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CHEMICAL PROFILE OF THE ANTIBACTERIAL COMPONENT FROM *LEPTOLYNGBYA* SP. HNBGU 002 ISOLATED FROM A HOT SPRING OF GARHWAL HIMALAYA

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ABSTRACT: Cyanobacteria are a well-established source of antibacterial compounds. However, the hot spring cyanobacterial strains are not properly explored for the purpose. The study investigates the antibacterial potential of the thermophilic oscillatoriales isolated from a hot spring of Garhwal Himalaya. Out of 48 endo-metabolite pools, extracted from 16 thermophilic cyanobacterial strains, tested for antibacterial activity against the bacterial pathogens, the DEEL (diethyl-ether extract of *Leptolyngbya* sp. HNBGU 002) was found to be active against *Staphylococcus aureus* ATCC 25923 with the minimum inhibitory concentration of 2.0 mg ml^{-1} and was able to disrupt the cell membrane function of bacteria as evidenced by an increase in membrane conductivity. In addition, DEEL was also found to be active against MRSA (methicillin-resistant *Staphylococcus aureus*) and VRE (vancomycin-resistant *Enterococcus faecium*) strains. Gas chromatography-mass spectrometry analysis revealed the hydrocarbons and phenolics as the major compounds present in the DEEL. Besides, a triazolo-pyrimidine (TP) derivative, 1; 7-methyl-6-nitro[1,2,4] triazolo[1,5-a] pyrimidin-5-ol, was also detected in the active fraction. The findings suggest that the antibacterial activity of DEEL may be attributed to the hydrocarbons, phenolics, the TP derivative (1), and their synergistic effects. To the best of our knowledge, being the first report on the occurrence of a TP derivative (1) from cyanobacterial sources, this is an interesting finding in view of pharmaceutical industries. The study suggests that the DEEL is a potential source of antibacterial metabolites and must be fractionated to characterize these compounds.

INTRODUCTION: Bacterial pathogens, particularly ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa,* and *Enterobacter* species) are causing a serious threat to the human population worldwide [1].

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The rapid development of antimicrobial resistance in these pathogens is worsening the situation and leading to the generation of new superbugs like VRE (Vancomycin-resistant Enterococci) and MRSA (Methicillin-resistant *Staphylococcus aureus*), included in the high-priority list of pathogens by World Health Organization [2].

On the other hand, the rate of development of new antimicrobial drugs is having a nearly static pace and thus tempting the scientists across the globe to screen various sources for finding the novel antimicrobial leads. Microbes have always been the source of new antimicrobial lead compounds since

the beginning of the last century. About 60% of the antimicrobial compounds have been isolated from microbial sources itself during 1981-2014 [3]. However, more than 90% of world's biodiversity is still to be tested for their bioactivity [4, 5]. Nowadays, endophytes, actinobacteria and cyanobacteria are being considered as most potential sources of antimicrobial compounds [6, 7].

Cyanobacteria, also known as blue-green algae, a group of gram-negative, oxygenic photosynthetic bacteria, have emerged as a potential source for antibiotic compounds in the last three decades [8-11]. However, majority of cyanobacterial strains studied for bioactivity belong to the order oscillatoriales and were isolated from marine habitats [12].

The natural cyanobacterial mats inhabiting thermal springs could be studied for its bioactivities and metabolite spectrum by few workers only in the current decade [13, 14]. Cyanobacteria from hot water springs could be a good source of thermostable bioactive metabolites, as they have refined their metabolic spectrum according to requirements for thermal adaption during their long evolutionary journey [15].

However, the bioactivity of laboratory-grown unialgal thermophilic cyanobacterial strains is least explored especially from the Himalayan region. Garhwal Himalaya endows a number of hot water springs containing thick mats and bloom of a diversified and substantial population of thermophilic freshwater cyanobacteria [16]. Therefore, the present investigation was carried out to determine the antibacterial activity of hot spring cyanobacterial strains isolated from Garhwal Himalaya against the multidrug-resistant clinical pathogens and chemical profiling of the antibacterial fraction by GC-MS analysis.

MATERIALS AND METHODS:

Collection of Cyanobacterial Sample: A total of sixty- eight cyanobacterial bloom and mat samples were collected in sterile polybags (HiMedia) from Taptkund hot spring, Badrinath (30°74'48" N and 79°49'18" E; elevation 3250 m) situated in Garhwal Himalaya, Uttarakhand, India in the month of April 2015. Samples were transported to the laboratory in sealed containers within 24 h and divided into three parts. A small portion of each sample was fixed in formalin immediately after reaching the laboratory.

The second portion was subjected to microscopy, and the third portion was processed for isolation of cyanobacteria.

Isolation and Cultivation of Cyanobacteria: The cyanobacterial bloom or mat samples were processed for isolation of cyanobacteria using standard microbiological techniques. The colonies were transferred alternatively in solid and liquid media for purification. The uni-algal isolates were cultivated in the 500 ml flasks containing 300 ml Castenholz-D medium and incubated in an incubator illuminated with fluorescent tubes (95 μmol photons m^{-2} s^{-1}) under 14:10 h light: dark cycle at 50 ± 2 °C [17]. All the chemicals used for the preparation of culture media were procured from HiMedia Laboratories Pvt. Ltd.

Identification of Cyanobacteria: The uni-algal isolates of cyanobacteria were identified on the basis of their morphological, morphometric features, and available literature [18, 19]. Morphometric analysis was performed using a light microscope (Olympus CX 21i), fitted with camera (Magnus 15F0419) and Magnus pro (version 3.7) software. The selected cyanobacterial isolate was subjected to partial 16S rDNA sequence homology analysis for molecular identification. Partial 16S rDNA sequence of this cyanobacterium was submitted to NCBI GenBank.

Biomass Harvesting and Preparation of Extracts: Cyanobacterial biomass was harvested in the late stationary phase of their growth by centrifugation at 4500 rpm for 20 min. The harvested biomass was dried at 50 ± 2 °C, powdered and extracted by the freeze-thaw method using three different solvents viz ethanol, methanol, and diethyl ether. The extracts were evaporated to dryness, re-dissolved in 10% DMSO (Di-methyl-sulphoxide), maintaining the concentration of 100 $mg.ml^{-1}$ and stored at 4 °C until further use. The extraction yield of different solvents was calculated using the formula:

$$\text{Yield (\%)} = (W_1 \times 100) / W_2$$

(Where, W_1 is the weight of dry extract and W_2 is the weight of dry biomass)

Bacterial Strains: Drug sensitive bacterial strains, *S. aureus* (*Staphylococcus aureus*) ATCC 25923 and *E. coli* (Escherichi coli) ATCC 25922 were obtained as gifts from Institute of Medical Sciences, Banaras Hindu University, India. The multidrug-resistant bacterial strains, MRSA and VRE were obtained from Veer Chandra Singh Garhwali Government Medical Science and Research Institute, Srinagar Garhwal, Uttarakhand India, and characterized in the laboratory on the basis of standard biochemical tests as well as their antibiotic resistance profile (antibiogram) [20].

