±0.58	11.33±0.58	10.00±0.57	ND	8.33±0.57	ND
	Aqueous residue	14.67±1.15	14.67±1.15	ND	ND	ND	ND	ND
L. latifolia	*n*-hexane	9.33±0.58	10.33±0.58	9.33±0.58	9.33±0.58	9.33±0.58	ND	ND
	Dichloromethane	18.67±0.57	12.33±0.58	10.33±1.15	9.33±0.58	13.33±0.58	18.67±0.57	ND
	Ethyl acetate	13.33±0.58	8.33±0.57	8.33±0.57	13.33±0.58	ND	ND	ND
	Aqueous residue	9.33±0.58	8.33±0.57	8.33±0.57	9.33±0.58	ND	9.33±0.58	ND
E. purpurea	*n*-hexane	12.33±0.58	14.67±1.15	14.67±1.15	9.33±0.58	14.67±1.15	ND	9.33±0.58
	Dichloromethane	14.67±1.15	9.33±0.58	13.33±0.58	13.33±0.58	14.67±1.15	9.33±0.58	9.33±0.58
	Ethyl acetate	9.33±0.58	9.33±0.58	18.67±0.57	14.67±1.15	9.33±0.58	9.33±0.58	14.67±1.15
	Aqueous residue	8.33±0.57	8.33±0.57	9.33±0.58	8.33±0.57	14.67±1.15	ND	9.33±0.58
P. abrotanoides	*n*-hexane	14.67±1.15	14.67±1.15	14.67±1.15	19.67±0.57	7.00±0.00	ND	8.33±0.57
	Dichloromethane	9.33±0.58	18.67±0.57	14.67±1.15	14.67±1.15	7.00±0.00	ND	8.33±0.57
	Ethyl acetate	9.33±0.58	9.33±0.58	ND	9.33±0.58	7.00±0.00	ND	9.33±0.58
	Aqueous residue	8.33±0.57	8.33±0.57	9.33±0.58	9.33±0.58	19.67±0.57	ND	8.33±0.57
A. tibetica	*n*-hexane	9.33±0.58	8.33±0.57	16.67±0.67	10.67±0.57	12.33±0.58	8.33±0.57	9.33±0.58
	Dichloromethane	11.33±0.58	12.33±0.58	13.33±0.58	10.67±0.57	10.67±0.57	8.33±0.57	8.33±0.57
	Ethyl acetate	9.33±0.58	8.33±0.57	16.67±0.67	8.33±0.57	10.67±0.57	8.33±0.57	ND
	Aqueous residue	9.33±0.58	9.33±0.58	10.67±0.57	8.33±0.57	9.33±0.58	11.33±0.58	11.33±0.58
Clove (*Syzygium aroma-ticum*)	Methanolic extract	22.33±0.58	16.67±1.15	15.67±0.57	15.33±0.57	22.33±0.57	15.33±0.57	15.67±0.57
Reference	Tetracycline c	22.33±0.58	19.33±0.58	18.33±0.58	20.33±0.58	NT	NT	NT
	Ketoconazole c	NT	NT	NT	NT	18.33±0.58	19.67±0.57	16.67±0.57

[a]Antimicrobial activity expressed as diameter of zone of inhibition in mm including 7 mm as diameter of the well. Values are mean inhibition zone (mm)±SD of three replicates, [b]All fractions were tested at 1 mg mL^{-1} concentrations, [c]30 µg tetracycline and ketoconazole discs were used as positive control, ND: Not detectable, NT: Not tested

Table 3: Minimum Inhibitory Concentration (MIC) of the most potent fraction (*n*-hexane) of *N. longibracteata* (NIH)

	[a]MIC	
Test organisms	NIH	Clove oil
E. coli (2461)	125	125
S. aureus (2413)	125	250
Acinetobacter sp. (2457)	500	250
Serratia sp. (2442)	250	250
Aspergillus niger (MTCC 872)	250	250
Fusarium moniliforme (MTCC 2088)	250	250
C. albicans (MTCC 227)	250	125

[a]MIC determined by microdilution method and expressed in µg mL^{-1}

Table 4: Major phytochemicals identified in the *n*-hexane fraction of *N. longibracteata* by GC-MS

