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ISOLATION, IDENTIFICATION AND SCREENING FOR BIOACTIVE COMPOUNDS WITH ANTIMICROBIAL ACTIVITIES FROM SUB-AERIAL CYANOBACTERIA OF EASTERN REGION, ODISHA

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Sub-aerial Cyanobacteria, Antimicrobial activity, Bioactive compounds, FTIR, LCMS

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ABSTRACT: Background and Objectives: Cyanobacteria are rich in various bioactive compounds. This study aims to screen the antimicrobial activity of sub-aerial Cyanobacteria isolated from sub-aerial habitats (Old monuments and building facades). **Materials and Methods:** Crude aqueous and organic extracts were screened for phytochemical constituents and tested for their antimicrobial efficacy against human pathogens. The crude extract that showed better antimicrobial activities were further fractionated by column chromatography and purified by TLC. The active bands were scraped out separately and concentrated in *vacuo* to isolate pure compounds. Each purified compounds dissolved in respective solvents, assayed for antimicrobial activities were further characterised through FTIR and LC-MS. **Results:** The results indicated the presence of many secondary metabolites. Of the isolated purified compounds, the compounds from three species, *viz. Scytonema hyalinum, Tolypothrix rechingeri,* and *Fischerella* species exhibited antimicrobial activities, whereas others showed antibacterial activity. The FTIR spectra revealed the peaks of carboxylic acid, alkaloids, and phenolic compounds. The chemical compounds were identified as 1,3-dihydroxycyclohexane [*Lyngbya kuetzingiana*], 4-(4-hydroxyphenyl) butanoic acid [*Nostoc linckia*], octahydro-1H-indole-2-carboxylic acid [*Scytonema* sp., *S. pseudoguyanensis*, *S. pseudohofmani*], ergost-5-en-3-ol [*Scytonema hyalinum*],7,11-dihydroxysolasodine [*Scytonema ocellatum*], 2, 4-Bis (2-methyl-2-propanyl)phenol-phosphorous acid [*Tolypothrix rechingeri*] and Pentaphenyl ferrocene carboxamide [*Fischerella* sp.] respectively. **Conclusion:** Though it is firsthand information regarding the isolation of sub-aerial Cyanobacteria, with a production of different bioactive chemical compounds, further investigation is required for use in pharmaceutical industries against costly harmful antibiotics and chemotherapeutics.

INTRODUCTION: The development of multi-drug resistance (MDR) among pathogens is a global concern today. This necessitates the demand for the discovery of the new source of chemical compounds as alternative antibiotics.

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In this regard, a large number of microbes are lagging behind which may produce potent and novel bioactive compound which can be used in the treatment of humans as well as animals. In this aspect, Cyanobacteria are considered to be one of the accessible organisms in the microbial world which are useful to humankind in several ways.

The use of Cyanobacteria to produce valuable chemicals and pharmaceutically applicable products is still little explored, and it seems a long way to go.

Modern research has focussed on a variety of bioactive compounds produced by Cyanobacteria from various biotypes, for example, marine [1], freshwater [2], terrestrial [3], hot spring [4], *etc*. Some of the lead compounds with antibacterial activities are bacteriocin, scytophytin, didehydromirabazole, bastadin, muscoride, noscomin, bis-x-butyrolactone, nostocin A, and lipids [5]. Butylated hydroxytoluene, hexadecanoic acid methyl ester, tjipanazole, ambiguines, fischerllin A, nostofungicidin, phytoalexin, hassalldin, scytophycin, hapalindole, *Y*-lactone, calothrixins [6] have been reported with antifungal activity. Thus screening efforts aimed to identify antimicrobial agents in sub-aerial Cyanobacteria which might reveal pharmaceutically important compounds.

Cyanobacteria are adaptive to the exterior surface of monuments and building facades which belong to a group of organisms known as "Extremophiles" due to their ability to endure extreme conditions. Few studies have yet been identified for the antimicrobial potential of sub-aerial Cyanobacteria isolates from this habitat [3], but no studies till date are adequate. In-state of Odisha, especially Bhubaneswar and Puri, known as temple city, rich in old cultural heritages including old/modern building is a home for diverse Cyanobacteria [7]. So far, very little work has been done on the antimicrobial properties of extremophilic Cyanobacteria. This prompted us to carry out this investigation to isolate and characterize different sub-aerial Cyanobacteria from the monument and building facades of Bhubaneswar and Puri and evaluate their antimicrobial activities against selected bacteria and fungi.