Antibacterial Assays: Total 48 different organic extracts obtained from 16 different thermophilic cyanobacterial isolates were tested for their antibacterial activity using agar well diffusion method [21]. Briefly, the bacterial suspensions containing (~10^8 cfu.ml^{-1}) were spread uniformly over the sterilized MHA (Mueller Hinton Agar) plates. The plates were dried for 30 min, and the wells of 08 mm diameter were made on the agar using agar borer. Test wells received 0.2 ml extracts, whereas the control wells received DMSO and chloramphenicol representing the negative and positive control, respectively. After addition of extracts in the well, the plates were first incubated at 4 °C for 4 h for the diffusion of extracts, followed by 20 h incubation at 37 ± 2 °C to observe the zones of bacterial growth inhibition.

The minimum inhibitory concentration (MIC) of the cyanobacterial extract was determined using macro broth dilution method as per CLSI guidelines [22]. The lowest concentration of the extract causing no visible growth was considered as MIC. The bacterial culture treated with MIC of the active extract was sub-cultured on fresh NA (Nutrient Agar) plates to observe the bacteriostatic/bactericidal nature of extract.

Effects of Crude Extract on Bacterial Membrane Conductivity: Membrane conductivity assay was carried out according to the method of Jiang *et al.,* [23,] with slight modifications. Briefly, the 03 ml MHB (Muller –Hinton Broth) containing 2% bacterial inoculums was mixed with MIC of the crude extract. The mixture was incubated at 37 ± 2 °C for 8 h, and the 0.5 ml aliquots were withdrawn from the mixture at the regular interval of 2 h. The cells were separated from the aliquots by centrifugation, and the supernatant was diluted 20 fold with PBS buffer (pH 7.4). The conductivity of the diluted samples was measured with a digital conductivity meter (EI-1504189). The bacterial culture treated with DMSO only was used as a control. Results were recorded and compared with the control.

GC-MS Analysis: The selected cyanobacterial extract with antibacterial activity, the diethyl-ether extract of *Leptolyngbya* sp. HNBGU 002 (DEEL), was subjected to analysis by the coupled GC-MS (Agilent mass-spectrometer USA, GC 7890B; MS 5977B MSD) instrument using the method of Abdel-Aal *et al.,* [24] with slight modification. The GC-MS instrument was operated with the following conditions; DB-5 MS (5% phenyl methyl polysiloxane) column with 30 m × 250 µm × 0.25 µm dimensions, solvent delay- 3 min, the injection volume- 1.0 µl, carrier gas - helium with the pulsed split-less mode at 3 ml min^{-1}. The total run time was 42.5 min, where the initial temperature was 60 °C with a rising rate of 8 °C min^{-1} and a maximum up to 280°C with a hold time of 10 min. The ion source temperature was set at 230 °C (maximum 250), and the quadruple temperature was set at 150 °C (maximum 200).

An inbuilt mass detector was used for the mass detection of the peaks. The detector was operated with the ionizing energy of 70 eV with the scanning range of 30-800 m/z values. The electron multiplier voltage (EM voltage) was maintained at 1065.7 with a gain factor of 1.00. Mass-Hunter / NIST17 software with a database library was used for the identification of the separated peaks.

Statistical Analysis: One-way ANOVA (Analysis of variance) followed by LSD (Least significant difference) was used for comparing the sizes of growth inhibition zone and the effects on bacterial membrane conductivity caused by different concentrations of the DEEL.

RESULTS: In the present study, a total of 68 cyanobacterial mat samples were collected from the hot water spring situated in Garhwal Himalaya. The temperature and pH of the hot water springs varied in the range of 52-68 °C and 6.2-6.8, respectively. Out of 68 fresh cyanobacterial mat samples collected, 26 different cyanobacterial morphotypes

including 01 chroococcales, 01 pleurocapsales, 19 oscillatoriales, 03 nostocales, 02 stigonematales, could be observed through light microscopy. Out of 19 oscillatoriales, only 16 morphotypes could be isolated in unialgal forms, cultivated and maintained in the laboratory.

TABLE 1: MORPHOLOGICAL AND MORPHOMETRIC FEATURES OF CYANOBACTERIA ISOLATED FROM TAPTKUND HOTSPRING, BADRINATH, UTTARAKHAND

