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ANTIMICROBIAL ACTIVITIES OF FRESHWATER CYANOBACTERIUM, NOSTOC SP. FROM TAMDIL WETLAND OF MIZORAM, INDIA: AN IDENTIFICATION OF BIOACTIVE COMPOUNDS BY GC-MS

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Key words:

Antimicrobial, Algicidal, Antibacterial, Antifungal, Chlorophycean, Cyanobacterium.

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ABSTRACT: A freshwater microalga, *Nostoc* sp. isolated from Tamdil Wetland of Mizoram was tested for its algicidal, antibacterial and antifungal activity. Extracts were prepared with methanol, ethanol:water and dichloromethane:isopropanol and tested for their antimicrobial activity against nine microorganisms comprising of three chlorophycean algae (*Chlorella vulgaris*, *Scenedesmus quadricauda* and *Selenastrum capricornatum*), one cyanobacterium (*Anabaena variabilis*), three bacterial strains (*Bacillus subtilis*, *Bacillus pumilus* and *Escherichia coli*) and two fungal strains (*Fusarium udum* and *Fusarium culmorum*). Among the three extracts tested the methanol extract was found most effective on algal test organisms. The highest (60%) inhibitory effect was observed against *A. variabilis* by methanol extract followed by 31% inhibition by dichloromethane:isopropanol. When tested on bacterial strains, the highest inhibition zone (23.67±1.58mm) was observed in *B. subtilis* by dichloromethane:isopropanol extract followed by ethanol:water extract. In case of fungal activity, the highest inhibition zone (11.00±0.58mm) was observed in *F. culmorum* by dichloromethane:isopropanol extract and the rest of the extracts showed almost similar effect. MICs of each extract on all the organisms varied. The extracts were further analysed by UV-VIS Spectrophotometer, which reveals the presence of active compounds. Among the three extracts, methanol indicated the highest percentage inhibition and also obtained highest absorption spectra. So, this extract was analysed by GC-MS and we identified the presence of main components in the extract as 1,2-Benzenedicarboxylic acid, mono (2-ethylhexyl) ester, Phytol, n-Hexadecanoic acid, 9,12-Octadecadienoic acid, etc. Further study for purification of the potent compound will explain their usefulness in pharmaceutical and biotechnological industry.

INTRODUCTION: Cyanobacteria or blue-green algae are morphologically diverse group of prokaryotic, photosynthetic organisms that flourish in diverse type of habitats. The evolutionary history of this group of organisms dates back to the Proterozoic era [1]. Most species of cyanobacteria are free-living, freshwater, marine or terrestrial and symbionts in association with other plants and lichens.

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Due to their occurrence in diverse habitats, these organisms are excellent materials for investigation by the ecologists, physiologists, biochemists, pharmacists and molecular biologists. Many metabolites with a diverse range of bioactivities have been reported in cyanobacteria [2].

These metabolites probably originated in cyanobacterial mats and were presumably responsible for regulation of communities. Productions of these metabolites are highly species and even strain dependent [3]. Various strains of cyanobacteria are known to produce intracellular and extracellular metabolites with diverse biological activities, such as antialgal, antibacterial and antiviral [4]. Antimicrobial activity depends on

both algal species and the solvents used for their extraction [5, 6]. Screening of cyanobacteria and algae for antibiotics and other pharmacologically active compounds have received considerable attention during the past few decades [7].

Nostoc has been shown as a good source of antifungal, algicide and cytotoxic metabolites [8]. Many different bioactive substances mainly peptides, alkaloids and phenolics have been identified in *Nostoc* species [9]. Cryptophycin-A and nostodione-A, which inhibit microtubule assembly and cyanovirin-N, which has anti-HIV property are detected in *Nostoc* strains [10]. 'Cyanobacterin' LU-1 and LU-2 have also been reported from *Nostoc linckia* CALA 892 and 893, respectively. Cyanobacterial metabolite known as algicide acts as light reactions of photosynthesis [11]. Flores and Wolk identified one species, *Nostoc* species 31, as good source of an anticyanobacterial compound named nostocyclamide [12]. This compound inhibited the growth of several cyanobacteria and chlorophyceans. The application of bioactive compounds derived from algae will prove beneficial and is much more effective as compared with traditional treatment methods [13]. Analytical methods play important roles in the discovery, development and manufacture of bioactive compounds [14].

The aim of the present study was to determine the antimicrobial activity of *Nostoc* extracts against algae, bacteria and fungi. Furthermore, GC-MS autogram for *Nostoc* sp. extracts was employed for preliminary detection of active constituents.

MATERIALS AND METHODS:

Culture and growth condition:

The *Nostoc* sp. was collected from Tamdil Wetland of Mizoram, India. It was isolated on agar plates by using standard methods of isolation and purification [15] and was grown axenically in 250-ml Erlenmeyer flasks containing 100 ml Chu-10 medium [16] at pH 7.0±0.2 in an air-conditioned culture room under the photoperiod of 12 hrs. Cultures were hand-shaken thrice daily with an interval of 2-3 hrs. The pure culture of *Nostoc*, is deposited at the Microalgal Culture Collection of Mizoram University (Accession No. MZUCC029).

Determination of specific growth rate, protein, chlorophyll a and carotenoid:

The growth rate of *Nostoc* was determined by spectrophotometric method and the specific growth rate was calculated according to Guillard [17]. 150 ml medium was inoculated to give final absorbance of 0.2 at 440 nm. The culture was kept under light for 16 days and at regular interval, 5 ml culture was removed and protein, chlorophyll a and carotenoids were determined. The protein content of the *Nostoc* was determined by Lowry's method using the standard BSA [18]. Quantitative estimation of chlorophyll *a* and carotenoid were performed by the method of Mackinney and Myers and Krantz [19, 20]. The cells were harvested by centrifugation and the pellet was suspended in 5 ml of 80% acetone. After overnight incubation in dark, the pellets were separated and the absorbance of the supernatant was measured at 665 and 480 nm using UV-VIS Spectrophotometer (Systronics, India, model: 117). The chlorophyll a and carotenoid were quantified as per the formula given below:

$$C = \frac{D}{d\alpha}$$

Where, α is the absorption coefficient (82.04 for Chlorophyll a and 200 for Carotenoid), D is the optical density, d is the inside path length of the spectrophotometer in cm (1cm) and C is the concentration of pigment in gl^{-1}.