MATERIALS AND METHODS:

Isolation, Identification and Culture Condition: The biofilms/crusts collected from various sub-aerial sites (*e.g.*, Stone monuments, caves, and building facades) were soaked in sterile distilled water and incubated under fluorescent light for 72 h and observed microscopically. Since the morphologically features needed for identification were not distinct even after prolonged soaking (up to 7 to 14days), a small amount of sample was transferred to BG-11 agar plates medium with and without nitrogen [8] (1.5% agar in the same medium) with the help of inoculation needle. Cultures were incubated at 28 ± 1°C under continuous fluorescent light at an intensity of 2000lux. After a period of 10 to 14 days of incubation, Cyanobacteria appeared in the culture were isolated using sub-culturing methods [9] and the axenic culture was cultured in a 500ml of BG-11 medium for biomass production, and were maintained under controlled conditions of room temperature (28 ± 1°C) and continuous illumination of 2000lux with 16:8 h light and dark regime in the laboratory. The cultures were identified through morphological variation studies following standard monographs [10, 11].

Microbial Strains: Both bacterial [*Bacillus subtilis* (MTCC121), *Pseudomonas aeruginosa* (MTCC741), *Staphylococcus aureus* (MTCC902), *Escherichia coli* (MTCC723)] and fungal strains [*Candida albicans* (MTCC4748) and *Epidermaphyton flocossum* (MTCC7880)] were procured from Microbial Type Culture Collection (IMTECH, India) and were used in the study.

Experimental Organisms: Ten axenic sub-aerial Cyanobacteria species belonging to five genera were identified and used in the experiments. These species are *Lyngbya kuetzingiana* (rock surface, Cave), *Tolypothrix rechingeri* (stone carving, Monument), *Scytonema* sp. (stone carving, Monument), *S. pseudoguyanensis* (stone surface, Temple), *S. pseudohofmani* (stone surface, Temple), *S. hyalinum* (limestone surface, Cave), *Nostoc linckia* and *Fischerella* sp. (cement wall surface, Building), and *Scytonema ocellatum* and *S. crispum* (lime-washed surface, Building wall), *etc*.

Preparation of Crude Extracts: After 32th days of incubation, in BG-11 broth, isolates were harvested with Whatman No.1 filter paper and shade dried for 30 min, and grinded to a fine powder with the help of a glass homogenizer. One gram of Cyanobacterial powder was extracted separately in a soxhlet apparatus using aqueous and different solvents like hexane, benzene, chloroform, ethyl acetate, acetone, and methanol, *etc*. The extracts were collected in a pre-sterilized air-tight container and preserved at 4 ºC for further characterizations.

Phytochemical Screening of the Crude Extracts: Phytochemical screening of the crude extracts for the detection of alkaloids, phenolic, flavonoids, quinones, glycosides, terpenoids, tannins, steroids, saponin, and other secondary metabolites was carried out by following the standard procedure [12].

Antimicrobial Efficacy and Screening of the Extracts: The crude extracts were further screened for antimicrobial activity through agar well diffusion technique [13] against the test pathogens. All the experiments were conducted in triplicates. Zone of inhibition was measured in mm, and the breakpoint for efficacy was taken as a zone of inhibition ≥ 10mm. Ciprofloxacin (10mg/ml) and Carbazine (10mg/ml) were used as controls.

Purification of Crude Extracts through Column Chromatography and Thin Layer Chromatography: The crude extracts that showed better antimicrobial efficacy during screening were further fractionated by column chromatography [14]. The fraction obtained from respective isolates were collected and tested for antimicrobial properties. The selected antimicrobial fractions were further purified by preparatory TLC using ethyl acetate and hexane (1:1) as the mobile phase, visualized under UV, the bands were marked, and their R_f values were determined. The active fractions were scrapped out separately, vacuum evaporated, and the pure compound was obtained in the powdered form, dissolved in 3ml respective solvents, and their bioactivity was checked out using agar well diffusion methods against the test pathogens as described earlier. The antimicrobial activity index (AI) of a respective active fraction was calculated using the formula: inhibition zone of sample/inhibition zone of the standard × 100 [15]. Further, the bioactivities of the active fractions were confirmed using the secondary screening technique (bioautography method) following method [16].