S. no.	Cyanobacterial strains	Characteristic feature observed through a light microscope			
		Structure, arrangement and cellular organization	Length and width (average with standard error)	Sheath	Cell content
1	*Leptolyngbya sp.* HNBGU 002	Unbranched, nearly straight, more or less parallel, end cell rounded, not capitates, not visible septa, not constricted	Filaments 1.88 ± 0.11 µm broad	Thin and diffluent	Mucilaginous and blue-green.
2	*Leptolyngbya* sp	Filament straight or slightly curved or bent, rigid, single and gregarious, not constricted at the cross walls and not granulated	Filament 3.11 ± 0.28 µm broad	Thick and colorless sheath observed	Pale blue-green
3	*Lyngbyalutea*	Gelatinous, leathery and coiled filament with rounded end cell, granulated cross walls	Filament 4.38 ± 0.32 µm broad	Thick and colourless sheath	Yellowish brown to olive-green
4	*Lyngbya mesotricha*	Unbranched filaments erect or more or less curved, not constricted at cross-walls, outer wall thick and ends not attenuated	Filaments 2.75±0.22 µm broad	Thin and colourless	Content pale blue green
5	*Mastigocladus* sp.	Branched filaments, irregular in shape and size, cell content homogeneous, constricted at cross walls ends not attenuated	Cells 2.36 ± 0.17 µm broad and 5.57 ± 0.26 µm long	Thick outer wall	Light blue green,irregular shape
6	*Oscillatoria jasorvensis*	Unbranched trichome, straight, somewhat bent at ends, not attenuated and without capitates, cross wall are not properly defined	Filaments 2.55 ± 0.34 µm broad	Not observed	Pale or yellowish blue green
7	*Oscillatoria unigranulata*	Unbranched, tenuous, straight or less curved, calyptras absent, not constricted at cross-wall, large granule at the centre of partition	Filaments 2.27 ± 0.21 µm broad	Not observed	Uniformly granular and blue green.
8	*Oscillatoria limnetica*	Unbranched filaments, straight, long, consisting of numerous cells, not attenuated and not capitates, apical cell straight and elongated	Cells 1.35 ± 0.21 µm broad and 3.16 ± 0.33 µm long	Without mucous sheath	Pale blue-green, with homogeneous content
9	*Phormidium tenue*	Unbranched filaments and straight at ends, elongated and rounded end cells	Cells 1.16 ± 0.17 µm broad and 2.63 ± 0.24 µm long	Wide, thin and diffluent sheath	Light blue green
10	*Phormidium usterii*	Unbranched trichome with intricately bent, thallus thick, mucilaginous, lacerated at margins, non-attenuated straight ends with broadly rounded ends	Filaments 3.14 ± 0.27µm broad	Sheath thin and diffluent	Content homogenous, blue green
11	*Phormidium* sp. 01	Unbranched filaments, straight and densely entangled, calyptras absent commonly visible constriction and attenuated ends	Cells 1.83 ± 0.23 µm broad	Sheath thin	Green filaments
12	*Phormidium* sp. 02	Unbranched filaments straight at ends, and densely entangled, calyptras absent, cross wall not commonly visible.	Cells 1.12 ± 0.18 µm broad and 2.56 ± 0.23 µm long	Present with the diffluent manner	Blue-green filaments
13	*Phormidium* sp. 03	Unbranched densely entangled filaments, moderate curved, distinctly constricted at the cross walls gradually towards ends	Filaments 2.54 ± 0.31 µm broad	Sheath thin and diffluent	Dark pale green cell content with
14	*Phormidium* sp. 04	Unbranched solitary filaments, homogenous cell content	1.77 ± 0.23 µm broad filament	Sheath diffluent	Bright blue green
15	*Symploca parietina*	Filaments fragile, tortuous, forming anastomosis, pale blue-green or yellowish, without calyptras, inconspicuous cross walls	Filaments are 2.81 ± 0.19 µm broad	Thin sheath observed	Trichomes pale yellowish green,
16	*Symploca thermalis*	Unbranched, constricted cross wall, end cell rounded, septate cell, divided by defined cross wall	Cells 1.25 ± 0.24 µm broad and 3.66 ± 0.29 µm long	Sheath very thin, sometimes slimy	Bright blue-green to blackish green

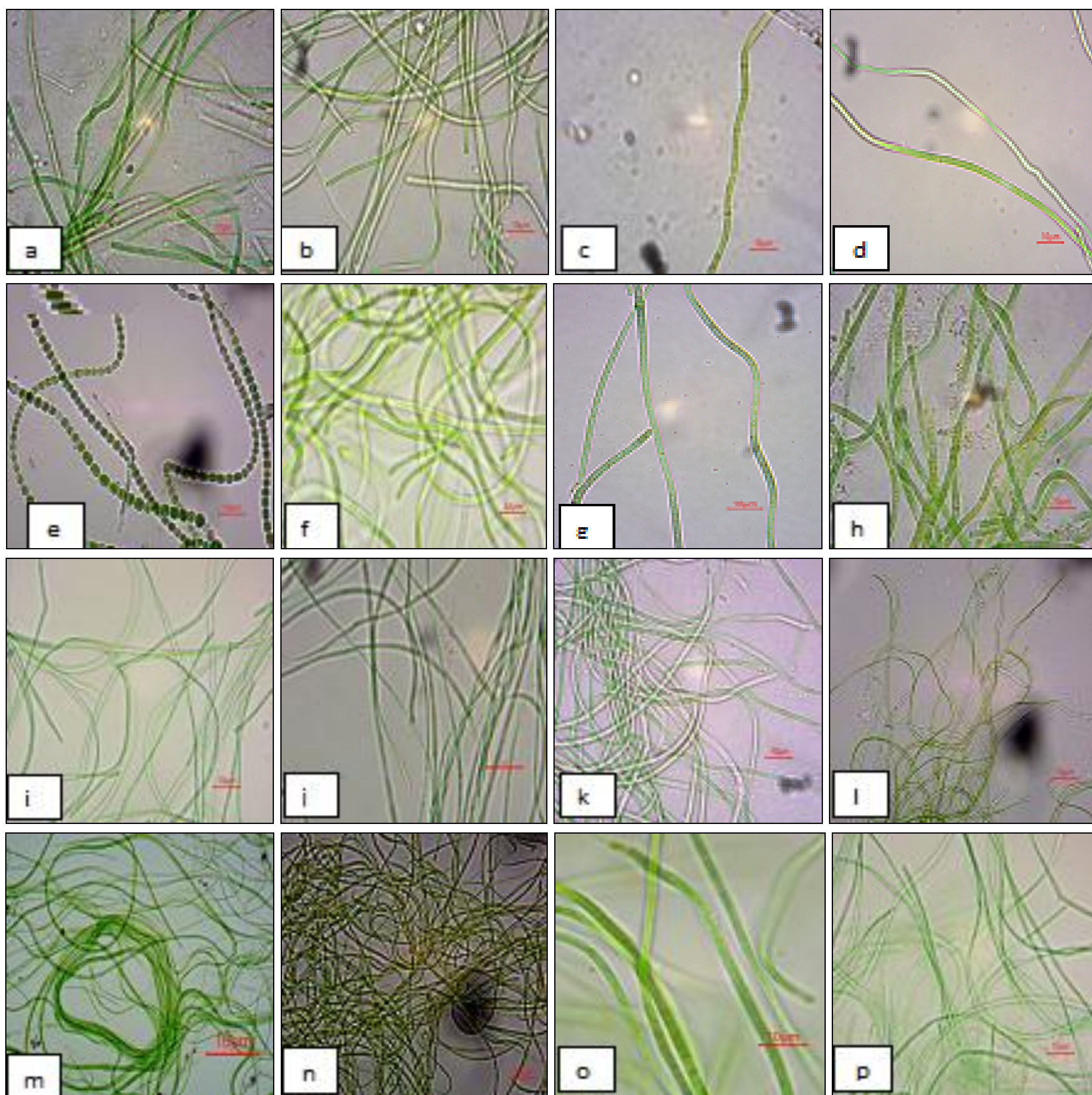

FIG. 1: PHOTOMICROGRAPHS OF CYANOBACTERIAL MORPHOTYPES ISOLATED FROM TAPTKUND HOT SPRING, BADRINATH. a - *Leptolyngbya* sp. HNBU 002, b - *Leptolyngbya* sp., c - *Lyngbya lutea*, d - *Lyngbya mesotricha*, e - *Mastigocladus* sp, f - *Oscillatoria jasorvensis*, g - *Oscillatoria unigranulata*, h - *Oscillatoria limnetica*, i - *Phormidium tenue* j - *Phormidium usterii*, k - *Phormidium* sp. 01, l-*Phormidium* sp. 02, m - *Phormidium* sp. 03, n - Phormidium sp. 04, o - Symploca *parietina*, p - *Symploca thermalis*