Peak	[a]Name of compound	Retention time	Area (%)	RI[b]	RI[c]	Activity reported
1	N-[(5-methyl-5-hexenyl)-2-oxy]pyridine-2(1H)-thione	16.14	2.73	1384	NA	Not reported
2	1-o-acetyl-2-azido-2,3,6-trideoxy-alpha-DL-ribo-hexopyranose	16.34	1.26	1391	NA	Reported in medicinal plants
3	Methyl-octadeca-13,14-dienoate	18.713	1.12	1484	NA	Antimicrobial
4	2-dodecanone	26.987	4.22	1852	1404	Antimicrobial, anthelmintic, hirudicide, nematicide
5	Methyl 2-[(aminosulfonyl)methyl]benzoate	27.54	0.45	1879	NA	Not reported
6	Hexadecanoic acid, methyl ester	28.623	17.38	1933	1933	Antibacterial, antioxidant, hypocholesterolemic, nematicide, hemolytic, 5 alpha reductase inhibitor
7	*n*-hexadecanoic acid	29.51	50.32	1979	1979	Antibacterial, antioxidant, hypocholesterolemic, nematicide
8	12-methyl-tetradecanoic acid methyl ester	32.44	6.07	2136	2037	Antibacterial, larvicidal and insect repellant
9	Pentadecanoic acid	33.15	8.75	2175	2720	Antibacterial and antifungal
10	2-propenyl (2,2-difluorocyclopropyl)methyl ether	33.613	0.08	2201	NA	Not reported
11	2,4-bis(n-hexyl)-1,3-dithiethane 1,1,3,3-tetraoxide	33.907	0.02	2218	NA	Not reported
12	1-chloroethyl acetate	35.337	0.04	2302	NA	Not reported
13	Tridecanoic acid, methyl ester	35.953	2.46	2339	1625	Antibacterial and antifungal
14	3-acetoxy-1,2,4-trioxolane	36.127	0.54	2350	NA	Not reported
15	2-furanpropanoic acid, tetrahydro-alpha.,5,5-trimethyl-	36.47	1.15	2370	NA	Antimicrobial
16	2-methyl-2-nitro-propan-1-ol	36.57	1.45	2376	900	Antimicrobial
17	Acetic acid, methyl ester	39.527	0.45	2539	NA	VOC emission from branches of plants
18	Bis(2-oxiranylmethyl) ether	43.41	0.08	2711	NA	Not reported
19	Acetonyl decyl ether	45.113	0.26	2810	NA	Flavor component found in tea, coffee and cocoa
20	N-heptyl disulphide	46.713	0.17	2910	NA	Antibacterial
21	Acetic acid, trichloro-, propyl ester	50.513	0.44	3100	1044	Not reported
22	(+)-3,4-dimethyl-3,4-epoxypentan-1-ol	57.897	0.57	3354	NA	Reported in medicinal plants

Components listed in order of elution, [a]Compounds identified through NIST11/Wiley8 Mass Spec library, [b]Retention index on SPB-5 column, [c]Retention index as compared to NIST library