Estimation of Lipid content:

0.06 g fresh weight of *Nostoc* was washed with 0.2% NaCl. The pellet was collected and suspended in 5 ml hot isopropanol. It was boiled for 3 min in order to inactivate the lipase activities. After cooling, the suspension was mixed with 5 ml chloroform and kept for 12 hrs at room temperature. 10 ml of water was added and the mixture was vortexed for 30 sec. The lower chloroform phase was collected. Chloroform was completely evaporated in a vacuum oven. 2 ml of acid dichromate reagent (stock) was added. After being heated for 30 min, 10 ml of water was added. Absorbance was recorded at 430 nm as previously described by Kalacheva *et al*., [21].

Estimation of Sugar content:

0.01g dry weight of *Nostoc* was mixed with 3 ml of 80% hot ethanol and kept at room temperature for

30 min. Subsequently, it was centrifuged at 5000 g for 10 min. Then, the supernatant was collected and used for estimation of soluble sugar contents as described by Gerhardt *et al*., [22].

Preparation of extracts:

The stationary phase culture was used for extraction of metabolites. After biomass was separated by centrifugation (4000 g for 10 min), the pellet was collected in a flask containing sterile Milli-Q water and kept at room temperature for 48 hrs to further stimulate the synthesis of secondary metabolites. Subsequently, the biomass was freeze dried at -20°C for 24 hrs [23]. 0.5 g freeze dried biomass was suspended in 10 ml of methanol and the cells were ruptured by ultrasonication (Sartorius, Germany; model: BBI 8535108) for 30 sec. The residues were separated by passing through Sephadex G column. The filtrates were used as methanol (100%) extract. Two other extracts, ethanol:water; 3:7 (v/v) and then dichloromethane:isopropanol (DCM:ISO) ; 1:1 (v/v) were also prepared from the remaining biomass.

Test Microorganisms

Three green algae (*Chlorella vulgaris*, *Scenedesmus quadricauda* and *Selenastrum capricornatum)*, one cyanobacterium (*Anabaena variabilis)*, two gram positive bacteria [*Bacillus subtilis* (strain ATCC 11774) and *Bacillus pumilus* (strain ATCC 14884)] and one gram negative bacteria [*Escherichia coli* (strain ATCC10536)] and two fungi (*Fusarium udum* and *Fusarium culmorum)* were used as target organisms for testing bioactivities. Algae were grown axenically in Chu-10 medium whereas bacterial strains were incubated in nutrient broth medium. Fungal strains were grown in potato dextrose agar (PDA) medium. The actively growing cultures of the organisms were used for various tests.

Tests for Bioactivities:

Algicidal assay:

Algal cultures of *Chlorella vulgaris*, *Scenedesmus quadricauda*, *Selenastrum capricornatum* and *Anabaena variabilis* were used to test potential bioactivities of *Nostoc* extracts. Different concentrations ranging from 25 µl to 100 µl of the extracts were added separately to 3 ml culture of *C. vulgaris*, *S. quadricauda*, *S. capricornutum* and *A. variabilis* (in triplicate each). After 4 days of incubation in light, the absorbance was recorded at 684 nm for *C. vulgaris*, 440 nm for *S. quaricauda* and *S. capricornutum*, and 417 nm for *A. variabilis* using spectrophotometer (Systronics, India, model: 117). Specific growth rate was calculated for treated and untreated cultures.

Anti-bacterial assay:

The antibacterial activities of different extracts were also tested using agar well diffusion assay as previously described [24]. Briefly, bacterial strains were inoculated in nutrient broth medium and then incubated at 35°C for 24 hrs. 0.5 ml of bacterial suspension was poured on sterilized nutrient agar plates and spread uniformly by using L-shape spreader. Plates were punched to make a well of 6 mm diameter with the help of sterile cork borer. Different concentrations (25 µl, 50 µl, 75 µl and 100 µl) of extracts were pipetted into the well and plates were incubated overnight at 35°C in an incubator. All the tests were done in triplicates. The plates were observed for the inhibition zone after 48-72 hrs.

Anti-fungal assay:

For antifungal assay, disc diffusion method was performed [25]. The fungal strains, (*Fusarium udum* and *Fusarium culmorum*) were inoculated on sterile petri dishes containing 10 ml of potato dextrose agar medium. The filter paper (8 mm diameter) were impregnated with 25 µl, 50 µl, 75 µl and 100 µl of the extracts and placed onto the agar plates previously inoculated with the fungal cultures by using a flamed forcep followed by gently pressing down to ensure contact [26]. The plates were incubated at 30^{o}C for 48 hrs, and diameters of the inhibition zones were measured.

Determination of Minimum Inhibitory Concentration (MIC):

Minimum inhibitory concentration (MIC) of different extracts was also evaluated using different concentrations (25 µl, 50 µl, 75 µl and 100 µl) of extracts against the selected microorganisms. These tests were done to determine the lowest concentration of algal extract that inhibits the growth of other organisms.

UV-Visible Spectrophotometric Analysis:
The absorption spectra of methanol, ethanol:water and dichloromethane:isopropanol extracts of *Nostoc* were determined using UV-Visible Spectrophotometer (HITACHI U4100L). The wavelength ranged from 200 to 750 nm [27].

GC-MS analysis of *Nostoc* extract:
Nostoc sp. crude methanol extract was dissolved in chloroform at the concentration of 10 mg/ml and was analysed by GC-MS -QP2010 Plus (Shimadzu, Japan). For GC-MS detection, an electron ionization system with ionization energy of 70 eV was used, we used helium as the carrier gas at a constant flow rate of 1.21 mL/min. The injector and MS transfer line temperature was set at 250°C and 260°C, respectively [28]. Compounds were identified based on the comparison of their relative retention time and mass spectra with those of the NIST, WILLY library data of the GC-MS system.

Statistical Analysis: The data obtained from the study were analysed statistically using the Analysis of Variance (ANOVA). The test was performed using Statistical 5.0 software at $p<0.05$ significant level.

RESULTS:
Growth pattern and determination of protein, chlorophyll a, carotenoid, lipid and sugar contents: The growth pattern of *Nostoc* species was determined by recording the absorbance of culture at 440 nm (**Fig. 1**). The growth of culture remained active upto 20 days, then entered into the stationary phase. The level of protein, chlorophyll *a* and carotenoid were found to be elevating till the stationary phase (**Fig. 2)** and monitored during the course of 15 days growth period. The general biochemical profile including fresh- and dry-weights, lipid content, protein content, sugar content, and photosynthetic parameters (Fv/Fm, Y(II), qP, qL, NPQ) are given in **Table 1**. The total protein, lipid and sugar contents were measured to be 0.148 mg ml^{-1}, 261.7 mg g^{-1} dry weight, 176.00 mg g^{-1} dry weight, respectively.