Identification of Oscillatoriales: The present study characterized the sixteen thermophilic cyanobacterial isolates of order oscillatoriales on the basis of morphological and morphometric features **Table 1**. The photomicrographs of these isolates are given in **Fig. 1**. The selected cyano-bacterial isolates subjected to 16S rDNA sequence homology analysis was identified as *Leptolyngbya* sp. HNBGU 002 and is publically available through NAIMCC (National Bureau of Agriculturally Important Microorganisms Culture Collection), India, under accession number NAIMCC-C-00335. The 16S rDNA sequence of the cyanobacterium is available at NCBI GenBank (Accession no MN817932). **Fig. 2** shows the photograph of *Leptolyngbya* sp. HNBGU 002 is growing in natural habitat, and its phylogenetic tree prepared on the basis of the 16S rDNA sequence.

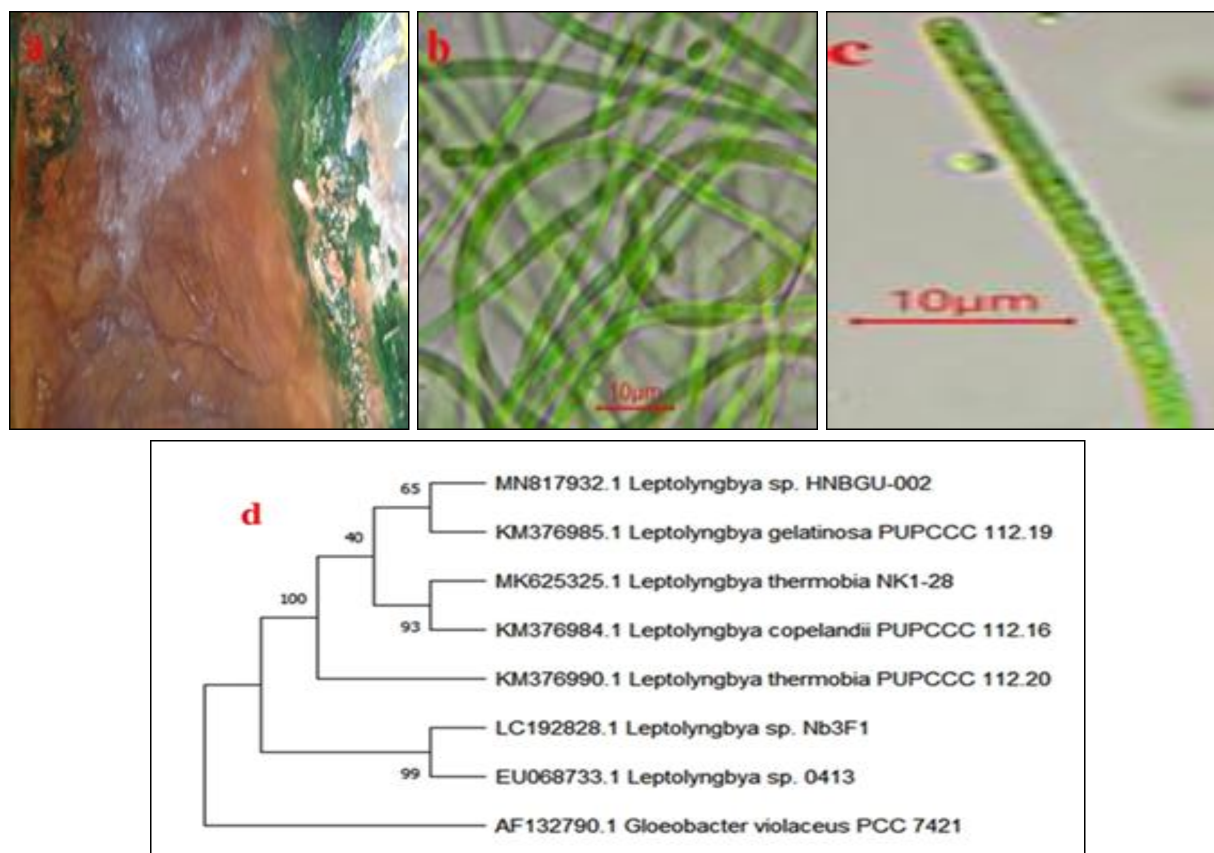

FIG. 2: MORPHOLOGY AND GENOLOGY OF *LEPTOLYNGBYA* SP. HNBGU 002; (a) Mat growing in the natural habitat, (b) photomicrograph of entangled mass, (c) photomicrograph of isolated filament, (d) Phylogenetic tree along with other selected *Leptolyngbya* strains based on partial 16S ribosomal DNA sequences. The accession number of the respective strains is given as prefix of their names. The 16S rDNA sequence of *Gloeobacter violaceus* PCC 7421 was used as an out-group. The evolutionary history was inferred using the UPGMA method. The sum of branch length is = 0.18591738. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches

Extraction of Endo-metabolites: The dried cyanobacterial biomass was extracted using three different organic solvents (methanol, ethanol, and diethyl ether).

The yield of extracts prepared with each solvent is given in **Table 2**. The maximum yield was obtained by methanol, whereas the minimum was with diethyl ether in all cases.

TABLE 2: YIELD OF CYANOBACTERIAL EXTRACTS PREPARED WITH DIFFERENT ORGANIC SOLVENTS

Cyanobacterial strains	Yield of Extracts* (mg/g of dry biomass)		
	Methanol extract	Ethanol extract	Diethyl-ether extract
Leptolyngbya sp.HNBGU 002	40.59±0.171#	24.54±0.227	11.38±0.303
Lyngbya kuetzingii	25.58±0.721	26.73±0.329	8.52±0.100
Lyngbya lutea	40.81±0.933	18.66±0.637	13.6±0.120
Lyngbya mesotricha	24.86±0.840	25.00±0.703	14.3±0.192
Mastigocladus sp.	44.99±0.458	26.92±0.395	11.5±0.330
Oscillatoria jasorvensis	36.99±1.071	25.29±0.221	10.56±0.177
Oscillatoria unigranulata	38.46±1.061	34.44±0.373	15.38±0.263
Oscillatoria limnetica	57.5±0.871	21.00±0.200	12.21±0.125
Phormidium tenue	37.02±0.611	24.99±0.395	12.5±0.308
Phormidium usterii	35.71±0.447	26.66±0.127	15.17±0.155
Phormidium sp. 01	41.39±0.594	24.16±0.536	11.66±0.437
Phormidium sp. 02	29.45±0.242	22.29±0.360	10.9±0.173
Phormidium sp. 03	27.85±0.898	26.36±0.178	9.28±0.223
Phormidium sp. 04	36.12±0.343	35.78±0.379	7.04±0.246
Symploca parietina	39.18±0.985	21.05±0.399	11.42±0.246
Symploca thermalis	39.4±0.676	24.44±0.452	11.11±0.110