Minimum Inhibitory Concentration (MIC): Different active compounds observed from the previous study were further used to determine the minimum inhibitory concentration (MIC). The MIC values were determined by the microdilution method as described by National Committee for Clinical Laboratory Standards guidelines (NCCLS) [17]. Compounds were serially diluted in order to obtain a range from 0.312mg/ml to 10mg/ml compounds in the respective solvent, in MHB and SDB for bacteria and fungi, respectively, in 96 well micro-titer plates. 20µl of overnight freshly grown pathogens were inoculated to each well. Wells, without extract, served as a negative control, and the antibacterial (Ciprofloxacin-10mg/ml) and antifungal (Carbazine-10mg/ml) agents were included in the assays as a positive control. The test plates were incubated at 37 ± 2°C and 28 ± 2°C for bacteria and fungi, respectively. After the incubation period, one loopful of the sample was sub-cultured onto NA and SDA plates for bacteria and fungi, respectively. A dilution where no growths of the pathogen were recorded is considered as the Minimum Inhibitory Concentration of the respective extract. The same procedure was followed for each extract and each organism separately.

Identification of Bioactive Compounds:

Fourier-Transform Infrared Spectroscopy: The antimicrobial compounds recorded with a highest antimicrobial index of different extracts were taken into consideration for identification of functional groups through FTIR analysis [18] using spectrophotometer (Nicolet 6700, USA and Perklin Elymer) in the range of wave number 500 to 4000 cm^{-1}.

Liquid Chromatography-Mass Spectroscopy: After FTIR analysis, the same fraction was further subjected to LCMS analysis for identification and characterization of bioactive compounds.

LC-MS analysis was carried out using High-Resolution Liquid Chromatography-Mass Spectrometer (1260 Infinity Binary LC coupled with 6530B accurate mass Q-TOF, Agilent Technologies, USA).

Statistical Analysis: All the data obtained from the experiments were expressed as mean ± SD of three replicates using One-way analysis of variance (ANOVA) through SPSS version 17.0 statistical software. P-value ≤0.05 was considered significant.

RESULTS: During the study, ten sub-aerial Cyanobacteria species, *viz*. [*Lyngbya kuetzingiana* (1), *Nostoc linckia* (1), six *Scytonema* of different species, *Scytonema* sp., *S. pseudoguyanensis*, *S. pseudohofmani*, *S. hyalinum*, *S. ocellatum*, *S. crispum*, *Tolypothrix rechingeri* (1) and *Fischerella* sp. (1)] inhabiting as microbial biofilms or crusts on different sub-aerial habitats of old and modern cultural heritages including building facades of the eastern region especially Puri and Bhubaneswar, in the state of Odisha were isolated and assayed for phytochemical screening, characterization, and

identification of bioactive compound with anti-microbial potential.

Phytochemical Screening: Aqueous and organic crude solvent extracts of the ten sub-aerial Cyanobacterial species were prepared as described earlier and subjected to phytochemical screening. The presence of various phytochemical constituents in different extracts of the isolates is presented in **Table 1**. Phytochemical screening revealed the presence of major secondary metabolites like alkaloids, phenolic, flavonoids, glycosides, quinones, and steroids in the extracts. None of the compounds were observed in aqueous, and ethyl acetate extract of all the isolates studied.