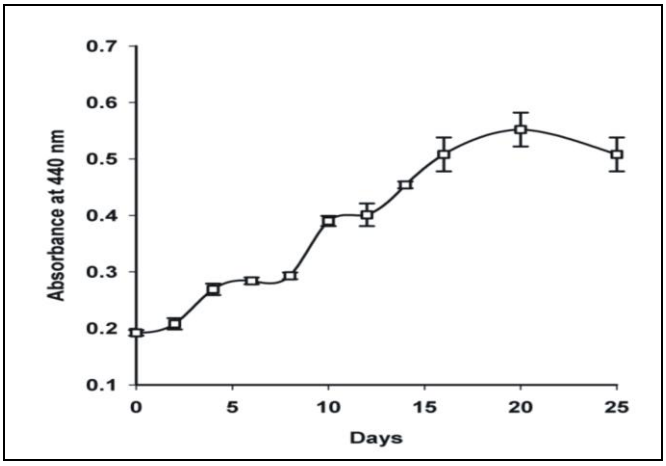


FIG. 1: GROWTH BEHAVIOR OF NOSTOC SPECIES. THE CYANOBACTERIUM WAS GROWN IN CHU-10 MEDIUM WITHOUT NITROGEN AND ABSORBANCE WAS RECORDED AT DIFFERENT DAYS. THE VALUES ARE MEAN OF THE THREE REPLICATES AND VERTICAL BARS INDICATES S.D.

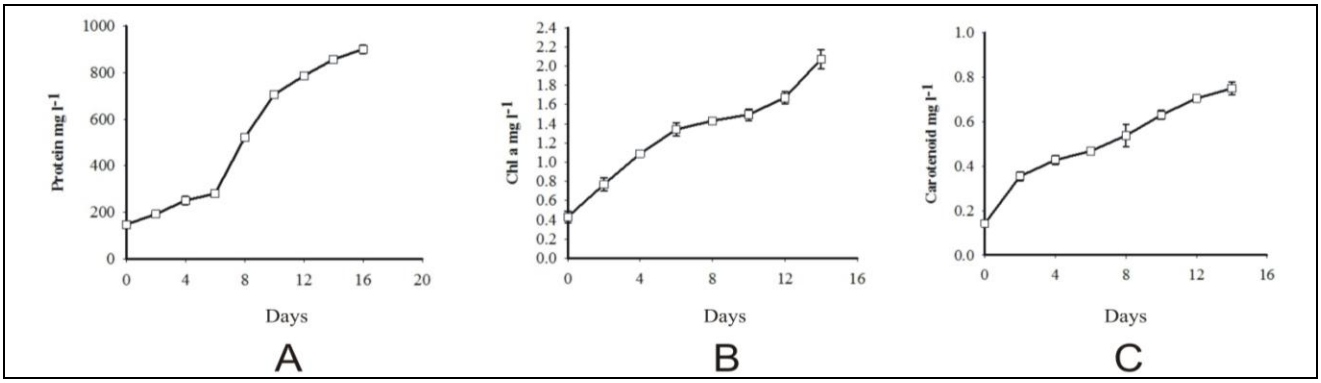


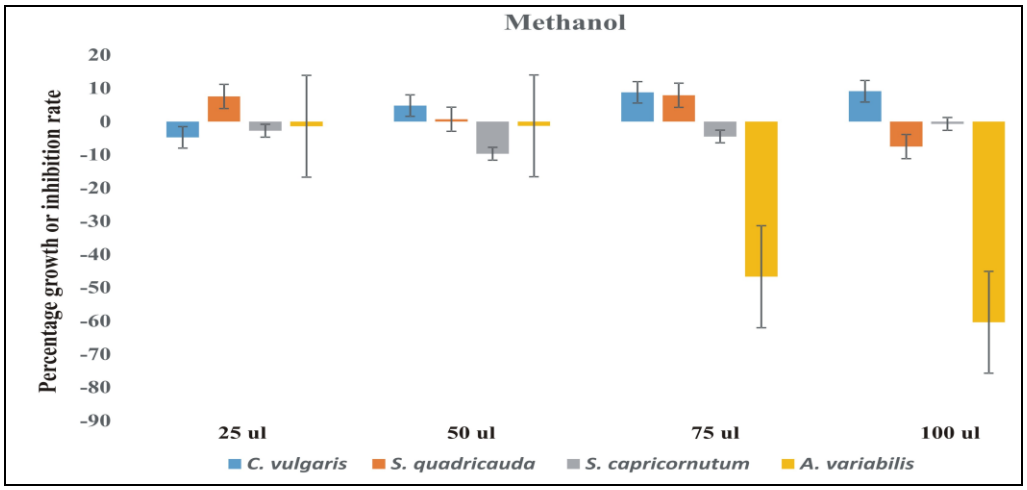
FIG.2: PROTEIN, CHLOROPHYLL a AND CAROTENOID CONTENT OF NOSTOC CELLS IN mgl-1 DURING THE GROWTH PERIOD OF 15 DAYS. VERTICAL BARS INDICATE S.D. OF MEAN VALUE OF THREE REPLICATES.

TABLE 1 : SOME CHARACTERISTICS OF NOSTOC SPECIES

Characteristics	Measured values
Fresh weight	2.670±0.161 gl^{-1}
Dry weight	0.33±0.1 g l^{-1}
Fresh weight : dry weight	0.125
Lipid content	261.7 mg g^{-1} dry weight
Total sugar	176.00 mg g^{-1} dry weight
Protein	0.148 mg ml^{-1} culture (OD at 440 nm = 0.2)
Photosynthesis	
Fv/Fm (max. PSII quantum yield)	0.294
Y(II) (effective PSII quantum yield)	0.296
qP (coefficient of photochemical quenching)	1.000
qL (coefficient of photochemical quenching)	1.000
NPQ (quantum yield of regulated energy dissipation)	0.000

Algicidal activity:

For antimicrobial activity assay by using three different extract solvents, methanol extract has maximum inhibitory effect against *A. variabilis* (60%). The inhibitory effect was noticed against both cyanobacterium and green algae tested in the present study. We also observed a dose dependent inhibitory response against the tested algae. The ethanol:water extract showed inhibitory effect against *C. vulgaris*, but surprisingly not against other two green algae tested. Instead, ethanol:water extract significantly stimulated the growth of *S. quadricauda*. The dichloromethane:isopropanol extract also inhibited almost all the test organisms. A maximum of 31% inhibition was observed against *A. variabilis*. Out of three extracts tested in the present study, methanol and dichloromethane : isopropanol extracts have more growth inhibitory effect than ethanol:water extract on algal test organisms (**Table 2; Fig. 3a, 3b** and **3c**).