#The values given represent the mean ± standard deviation (n=3)

TABLE 3: ANTIBACTERIAL ACTIVITY OF CHLORAMPHENICOL (30 µg) AND CYANOBACTERIAL EXTRACTS (2.0 mg) AGAINST DRUG SENSITIVE CLINICAL PATHOGENS

S. no.	Name of drug/cyanobacterial extract (dose)		Sizes of Growth Inhibition zone (mm) against	
			S. aureus ATCC 25923	*E. coli* ATCC 25922
1	Chloramphenicol (30 µg)		22± 0.33#	24± 0.33
2	*Leptolyngbya* sp. HNBGU 002	Ethanol	ND*	ND
		Methanol	15.2± 0.84	ND
		Di-ethyl ether	17.8 ± 0.58	14.2± 0.84
3	*Lyngbya kuetzingii*	Ethanol	ND	ND
		Methanol	9.6± 0.89	ND
		Di-ethyl ether	16± 0.71	ND
4	*Lyngbya lutea*	Ethanol	ND	ND
		Methanol	ND	ND
		Di-ethyl ether	15.4± 0.55	ND
5	*Lyngbya mesotricha*	Ethanol	ND	ND
		Methanol	12.4± 0.55	ND
		Di-ethyl ether	16.2± 0.84	ND
6	*Mastigocladus* sp.	Ethanol	ND	ND
		Methanol	12.2± 0.45	ND
		Di-ethyl ether	16.25± 0.50	9.8± 0.84
7	*Oscillatoria jasorvensis*	Ethanol	ND	ND
		Methanol	8.8± 0.84	ND
		Di-ethyl ether	16.5± 0.50	8.6± 0.55
8	*Oscillatoria unigranulata*	Ethanol	ND	ND
		Methanol	9.2± 1.09	ND
		Di-ethyl ether	14.6±0. 89	ND
9	*Oscillatoria limnetica*	Ethanol	ND	ND
		Methanol	ND	ND
		Di-ethyl ether	ND	ND
10	*Phormidium* sp. 1	Ethanol	ND	ND
		Methanol	ND	ND
		Di-ethyl ether	ND	ND
11	*Phormidium* sp. 2	Ethanol	ND	ND
		Methanol	10± 0.71	ND
		Di-ethyl ether	14.8± 0.45	ND
12	*Phormidium* sp. 3	Ethanol	ND	ND
		Methanol	15.2± 0.45	ND
		Di-ethyl ether	16.8± 0.45	ND
13	*Phormidium* sp. 4	Ethanol	ND	ND
		Methanol	15.8± 0.45	ND
		Di-ethyl ether	14.6± 0.89	ND
14	*Phormidium tenue*	Ethanol	ND	ND
		Methanol	10.2±0.45	ND
		Di-ethyl ether	ND	ND
15	*Phormidium usterii*	Ethanol	ND	ND
		Methanol	12.6± 0.55	ND
		Di-ethyl ether	15± 0.71	ND
16	*Symploca parietina*	Ethanol	ND	ND
		Methanol	13.6± 0.55	ND
		Diethyl ether	12.25± 0.50	11.2± 0.50
17	*Symploca thermalis*	Ethanol	ND	ND
		Methanol	ND	ND
		Diethyl ether	ND	ND

#The values given represent the mean ± standard deviation (n=3), *ND= Not detected

Screening of Cyanobacterial Extracts for Antibacterial Activity: Table 3 shows sizes of bacterial growth inhibition zone caused by different cyanobacterial extracts against a gram positive and a gram negative pathogen. Out of the 48 extracts tested for their antibacterial activity, 12 extracts prepared with diethyl-ether (polarity 2.8) exhibited antibacterial activity against the antibiotic sensitive strain of gram-positive pathogen, *S. aureus* ATCC 25923, whereas four diethyl-ether extracts

exhibited activity against antibiotic sensitive strain of gram negative pathogen, *E. coli* ATCC 25922 at the dose of 2.0 mg per well. Based on the size of bacterial growth inhibition zones, the most effective antibacterial activity was observed in DEEL against the gram-positive pathogen, *S. aureus* ATCC 25923.

Effects of DEEL on the Membrane Conductivity: Fig. 3 shows the changes in cell membrane conductivity of *S. aureus* ATCC 25923 treated with MIC of DEEL as compared to the untreated control. A significant increase in membrane conductivity as compared to control was observed after 2 h of exposure with the extract. After that, only a slight increase was observed in the conductivity values up to 8 h. However, the conductivity value of the treated culture was always higher than that of the control.

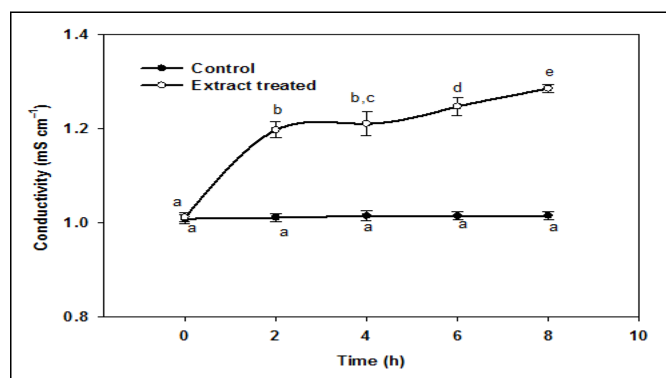

FIG. 3: EFFECT OF DEEL (DIETHYL-ETHER EXTRACT OF *LEPTOLYNGBYA* SP. HNBGU 002) AT THE DOSE OF 2.0 mg.ml^{-1} ON MEMBRANE CONDUCTIVITY OF *S. AUREUS* ATCC 25293. The values marked with the different letters are significantly different from each other ($p<0.05$)

TABLE 4: RESISTANCE PROFILE (ANTIBIOGRAM) OF MULTI DRUG RESISTANT BACTERIAL STRAINS (RED COLOUR REPRESENTS THE RESISTANCE AND GREEN COLOR REPRESENTS SENSITIVE TO THE PARTICULAR ANTIBIOTICS) ACCORDING TO CLSI 2018