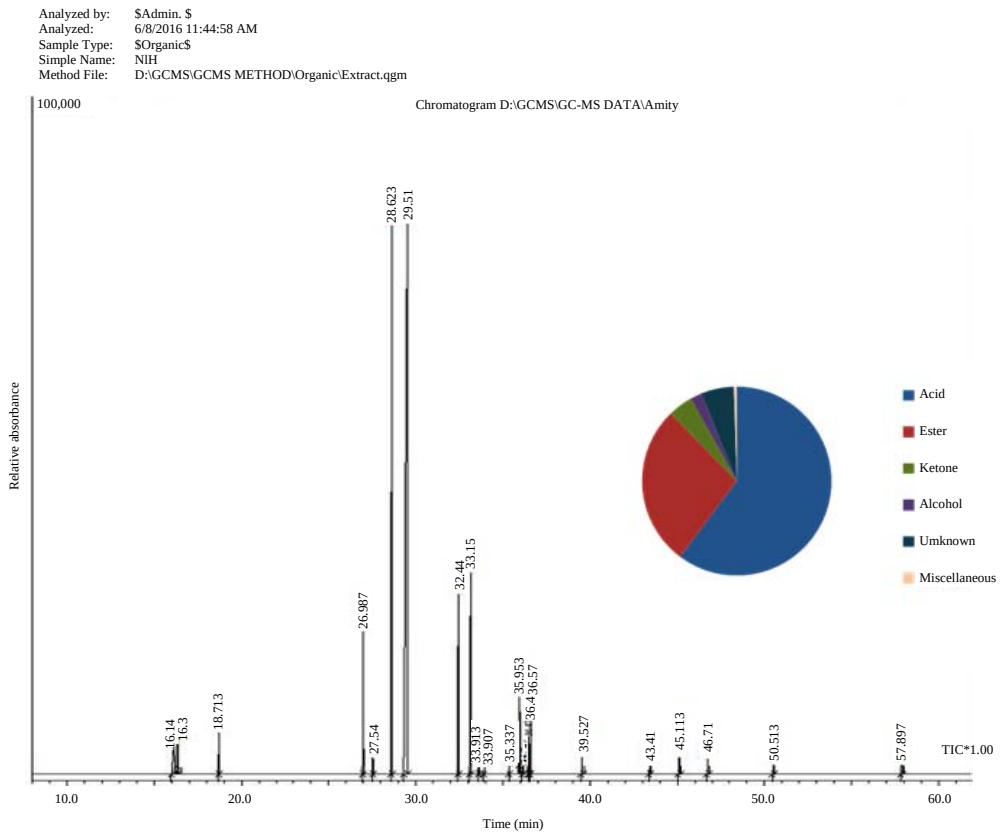

Fig. 1: GC-MS of n-hex fraction of *N. longibracteata*
The compounds were classified on the basis of chemotypes and represented as pie chart

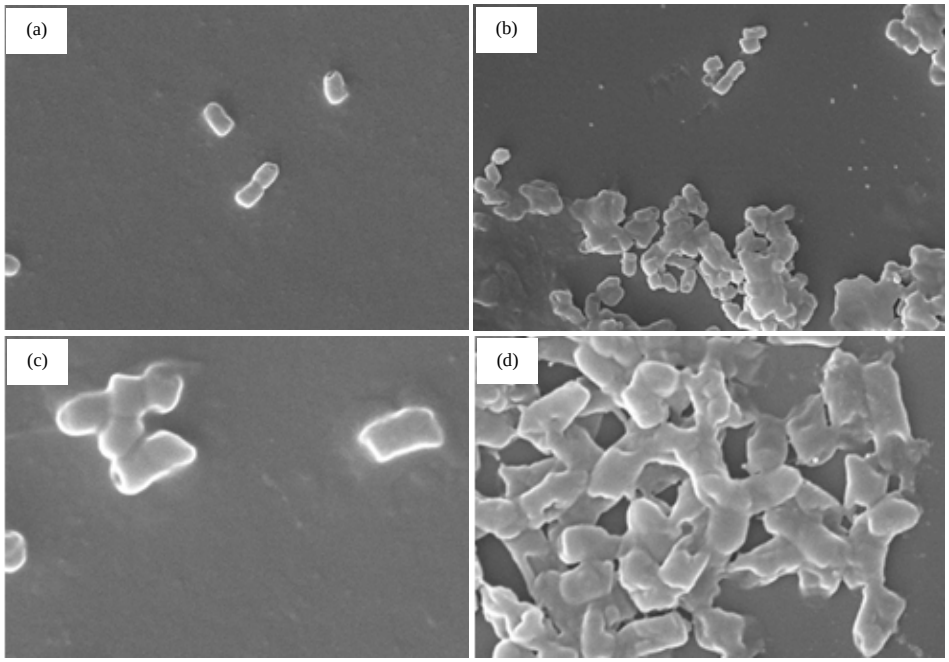

Fig. 2(a-d): Scanning electron micrograph of *E. coli* treated with *n*-hexane fraction of *N. longibracteata* (NIH). (a, b) Untreated cells appeared smooth and intact and (c, d) NIH treated cells are swollen and have distorted cell morphology magnification of (a, c) 10000X and (b, c) 25000X

DISCUSSION

The phytochemical analysis data provides an overview of the chemical classes and their relative proportion in a fraction. This knowledge can serve as a background to select isolation strategy of the active ingredients. Several secondary metabolites from plants have been reported as potential antimicrobial agents including alkaloids, flavonoids, tannins and terpenes[17]. Phlobatannins and tannins have been reported to possess significant antibacterial activity in particular against *E. coli* and *S. aureus*[18]. Similarly, saponins which are made up of carbohydrates linked to steroids or terpenes have also demonstrated antibacterial activity against *S. aureus*[19]. Presence of saponins, phlobatannins and steroids in the most active fraction indicates that the observed antimicrobial activity of the most active fraction NlH could be due to the presence of these secondary metabolites.

The antibacterial screening conducted with 28 fractions of seven plants resulted in the identification of NlH as the most active fraction with significant activity against *S. aureus*, *Serratia* sp., *Acinetobacter* sp. and ampicillin and chloramphenicol resistant *E. coli* with MIC in the range of 125-500 μg mL^{-1}. The screening result indicated that NlH is equally effective against Gram positive as well as Gram negative microorganisms. The antifungal screening outcome showed that NlH possesses substantial antifungal activity with MIC 250 μg mL^{-1} against filamentous fungi, in particular *Aspergillus* species, known for mycotoxin production and food spoilage. The MIC values are comparable to that of *Ocimum basilicum* (300 μg mL^{-1}) and lower to that of *Withania somnifera* (600 μg mL^{-1}) which are reported to have strong antifungal activity against *Aspergillus* species[20].