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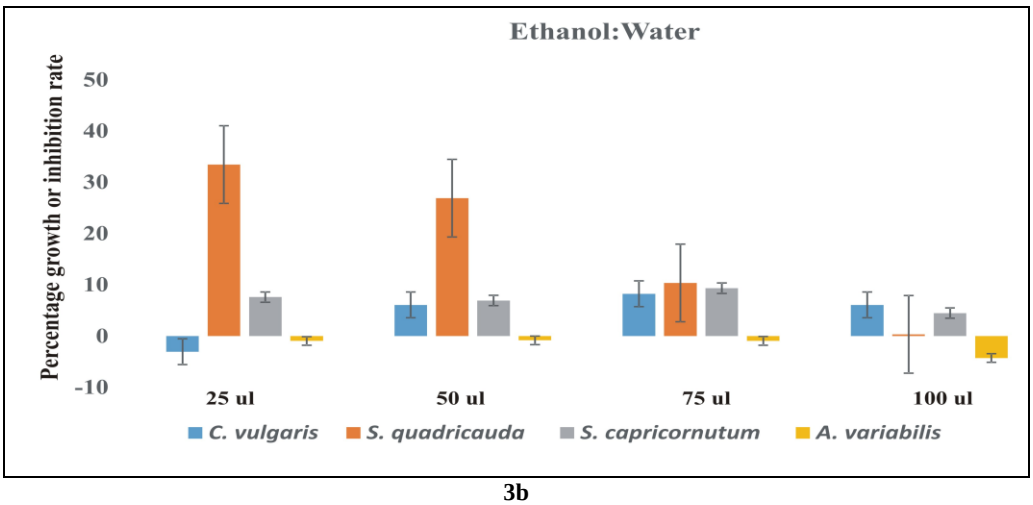

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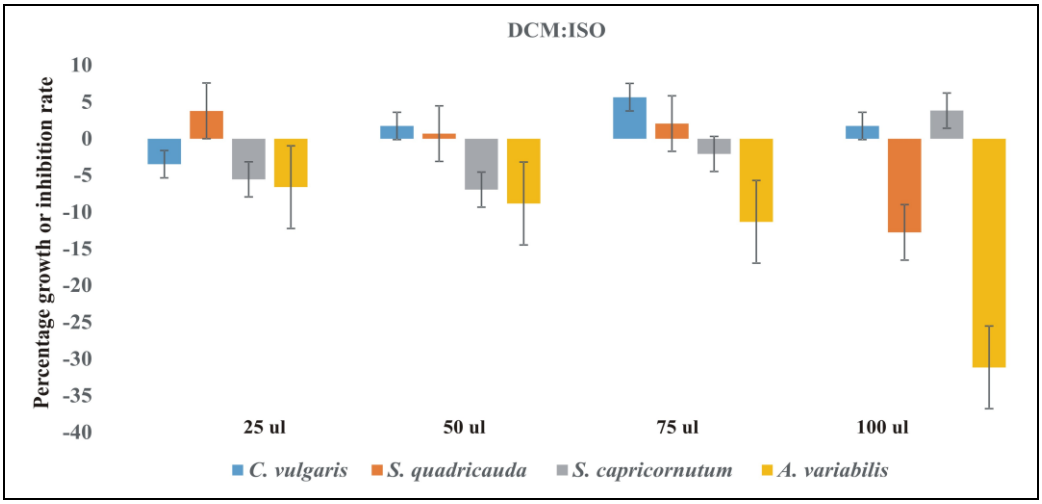


3c

FIG. 3: PERCENTAGE GROWTH OR INHIBITION OF *C. VULGARIS*, *S. QUADRICAUDA*, *S. CAPRICORNUTUM* AND *A. VARIABILIS* BY THE NOSTOC EXTRACTS PREPARED IN a) METHANOL b) ETHANOL:WATER AND c) DICHLOROMETHANE:ISOPROPANOL (DCM:ISO) IN VARIOUS CONCENTRATIONS.

TABLE 2: ANTIALGAL TEST OF EXTRACTS OF NOSTOC WITH FOUR DIFFERENT CONCENTRATIONS.

Extracts	Target organisms	Untreated (μd^{-1})	Specific growth pattern with percentage effect in different concentrations extracts (µL)			
			25	50	75	100
Methanol	*C. vilgaris*	0.230±0.001	0.219±0.003 (-4.78)	0.223±0.004 (+4.79)	0.241±0.007 (+8.70)	0.243±0.003 (+9.13)
	S. quadricauda	0.290±0.001	0.312±0.004 (+7.59)	0.298±0.002 (+0.69)	0.295±0.002 (+7.93)	0.222±0.002 (-7.54)
	S. capricornutum	0.289±0.003	0.281±0.004 (-2.77)	0.254±0.004 (-9.71)	0.252±0.004 (-4.5)	0.248±0.004 (-0.71)
	A. variabilis	0.212±0.003	0.184±0.002 (-1.41)	0.180±0.00 (-1.29)	0.078±0.007 (-46.71)	0.157±0.002 (-60.41)
Ethanol: water	*C. vilgaris*	0.230±0.001	0.223±0.007 (-3.04)	0.226±0.004 (+6.09)	0.242±0.003 (+8.26)	0.250±0.006 (+6.08)
	S. quadricauda	0.290±0.001	0.387±0.003 (+33.45)	0.378±0.003 (+26.9)	0.348±0.004 (+10.35)	0.335±0.005 (+0.34)
	S. capricornutum	0.289±0.003	0.311±0.004 (+7.61)	0.316±0.002 (+6.94)	0.340±0.003 (+9.35)	0.345±0.005 (+4.48)
	A. variabilis	0.212±0.003	0.185±0.005 (-0.94)	0.181±0.001 (-0.82)	0.179±0.005 (-0.93)	0.151±0.004 (-4.27)
DCM:ISO	*C. vilgaris*	0.230±0.001	0.222±0.005 (-3.48)	0.216±0.004 (+1.74)	0.212±0.006 (+5.65)	0.200±0.004 (+1.74)
	S. quadricauda	0.290±0.001	0.301±0.001 (+3.79)	0.282±0.004 (+0.69)	0.268±0.002 (+2.07)	0.207±0.002 (-12.76)
	S. capricornutum	0.289±0.003	0.273±0.005 (-5.54)	0.262±0.004 (-6.94)	0.259±0.005 (-2.08)	0.257±0.004 (+3.83)
	A. variabilis	0.212±0.003	0.173±0.002 (-6.60)	0.164±0.003 (-8.84)	0.153±0.005 (-11.33)	0.094±0.005 (-31.16)

N.B. (-) and (+) indicate inhibition and stimulation in % growth rate, respectively. The values are corrected for solvent effect (data are not shown).