Antimicrobial Activity Studies: The organic crude extracts of the ten sub-aerial Cyanobacteria showed antimicrobial activities in terms of zone of inhibitions against the reference pathogenic bacterial and fungal strains during screening. Most of the antimicrobial activity was detected with the extract of relatively polar solvents. In two species, the antibacterial activity was observed in a non-polar solvent like hexane and benzene in *Fischerella* sp. and chloroform in *Tolypothrix rechingeri*. None of the sub-aerial Cyanobacteria showed bioactivity in aqueous as well as ethyl acetate extracts. The crude extract that showed better antimicrobial activities, further fractionated by column chromatography and purified by TLC. The active band on TLC were scrapped out separately, vacuum evaporated and pure compound is obtained in the powdered form, dissolved in 3ml respective solvents, and were checked for antimicrobial activities. The anti-microbial activities (zone of inhibition and respective activity index) of each ten isolates against pathogens were summarised in **Table 2**, **Fig. 1-5**. Results obtained from isolated compounds indicated that out of the ten isolates, three compounds (A_2, M_5 and A_2) respectively isolated separately from three sub-aerial Cyanobacteria species, *Scytonema hyalinum*, *Tolypothrix rechingeri*, and *Fischerella* species showed considerable inhibition zone respectively against target bacterial and fungal pathogens. It was noted that the extracts significantly inhibited gram-negative bacteria, whereas selectively inhibited gram-positive bacterial and fungal strains. Most activities were observed in the case of acetone dissolved compounds (*Scytonema* sp., *S. hyalinum*, *S. ocellatum*, *Fischerella* sp.), and methanol compounds (*Lyngbya kuetzingiana*, *Nostoc linckia*, *Scytonema pseudoguyanensis*, *S. pseudohofmani*, and *Tolypothrix rechingeri*) was found to be effective against *E. coli* and *P. aeruginosa* (accept) *Fischerella* sp. and *Tolypothrix rechingeri* **Table 2**; **Fig. 1-5**.

TABLE 1: PHYTOCHEMICAL SCREENING OF THE SUB-AERIAL CYANOBACTERIA CRUDE EXTRACTS

Experimental Organisms	Different Organic Extracts	Phytochemical constituents								
		Alkaloids	Phenol	Flavonoids	Quinines	Glycosides	Terpenoids	Tannins	Steroids	Saponin
Lyngbya kuetzingiana	Aqueous	-	-	-	-	-	-	-	-	-
	Hexane	+	+	+	-	-	-	-	-	-
	Benzene	+	+	+	-	-	-	-	-	-
	Chloroform	+	+	+	-	-	-	-	-	-
	Ethyl acetate	+	+	+	-	-	-	-	-	-
	Acetone	++	++	++	-	-	-	-	-	-
	Methanol	++	++	++	-	-	-	-	-	-
Nostoc linckia	Aqueous	-	-	-	-	-	-	-	-	-
	Hexane	+	+	+	-	-	-	-	-	-
	Benzene	+	+	+	-	-	-	-	-	-
	Chloroform	+	+	+	-	-	-	-	-	-
	Ethyl acetate	-	-	-	-	-	-	-	-	-
	Acetone	++	++	++	-	-	-	-	-	-
	Methanol	++	++	++	-	-	-	-	-	-
Scytonema sp.	Aqueous	-	-	-	-	-	-	-	-	-
	Hexane	+	+	-	-	-	-	-	-	-
	Benzene	+	+	-	-	-	-	-	-	-