S. no.	Standard antibiotics (µg)	Zone of inhibitions (mm)	
		MRSA	VRE
1	Cholramphenicol (C 30)	20.33±0.47 #	18.33±0.47
2	Amikacin (AK 10)	17.33±0.92	18.66±0.94
3	Ciprofloxacin (CIP 05)	14.33±0.47	12.33±0.47
4	Gentamycin (GEN 10)	12.33±0.47	12.33±0.47
5	Cefotaxime (CX 30)	ND*	ND
6	Ceftriaxone (CTR 30)	ND	ND
7	Vancomycin (VA 30)	ND	ND
8	Cefuroxime (CFM 10)	ND	ND
9	Amoxicillin/Clavulanate (2:1) (AMC 30)	ND	ND
10	Teicoplanin (TEI 30)	ND	ND
11	Erythromycin (E 15)	ND	ND
12	Clindamycin (CD 02)	ND	ND
13	Co-trimaxazole (COT 25)	ND	ND
14	Tetracycline (TE 30)	ND	ND
15	Linezolid (LZ 30)	ND	ND

#The values given represent the mean ± standard deviation (n=3),*ND= Not detected

Antibiogram of Multidrug-Resistant Pathogens: Table 4 shows the antibiogram of multidrug resistant (MDR) bacterial pathogens, namely methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant *E. faecium* (VRE) used in the study. The results indicate that these pathogens are resistant to most of the antibiotics commonly used in public health settings. The MRSA showed the sensitivity only to the chloramphenicol, which is bacteriostatic in nature.

Anti-MRSA and Anti-VRE activity of DEEL: Fig. 4 shows the sizes of growth inhibition zones caused by different concentrations of the DEEL and chloramphenicol (30 µg), against drug-resistant pathogens, MRSA and VRE. The results revealed the dose-dependent increase in the size of bacterial growth inhibition zones ($p<0.05$). The extract at the concentration of 2.5 mg exhibited the size of growth inhibition zones of 16.25 mm and 21.25 mm against MRSA and VRE, respectively.

The quantitative bioassay with the same extract revealed the minimum inhibitory concentration (MIC) of 2.5 mg ml^{-1} against both the multidrug resistant pathogens.

Further, the subculture of the pathogens treated with the extract at the concentration equivalent to MIC indicated the bactericidal nature of the extract **Fig. 5**.

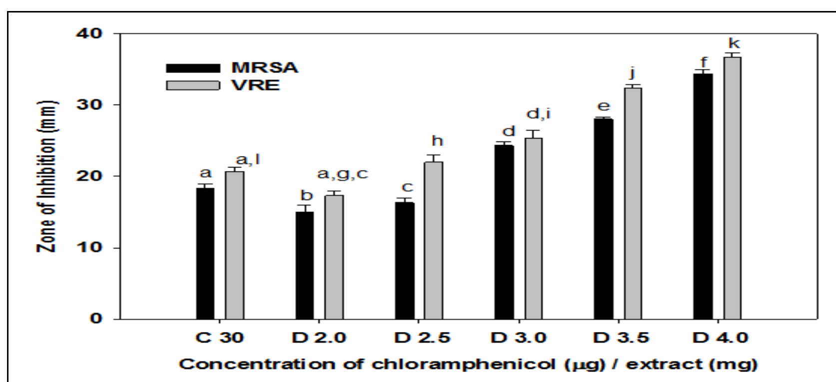

FIG. 4: ANTIBACTERIAL ACTIVITY OF CHLORAMPHENICOL (C30 = 30 µg) AND DIFFERENT CONCENTRATION (D 2.0, D 2.5, D 3.0, D 3.5, D 4.0 = 2.0, 2.5, 3.0, 3.5, AND 4.0 mg, RESPECTIVELY) OF THE DEEL. Error bars represent the standard deviation from the mean value (n=3). The values marked with the different letters are significantly different from each other ($p<0.05$)

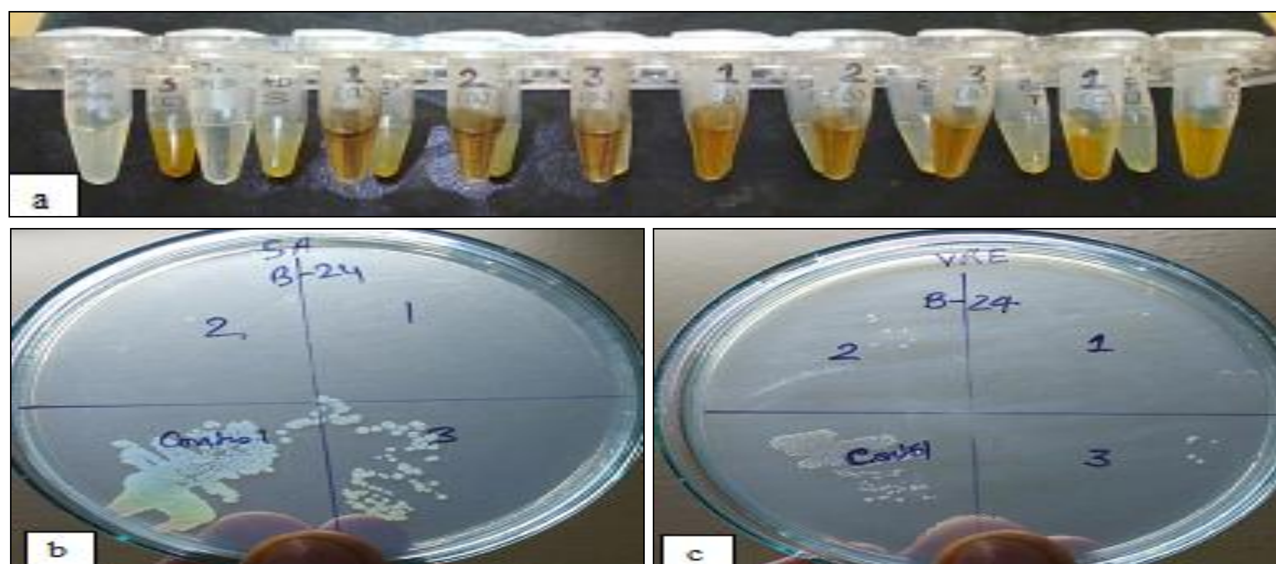

FIG. 5: MIC DETERMINATION OF DEEL AGAINST MRSA AND VRE (a-macro broth dilution tubes, b- MRSA and c-VRE subculture plates)

Chemical Profile of the DEEL: Fig. 6 shows the GC-MS chromatogram of the selected cyano-bacterial extract, DEEL. The RT (retention time) of the peaks observed in the chromatogram, major compounds retrieved from NIST mass spectral library showing ≥90% similarity index with the peaks and their corresponding molecular weight are enlisted in **Table 5**. Some of them appear more than once, although their selection criteria stick to retention time and similarity index. Hydrocarbons, its halo derivatives, esters, phenolic compounds, and 1, 2, 4-Triazolo [1,5-a] pyrimidine derivatives are the major compounds observed.