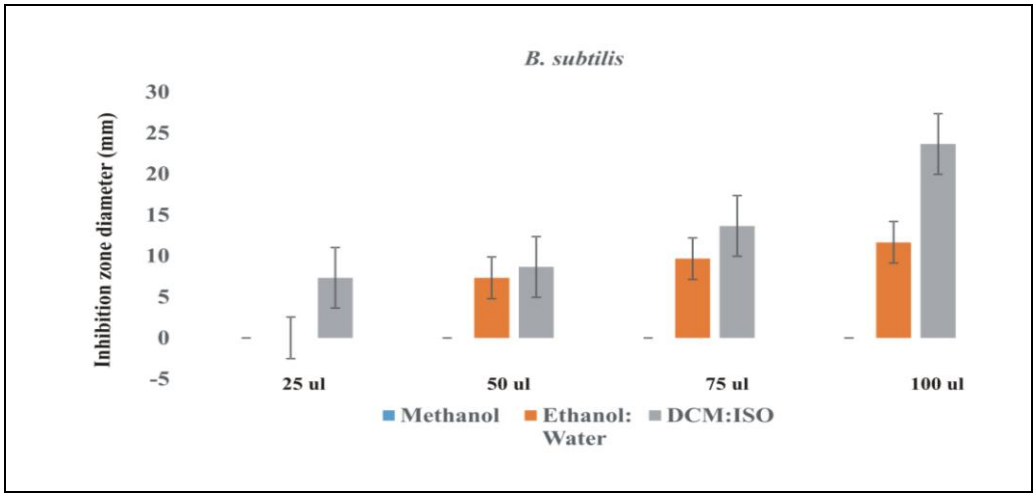
Anti-bacterial activity:

In antibacterial assay, both ethanol:water and dichloromethane:isopropanol extracts inhibited the growth of *B. subtilis*, while only dichloromethane:isopropanol extract inhibited the growth of *B. pumilus*. Methanol extract did not show any effect on all the bacterial strains. However, all the three extracts have no effect on *E. coli*. The highest inhibition zone in *B. subtilis* by 100µl of dichloromethane:isopropanol extracts extract was 23.67±1.53 (**Fig. 4a**), followed by 14.00±1.00 (**Fig. 4b**) in *B. pumilus* **(Table 3)**. As the concentration of the extracts increases, the zone of inhibition become higher.

TABLE 3: ANTIBACTERIAL ACTIVITIES OF NOSTOC EXTRACTS

Bacterial strains	Extracts	Inhibition zone diameter with different concentrations of *Nostoc* extracts (in mm)			
		25µl	50 µl	75 µl	100 µl
Bacillus subtilis	Methanol	-	-	-	-
	Ethanol:water	-	7.33±0.58	9.67±1.15	11.67±2.88
	DCM:ISO	7.33±0.58	8.67±0.58	13.67±1.15	23.67±1.53
Bacillus pumilus	Methanol	-	-	-	-
	Ethanol:water	-	-	-	-
	DCM:ISO	10.67±0.58	11.33±0.58	12.33±0.58	14.00±1.00
Escherichia coli	Methanol	-	-	-	-
	Ethanol:water	-	-	-	-
	DCM:ISO	-	-	-	-

('-' sign indicates no effect, there was no effect of extracts on *E. coli*)



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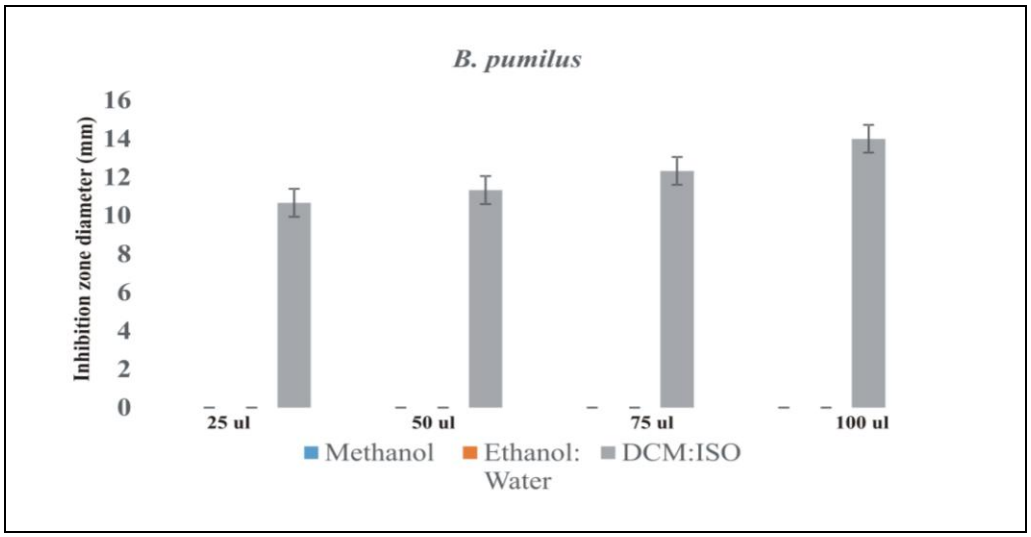

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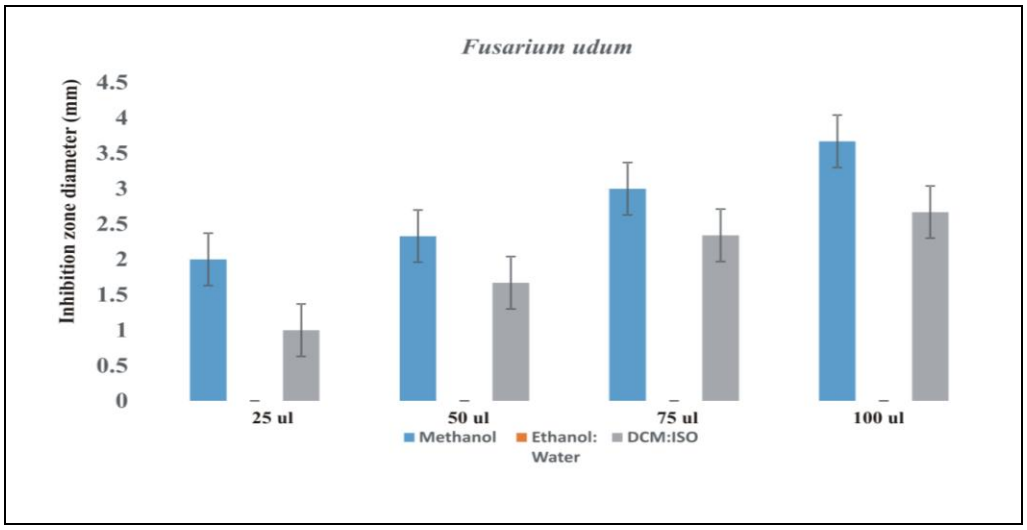
FIG. 4: INHIBITION ZONE DIAMETER OF a) B. SUBTILIS AND b) B. PUMILUS BY THE NOSTOC EXTRACT PREPARED IN METHANOL, ETHANOL:WATER AND DICHLOROMETHANE:ISOPROPANOL (DCM:ISO).