The antibacterial activity of NlH was comparable to that of clove oil, a well-established antimicrobial agent used in various food preparations and herbal medicines[21,22]. The DCM fractions of *D. brunonianum*, *L. latifolium* and *E. purpurea* also demonstrated moderate to high antibacterial activity however, we considered *n*-hexane fraction of *N. longibracteata* for further analysis, considering its common biological functionality.

Phytochemical profiling of NlH by GC-MS resulted in the identification of fourteen compounds where, *n*-hexadecanoic acid and methyl hexadecanoate represented 67.7% of the total constituents.

A large number of secondary metabolites including, nepetalactones, monoterpenes (iridoids and their glucosides), diterpenes, triterpenes, phenolics and flavonoids have been reported from the genus *Nepeta*[23-25], however, none of the reported nepetalactones and monoterpenes were identified in the active fraction. Moreover, the present investigation demonstrated that, there is striking differences in phytochemical profile of the essential oils isolated from various *Nepeta* species collected from Jammu and Kashmir region[26]. Interestingly, occurrence of almost negligible antioxidant activity in NlH[27] as determined by free radical scavenging assay suggested that, bioassay based fractionation could successfully concentrate antimicrobial activity in a particular fraction. Similar results have been obtained in a previous study on *Nepeta deflersiana* where the non-polar fraction had significant antibacterial activity but moderate antioxidant activity[28]. Various publications on *Nepeta* volatile oil, clearly demonstrate that chemical polymorphism is common characteristic of this species and oil composition varies on a number of factors including geographical, climatic conditions and genetic differences[29]. Might be, the extreme climatic condition of Ladakh does not facilitate to produce nepetalactones and monoterpenes which is also not found in other *Nepeta* species of the Himalayas. Cell cytotoxicity of the active fraction was evaluated against chick embryo culture based fibroblast cells and was well within the permissible limit. The presence of large amount of fatty acid and ester in NlH prompted us to study effect of the fraction on the cell morphology of *E. coli* through SEM. The untreated *E. coli* cells appeared intact, rod shaped, having smooth surface and separated from each other while the NlH treated cells appeared to be swollen, aggregated and partially deformed. Figure 2c and d indicated that the bioactive fraction induced potential morphological changes in the treated cells with respect to the untreated negative control (Fig. 2a, b). It also appeared that the cytoplasmic material of the bacterial cells had leaked and the aggregate cells had multiple blisters on the cell surface. Similar observations indicating the distortion and aggregation of bacterial cells as a stress response to exposure to antimicrobial compounds has also been reported previously[30]. The present study confirms the broad-spectrum antibacterial nature of NlH as well as its membrane damaging property. The main component of NlH constitutes of Free Fatty Acids (FFA) and their esters. These have been reported to possess antibacterial properties, typically broad spectrum, comparable to natural antimicrobial peptides (AMPs)[31]. Even though their precise mode of action is complex and unclear, their primary target appears to be cell membrane. Due to their amphipathic nature, FFA can interact with the cell membrane and can create transient or permanent pore of variable size. The increased permeability of the membrane by the insertion of FFAs allows internal contents to leak from the cell, which can cause growth inhibition or even death[32]. The use of FFA is safe and finds wide applications in the field of medicine,

agriculture and food preservation due to its broad spectrum and non-specific mode of action. Moreover, the evolution of inducible FFA-resistant phenotypes is less problematic than with conventional antibiotics[33]. In addition, the membrane permeabilizing activity of NlH, as demonstrated in this study, may be exploited in future to increase the efficacy of conventional antibiotics by enhancing their cellular uptake. Such a strategy holds immense potential in the current scenario of continuous emergence of antibiotics resistant microorganisms.

CONCLUSION

Summarizing the results, bioassay guided fractionation identified *n*-hexane fraction of *N. longibracteata* as the most active fraction in terms of antimicrobial activity against MDR pathogens. The GC-MS profile was distinctive in nature which could be used further, to differentiate *Nepeta longibracteata* from other *Nepeta* species. The antibacterial activity of NlH was attributed by the degradation of bacterial cell wall, which could be beneficial for commercialization of the fraction in herbal medicine and food preservation.

SIGNIFICANCE STATEMENTS

- This study discovers the *n*-hexane fraction of *Nepeta longibracteata* (NlH) exhibited most significant antibacterial activity against *E. coli* and *S. aureus* comparable to that of clove oil
- SEM analysis revealed morphological changes induced in bacteria by NlH

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