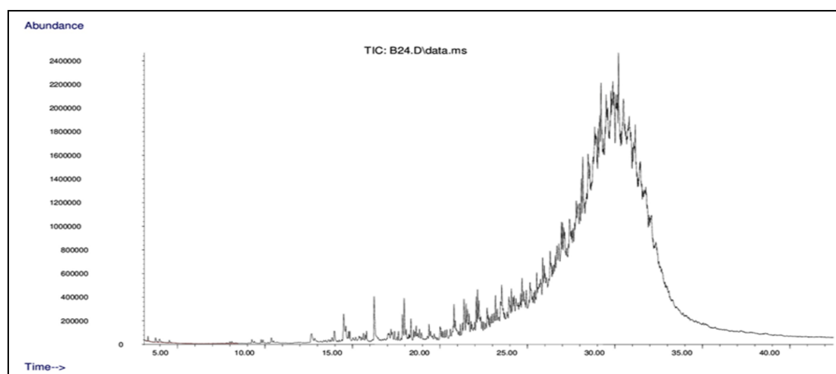

FIG. 6: GC-MS CHROMATOGRAM OF DEEL

TABLE 5: COMPOUNDS IDENTIFIED IN GC-MS ANALYSIS OF DEEL

S. no.	Retention time (min)	Peak area %	Compounds	Similarity (%)	Molecular Weight (amu)
1	3.75	0.02	Ethylbenzene	94	106.078
2	3.985	0.02	o-Xylene	94	106.078
3	12.667	0.09	Dodecane	95	170.203
4	14.504	0.18	Tridecane	97	184.219
5	15.813	0.05	Dodecane, 2,6,10-trimethyl-	91	212.25
6	16.249	0.3	Tetradecane	98	198.235
7	17.281	0.06	Nonadecane, 9-methyl-	91	282.329
8	17.868	0.1	Pentadecane	98	212.25
9	17.969	0.46	2,5-bis(1,1-dimethylethyl)- phenol	91	206.167
10	17.969	0.17	2,4-Di-tert-butylphenol	97	206.167
11	19.286	0.03	Cetene	96	224.25
12	19.378	0.06	Hexadecane	97	226.266
13	19.471	0.06	10-Methylnonadecane	91	282.329
14	20.293	0.04	Methoxyacetic acid, 2-tetradecyl ester	91	286.251
15	20.813	0.21	Heptadecane	97	240.282
16	20.888	0.12	Tetracosane	90	338.391
17	21.383	0.23	Dodecane, 4,6-dimethyl-	92	198.235
18	23.531	0.31	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	96	276.173
19	23.841	0.25	1-Eicosene	90	280.313
20	24.353	0.41	Eicosane	90	282.329
21	24.621	0.3	1-Octadecene	95	252.282
22	24.705	0.31	Tritetracontane	91	604.689
23	24.797	0.51	Nonadecane	92	268.313
24	25.284	0.3	[1,2,4]Triazolo[1,5-a]pyrimidin-5-ol, 7-methyl-6-nitro-	90	195.039
25	25.687	0.53	Nonahexacontanoic acid	93	999.07
26	26.517	0.42	1-Nonadecene	94	266.297
27	26.802	0.82	Ethanol, 2-(octadecyloxy)-	93	314.318
28	27.415	1.34	Heneicosane	92	296.344
29	27.616	0.66	1-Docosene	95	308.344
30	28.002	0.79	Tetrapentacontane, 1,54-dibromo-	93	914.682
31	28.094	0.97	Nonadecane, 1-chloro-	93	302.274
32	29.042	1.1	1-Tetracosene	95	336.376
33	29.218	2.6	Docosane	96	310.36
34	29.512	2.81	Tricosane	96	324.376
35	30.703	0.65	1-Hexacosene	95	364.407
36	31.433	2.73	Hexacosane	96	366.423
37	32.347	1.37	Octadecane, 1-iodo-	95	380.194

DISCUSSION: Unlike the previous studies on bioactivity of natural cyanobacterial mat grown in hot water springs, the present investigation was carried out to test the antibacterial potential of hot spring cyanobacterial isolates grown in the laboratory in unialgal form. Thus, the present study offers an advantage of the reproducibility of the same metabolites by the test organism under defined cultivation conditions.

As far as the thermal springs of the Himalayan region is concerned, cyanobacterial diversity has been reported by few workers only [16]. The present study revealed the dominance of oscillatoriales comprising mainly with strains of *Leptolyngbya*, *Oscillatoria*, and *Phormidium* genera in the unexplored Badrinath hot spring, situated at the elevation of 3250 m in western Himalaya, India. The members of oscillatoriales were identified on the basis of their characteristic morphological features such as uniseriate, cylindrical, isopolar, unbranched trichomes with undifferentiated cells [18, 25]. Our results are in consonance with the recent study by Singh *et al.,* [16] which reported the dominance of oscillatoriales in nine different hot water springs situated in Indian Himalayan region.

Oscillatoriales have been reported to produce about half of the known bioactive metabolites from the cyanobacterial origin [26]. Among oscillatoriales, the

most prolific producer strains of bioactive metabolites belong to the genera *Lyngbya, Oscillatoria, Phormidium* [8, 12]. Error! Reference source not found. Present study also revealed the antibacterial activity in non-polar (diethyl-ether) extracts of 75% strains tested against the *S. aureus*. The diethyl-ether extracts of all the strains exhibited better inhibition activity in comparison to that of polar (ethanol and methanol) extracts. However, the extraction yields were lesser in the case of non-polar solvents as compared to the polar ones. A previous study conducted with hot water cyanobacterial mats from the natural conditions also revealed the highest antimicrobial potential of non-polar extracts as compared to polar extracts [13]. Convincingly, diethyl-ether is an excellent solvent for the extraction of antimicrobial metabolites from the thermotolerant cyanobacterial species. Among the non-polar extracts, DEEL exhibited the most effective antibacterial activity. The results are in good agreement with the previous studies, which reported the antibacterial activity in non-polar extracts of hot spring *Phormidium* strains [13, 27].