Anti-fungal activity:

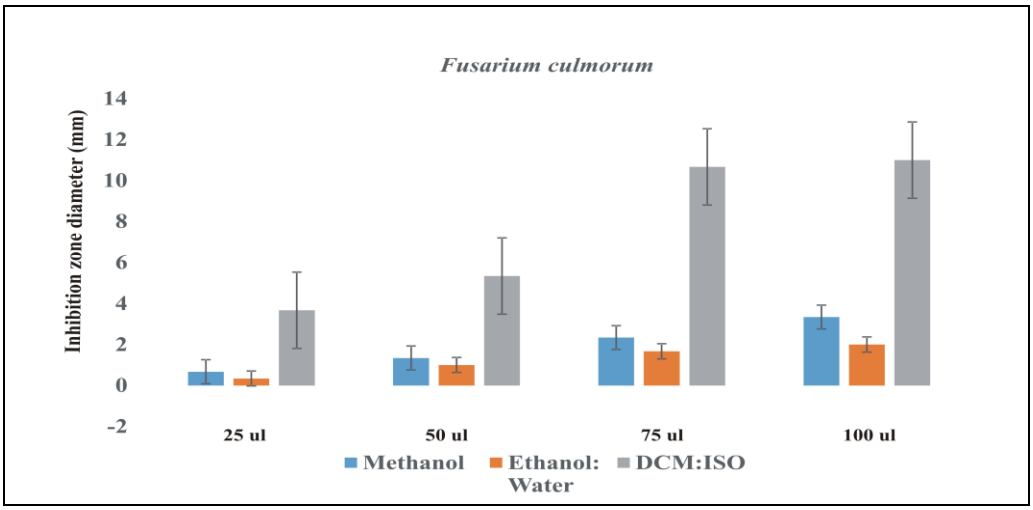
Table 4, **Fig. 5a** and **5b** show the antifungal assay. In this case, the three extracts inhibited the growth of *Fusarium culmorum,* but ethanol:water extract had no effect against *Fusarium udum*. In *F. culmorum,* the dichloromethane:isopropanol extract showed higher inhibitory zone than the other extracts.

TABLE 4: EFFECT OF NOSTOC EXTRACTS ON FUNGAL STRAINS.

Fungal strains	Extracts	Inhibition zone diameter in mm with different concentrations of *Nostoc* extracts			
		25µl	50 µl	75 µl	100 µl
Fusarium udum	Methanol	2.00±0.00	2.33±0.58	3.00±0.56	3.67±1.16
	Ethanol:water	-	-	-	-
	DCM:ISO	1.00±0.58	1.67±0.00	2.34±0.58	2.67±2.65
Fusarium culmorum	Methanol	0.67±0.00	1.34±0.58	2.34±0.58	3.34±1.53
	Ethanol:water	0.34±0.58	1.00±0.58	1.67±1.73	2.00±1.15
	DCM:ISO	3.67±0.00	5.34±1.53	10.67±0.00	11.00±0.58



5a



5b

FIG. 5: INHIBITION ZONE DIAMETER OF a) F. UDUM AND b) F. CULMORUM BY THE NOSTOC EXTRACTS PREPARED IN METHANOL, ETHANOL:WATER AND DICHLOROMETHANE:ISOPROPANOL (DCM:ISO).

Minimum inhibitory concentration

Minimum inhibitory concentrations of all the three extracts were varied (**Table 5**). 25 µl of all the extracts showed inhibitory effect against *C. vulgaris*. 100 µl of methanol and dichloromethane: isopropanol extracts inhibited the growth *S. quadricauda*. 75 µl of extract prepared in methanol has shown significant inhibition of growth of *S. capricornutum* and *A. variabilis*. 25 µl of dichloromethane:isopropanol extract also inhibited the growth of *S. capricornutum*, *A. variabilis*, *B. subtilis* and *B. pumilus*. The MIC of ethanol: water extract on *A. variabilis* and *B. subtilis* was 100 µl and 50 µl, respectively. All the extracts showed their MIC at 25 µl on the fungal strains tested.

TABLE 5: MINIMUM INHIBITORY CONCENTRATIONS OF EXTRACTS OF NOSTOC ON TEST ORGASNISMS.

Target organisms	Extracts		
	Methanol	Ethanol:water	Dichloromethane:isopropanol
C. vilgaris	25 µl	25 µl	25 µl
S.quadricauda	100 µl	-	100 µl
S. capricornutum	75µl	-	25 µl
A. variabilis	75 µl	100 µl	25 µl
B. subtilis	-	50 µl	25 µl
B. pumilus	-	-	25µl
E.coli	-	-	-
F. udum	25 µl	-	25 µl
F. cornitum	25 µl	25 µl	25 µl

UV-Visible spectrophotometric analysis:

From this analysis, several peaks were observed in different wavelengths, which indicate the presence of active compounds in the extracts of *Nostoc*.

Among the extracts of *Nostoc*, the highest numbers of peaks were found in methanol extract which was followed by dichloromethane: isopropanol extract and lastly ethanol: water extract **(Fig. 6).**

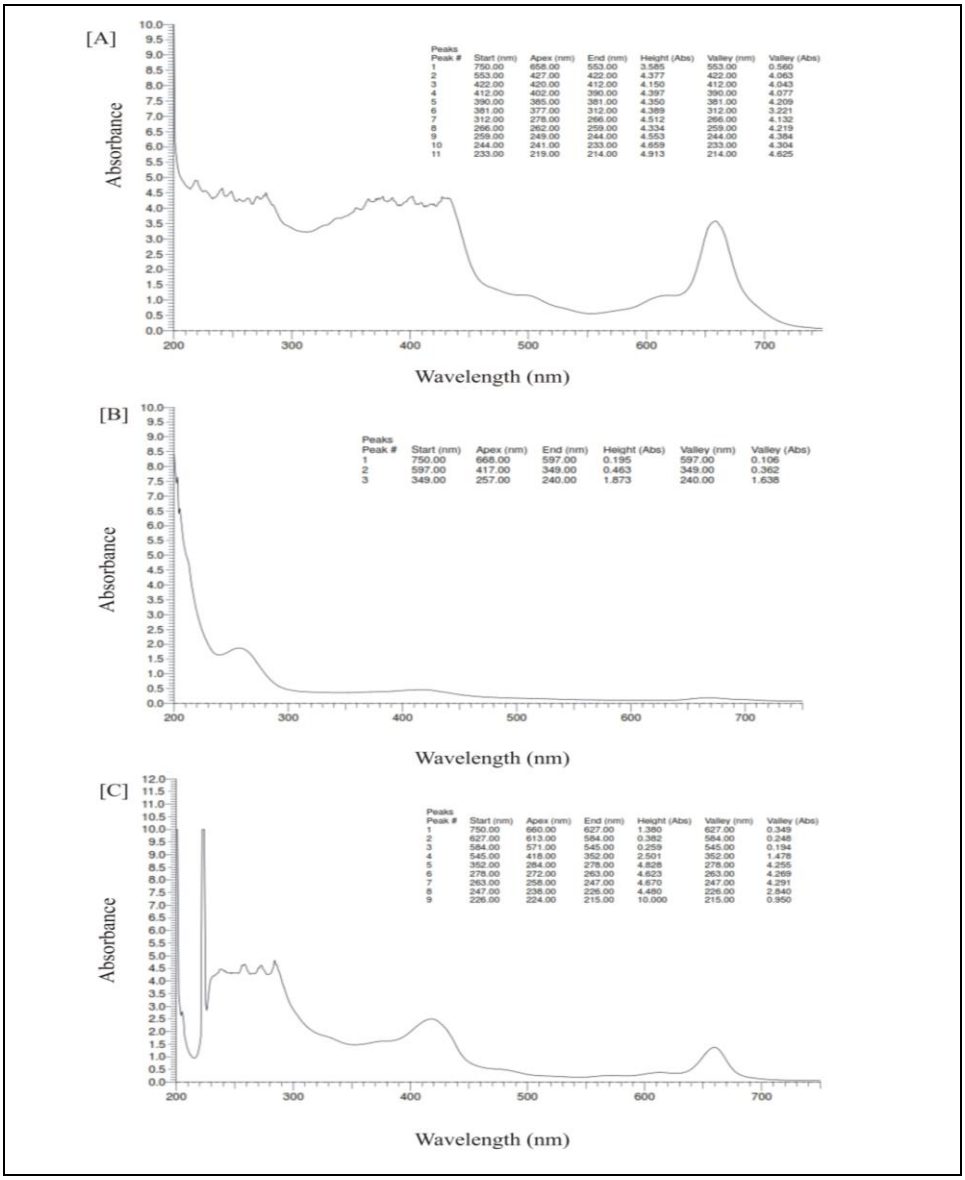


FIG. 6: UV-VISIBLE SPECTRA OF METHANOL [A], ETHANOL: WATER (3:7) [B] AND DICHLOROMETHANE: ISOPROPANOL (1:1) [C] EXTRACTS OF NOSTOC SPECIES. THE MAJOR PEAKS DETECTED ARE SHOWN INSIDE THE FIGURE FOR RESPECTIVE EXTRACTS.

GC-MS analysis of Nostoc sp. extract:

GC-MS analysis of the *Nostoc* extract prepared in methanol has identified 32 active compounds. Some of the few compounds are 2,4,4-Trimethyl-1-hexene, Octadecane, 2,6,10-Ttrimethyl-14-Ethylene-14-Pentadecane, n-Hexadecanoic acid, 9,12-Octadecadienoic acid, 9,12-Octadecadienoyl chloride, Phytol, 1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester and Vitamin E **(Table 6**). The chromatogram of the GC-MS is presented in **Fig. 7**.

TABLE 6: SELECTED LIST OF COMPOUNDS IDENTIFIED BY GC-MS IN METHANOL EXTRACT OF NOSTOC SPECIES

	RT	Name of compounds	Molecular Formula	Molecular Weight	Composition (%)
A	10.764	2,4,4-Trimethyl-1-hexene	C_9H_{18}	126	2.56
B	12.944	Octadecane	$C_{18}H_{38}$	254	5.73
C	13.999	2,6,10-Trimethyl,14-ethylene-14-pentadecane	$C_{20}H_{38}$	278	6.39
D	15.255	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256	5.53
E	16.538	9,12-Octadecadienoic acid	$C_{18}H_{32}O_2$	280	5.24
F	16.608	9,12-Octadecadienoyl chloride	$C_{18}H_{31}ClO$	298	8.64
G	16.722	Phytol	$C_{20}H_{40}O$	296	13.92
H	20.412	1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester	$C_{16}H_{22}O_4$	278	20.13
I	26.857	Vitamin E	$C_{29}H_{50}O_2$	430	1.89