Any chemical(s) have the specific mechanism of action which can be elucidated by its effects on the target site. The bacterial cell membrane is the most common target of antibacterial drugs. The present investigation exhibited that DEEL contributed to changes in the conductivity of *S. aureus* membrane which stabilized after 2 h of exposure with the extract. This indicates that the extract caused the slight leakage in the membrane and the intra-cellular fluid, particularly the charged particles, leaked out into the supernatant solution, which increased the conductivity of the membrane. The movement of charged particles ceased after sometime due to the equal concentration of charged particles inside and outside the cells [23]. Similar observations were also made by Wu *et al.,* [28] (2018), when they have treated the *S. aureus* with 4, 4-trisulfanediylbis. Thus, the DEEL extract must have a group of compounds that retarded the bacterial membrane functions.

The study also revealed that the DEEL has significant antibacterial activity against the drug resistant clinical pathogens, including the MRSA and VRE. To the best of our knowledge, this is the first report on antibacterial activity of diethyl-ether extract prepared with laboratory-grown biomass of unialgal thermophilic *Leptolyngbya* isolate against the drug-resistant clinical pathogens like MRSA and VRE. These two multi-drug resistant pathogens cause major public health threats across the world and imposed high morbidity and mortality rates [29]. On the other side, the physicians are facing limited choices of currently available drugs, like bacteriostatic chloramphenicol, against the infections caused by these pathogens [30]. The sizes of bacterial growth inhibition zone caused by the DEEL (2.5 mg) were observed to be comparable to that caused by chloramphenicol (30 µg). Luesch *et al.,* [31] have shown that MIC of pure bioactive constituents lies around 0.25% as compared to that of the crude extract. Thus, the DEEL offers a new source for the discovery of urgently needed bactericidal medicines against the superbugs, MRSA and VRE, listed in the WHO's priority list.

Further, DEEL was subjected to GC-MS analysis, a technique used for the identification of non-polar and volatile component of the crude extracts. This analysis revealed that the active extract has the versatile chemical profile, including the higher percentage (~30%) of hydrocarbons, their esters, and halogen derivatives followed by tris-butyl phenolics and other compounds. Similar to our findings, previous studies have also reported the hydrocarbons as principal volatile components in the non-polar extracts of cyanobacteria and algae having antibacterial activity [13, 32, 33]. However, these major compounds (hydrocarbons) are also known for non-selective cytotoxicity, henceforth cannot be developed as antimicrobial drugs [34]. The second major group of compounds observed in the extract was tert-butyl phenolics. The compounds belonging to this chemical class have been reported previously for their antibacterial, antioxidant, cytotoxicity and antifungal activity [35]. A compound, 2, 5-bis(1,1-dimethylethyl) phenol, found in our study has been recently reported from the non-polar extract of *Streptomyces* sp. PWS 52 having antibacterial activity against MRSA [36].

Besides, hydrocarbons and phenolics, a peak (RT 25.284), observed in the GC-MS spectrum of the DEEL, exhibited <75% similarity with all the hits in NIST 17 mass spectral library except a chemically synthesized compound, 1; 7-methyl-6-nitro [1, 2, 4]triazolo[1,5-a] pyrimidin-5-ol, at CAS number 056424-00-1, which shows 90% similarity

with the test peak. The compound 1is a derivative of the parent compound, [1, 2, 4] triazolo[1,5-a] pyrimidine (TP) **Fig. 7a**. The derivatives of TP are being chemically synthesized in the laboratory since 1909 and are rarely isolated from nature [37]. The only natural TP derivative, 2; essramycin **Fig. 7b**, has been isolated from a marine organism, Streptomyces sp. Merv 810238. Thus, this study first time reports any TP derivative from hot spring cyanobacterium.

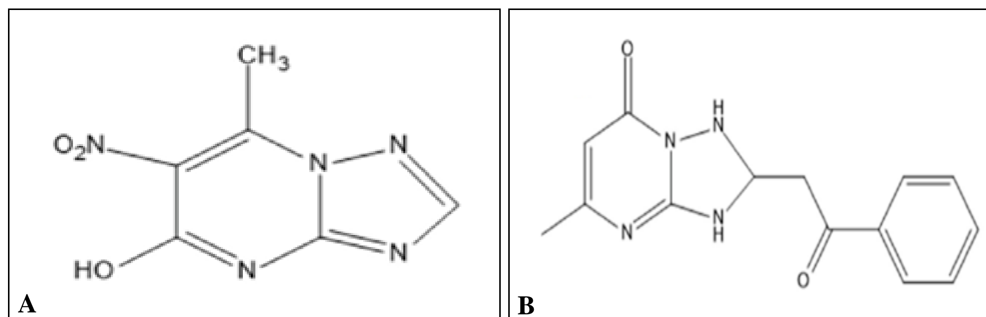

FIG. 7: STRUCTURE OF (A) 1; 7-METHYL-6-NITRO[1,2,4] TRIAZOLO[1,5-A] PYRIMIDIN-5-OL) IDENTIFIED IN DEEL (B). 2; ESSRAMYCIN; (5-METHYL-2-PHENACYL-1H- [1,2,4] TRIAZOLO [1,5-A] PYRIMIDINE-7-ONE)

The chemically synthesized TP derivatives have shown various bioactivities as antibacterial, anti-fungal, antiparasitic, antiviral, antipyretic, anti-cancer, CDK inhibitors and metal chelating [37, 39]. Recent study reported the antibacterial activity of TP derivatives against MRSA [40]. Another TP derivative was found to be active against VRE by interfering with the cell wall biosynthesis [41]. The 2; essramycin, of natural origin, also exhibited broad-spectrum antibacterial activity [38]. However, chemically synthesized isotopes of compound 2 did not show any antibacterial activity [42], which indicates the significant difference in the bioactivity of chemically synthesized and natural isotopes of the same compound. Therefore, compound 1 needs to be isolated from the crude extract of the test organism and tested for its antibacterial activity. The present study enlisted the compounds represented by peaks having similarity index ≥90 to the already known compounds, available in the NIST 17 mass spectral library. These compounds represent ~10% of the complete metabolite spectrum of the tested extract, DEEL. Rest of the peaks (similarity index <90) were considered as unidentified compounds. Isolation and identification of these unidentified compounds may result in the discovery of new bioactive compounds.

CONCLUSION: The study concludes that the non-polar extract of Leptolyngbya sp. HNBGU 002 is a rich source of antibacterial compounds having activity against multidrug-resistant clinical pathogens, including MRSA and VRE. The extract is having some compound(s) causing leakage in bacterial cell membranes. The antibacterial activity of the extract may be due to the individual or synergistic effects of the hydrocarbons, phenolics and the TP derivative, 1, present in the extract, DEEL. Compound 1, is being reported from cyanobacterial origin for the first time, which is an interesting finding in view of the industrial production of these pharmaceutically important compounds. Thus, the study provides a new source of antibacterial compounds that need to be purified, identified, and characterized.

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