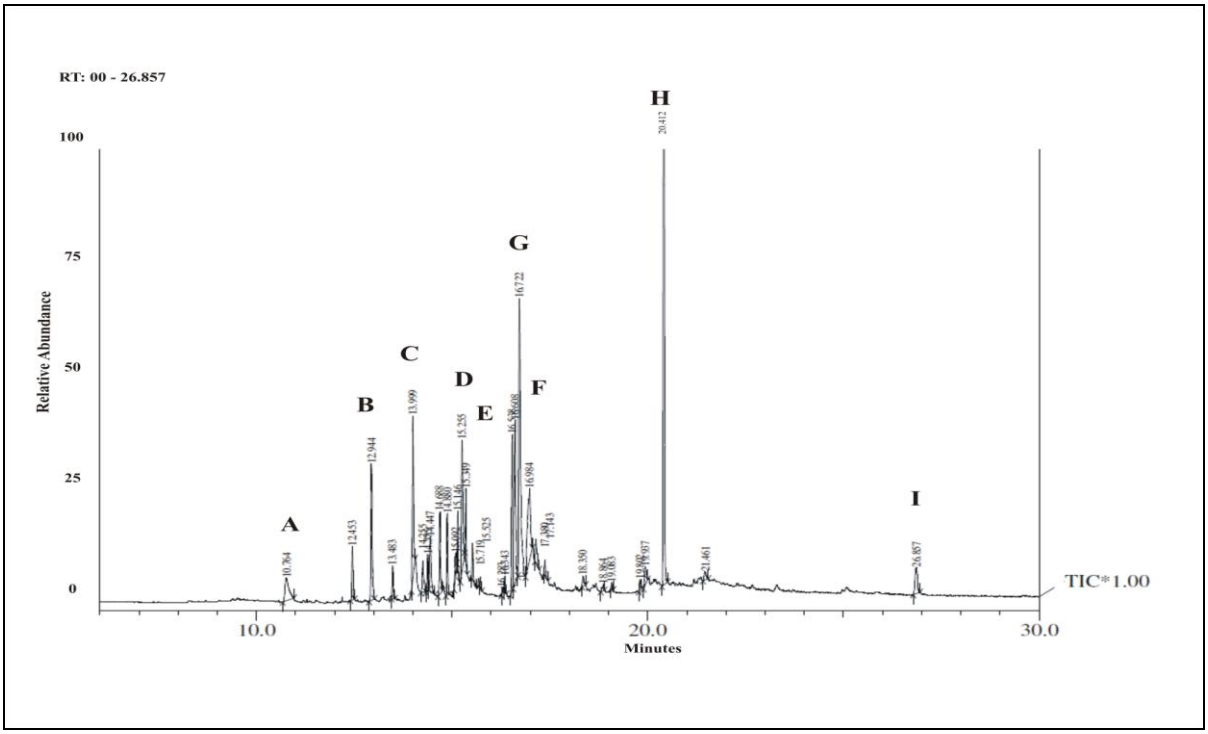


FIG. 7: **GAS CHROMATOGRAPGIC PROFILE OF THE MAJOR CONSTITUENTS OF NOSTOC SPECIES**

DISCUSSION: The present study has clearly shown the potential antimicrobial activity of *Nostoc* sp. Though the extracts prepared in different solvents showed variation in their inhibition properties in different test organisms of algae, bacteria and fungi, we have observed the potential pharmacological properties. Maximum growth inhibition was shown by the extract prepared in methanol (46.71% - 60.41%) followed by dichloromethane:isopropanol extract (11.33% - 31.16%) against *A. variabilis.* The growth inhibition of *A. variabilis* by the extracts of *Nostoc* suggests the possible presence of algicidal metabolites in *Nostoc*. In a similar study, it inhibited the growth of *Nannochloris*, *Ankistrodesmus* and *Scenedesmus* [29]. *Nostoc*

possesses algicidal compounds like Crytophycin-A and nestodione-A, hexapeptides, Cyanobacterin LU-1 and LU-2 [30]. Compounds isolated from cyanobacteria act on the photosynthetic electron transport chain (ETC), proton coupling, pigment synthesis and photo-oxidative effects and synthesis of amino acids [31]. It also reacts with cell membrane molecules thereby inhibiting the growth [32]. Another very interesting observation was that the extract prepared in ethanol:water stimulated the growth of *S. quadricauda* (10.35% - 33.45%) and *S. capricornutum* (4.48% - 9.35%). Phytol and Vitamin E identified by GC-MS might have played key role in growth stimulation. Our observation is in corroboration with an earlier report on stimulation of growth of few phytoplanktons treated with extracts prepared from cyanobacteria [33]. Phytohormones like auxins, gibberellins and cytokinins are present in the extract, and may act on the different metabolic pathways. The target algae have ability to evolve pathways to effectively metabolize the compounds like allelochemical-like compounds [34]. We are yet unable to answer to this stimulatory mechanism. The bioactive compounds are associated with positive nutritional and adaptation mechanisms of target organism [35]. Our findings of algicidal assays using different solvents suggest that stimulation or inhibition of growth is not solvent specific, but a target (organism) specific.

The antibacterial activity was done in three widely used bacterial strains, such as *B. subtilis, B. pumilus* and *E. coli*. Out of the three extracts, two extracts prepared in ethanol:water and dichloromethane:isopropanol showed inhibition of growth of *B. subtilis* (7.33-23.67 mm). *Nostoc* extract prepared in dichloromethane:isopropanol inhibited the growth of *B. pumilus* (10.67-14.00 mm). 2, 6, 10 - Trimethyl, 14 – ethylene - 14 - pentadecane and 1,2-Benzenedicarboxylic acid were identified by GC-MS. They are well known for antibacterial activity [36]. Asthana *et al*., identified naphthalene compound from *Nostoc* CCC 537 which has antibacterial property [37].

In a similar study, Madhumati *et al*., showed growth inhibition of *B. subtilis* by the extract of cyanobacteria *Oscillatoria latevirens* (2.2 cm) and *Lyngbya martensiana* (2.5 cm) [38]. Methanol extract of *Nostoc* showed the growth inhibition of *B. subtilis* (17%) [28]. Our findings suggest the presence of bioactive compounds which interfere with the cellular and metabolic activity. Diterpenoid, isolated from *Nostoc commune*, was identified to have an antibacterial activity [39]. Another compound, Nostocyclamide M, also showed allelopathic effect [40]. However, all the three extracts showed no effect against *E. coli.* In another study, the *Nostoc commune* extract prepared in methanol inhibited the growth of *Staphylococcus, B. subtilis, B. cereus, B. pumilus* and *E. coli* [41]. There is also report for antimicrobial properties of ethanol extract against *E. coli* [42]. This discrepancy is explained by the genetic and physiological variations within the particular cyanobacterial strains.

Our present study has clearly shown antifungal activity of *Nostoc.* All the extracts of *Nostoc* inhibited the growth of *F. culmorum*, whereas methanol and dichloromethane:isopropanol extracts inhibited *F. udum* only. But, antifungal activity was found with the methanol extract on the two species as observed by Arun *et al*., [43]. Nostofungicidine, a lipopeptide compound, isolated from *Nostoc commune* has antifungal activity [44]. Growth of fungus, *Aspergillus niger* was inhibited by *Nostoc* extract [28]. A polyunsaturated fatty acid hexadecatrienoic acid n4 has an antibacterial activity [45]. Antimicrobial effects are related with the compounds of hydrogen peroxide, terpenoid, bromoether and volatile fatty acids compounds isolated from algae [46]. Phenols, plasticizer compound, phytol (acyclic diterpene alcohol) and flavonoid are abundant in methanol extract, while plasticizer compound, phytol, alkenes and ester are abundant in the acetone extract of *Nostoc* [28]. We are also looking for the bioactive compounds identified by GC-MS in different extracts, which is under validation phase.

The present findings on the overall antimicrobial activities clearly revealed that among the three different extracts of *Nostoc*, the methanol and dichloromethane:isopropanol extracts were more effective than the ethanol:water extract. When the extracts were analysed in UV-Visible spectra, methanol extract showed highest number of peaks which represents the presence of more active

compounds in the extract that are responsible for antimicrobial activity.

C**ONCLUSION:** From this study, we conclude that *Nostoc* has antimicrobial activities against both prokaryotic and eukaryotic target organisms (algae, bacteria and fungi). Further identification of the compounds in *Nostoc* sp. extracts followed by their phytochemical studies can elucidate the groups of compounds associated with such antimicrobial activity. Use of modern technology in breeding the culture will also increase the quality and quantity of algal production. *Nostoc* sp. has potential pharmacologic applications.

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