	Chloroform	+	+	-	-	-	-	-	-	-
	Ethyl acetate	+	+	-	-	-	-	-	-	-
	Acetone	+++	+++	-	-	-	-	-	-	-
	Methanol	+++	+++	-	-	-	-	-	-	-
Scytonema pseudoguyanensis	Aqueous	-	-	-	-	-	-	-	-	-
	Hexane	+	+	+	-	+	-	-	-	-
	Benzene	+	+	+	-	-	-	-	-	-
	Chloroform	++	++	+	-	-	-	-	-	-
	Ethyl acetate	+	+	+	-	-	-	-	-	-
	Acetone	+++	++	++	-	++	-	-	-	-
	Methanol	+++	+++	+++	-	++	-	-	-	-
Scytonema pseudohofmani	Aqueous	-	-	-	-	-	-	-	-	-
	Hexane	+	+	-	-	+	-	-	-	-
	Benzene	+	+	-	-	-	-	-	-	-
	Chloroform	+	+	-	-	-	-	-	-	-
	Ethyl acetate	+	+	-	-	-	-	-	-	-
	Acetone	++	++	-	-	++	-	-	-	-
	Methanol	++	++	-	-	++	-	-	-	-
Scytonema hyalinum	Aqueous	-	-	-	-	-	-	-	-	-
	Hexane	+	+	++	+	-	-	-	-	-
	Benzene	+	+	++	+	-	-	-	-	-
	Chloroform	+	++	++	+	-	-	-	-	-
	Ethyl acetate	-	-	-	-	-	-	-	-	-
	Acetone	+++	++	++	+	+	-	-	-	-
	Methanol	+++	++	++	+	+	-	-	-	-
Scytonema ocellatum	Aqueous	-	-	-	-	-	-	-	-	-
	Hexane	+	+	-	-	-	-	-	-	-
	Benzene	+	+	-	-	-	-	-	-	-
	Chloroform	+	+	-	-	-	-	-	-	-
	Ethyl acetate	-	-	-	-	-	-	-	-	-
	Acetone	+++	+++	++	-	+	-	-	+	-
	Methanol	+++	+++	++	-	+	-	-	+	-
Scytonema crispum	Aqueous	-	-	-	-	-	-	-	-	-
	Hexane	+	+	-	-	-	-	-	-	-
	Benzene	+	+	-	-	-	-	-	-	-
	Chloroform	++	++	-	-	-	-	-	-	-
	Ethyl acetate	-	-	-	-	-	-	-	-	-
	Acetone	++	++	++	-	+	-	-	+	-
	Methanol	++	++	++	-	+	-	-	+	-
Tolypothrix rechingeri	Aqueous	-	-	-	-	-	-	-	-	-
	Hexane	+	+	+	-	-	-	-	-	-
	Benzene	+	+	+	-	-	-	-	-	-
	Chloroform	++	++	++	-	-	-	-	-	-
	Ethyl acetate	-	-	-	-	-	-	-	-	-
	Acetone	+++	++	++	+	+	-	-	-	-
	Methanol	++	++	++	-	-	-	-	-	-
Fischerella sp.	Aqueous	-	-	-	-	-	-	-	-	-
	Hexane	+	+	++	+	+	-	-	-	-
	Benzene	+	+	+	+	+	-	-	-	-
	Chloroform	+	+++	++	+	+	-	-	-	-
	Ethyl acetate	-	-	-	-	-	-	-	-	-
	Acetone	+++	++	++	++	++	-	-	-	-
	Methanol	+++	+++	+++	++	++	-	-	-	-

*Keys: +++ = indicate prominent; + = indicates slightly and - = indicates absence

Few isolated compounds (M_3, $M_{3,}$ and M_2) from the methanol extracts of *Lyngbya kuetzingiana*, *Scytonema pseudoguyanensis*, and *S. pseudo-hofmani* respectively showed positive response against *Bacillus subtilis* and four compounds M_3 (*Lyngbya kuetzingiana*), M_2 (*Nostoc linckia*), A_2 (*Scytonema hyalinum*) and M_5 (*Tolypothrix rechingeri*) against *Staphylococcus aureus* respectively. However, the compound (A_2) of acetone extracts of *Fischerella* species showed the highest inhibition zone (Complete inhibition) against *E. coli*. Concerning to fungal strains, out of isolated compounds from ten species, the compound (A_2) of acetone extract of *Fischerella* species was found to be active against *C. albicans* whereas, *Scytonema hyalinum* and *Tolypothrix rechingeri* also exhibited activity against both the fungal strains studied.

Each fraction had one active band obtained on TLC plates against target pathogenic strains. The bioautography was showed with one major active band separately obtained from hexane, benzene, chloroform, acetone, and methanol extract of ten isolates against respective pathogens with different R_f values **Table 2**.

The positive controls, ciprofloxacin and carbazine used in this study, showed better antimicrobial activities in comparison to the compound extracts studied **Fig. 5**. However, *Fischerella* species isolated compound showed better activities against *E. coli*, completely inhibiting the growth of the test organism in comparison to ciprofloxacin (28mm, zone of inhibition, **Table 2**; **Fig. 4-5**.

TABLE 2: ANTIMICROBIAL ACTIVITY OF ACTIVE FRACTIONS OF SUB-AERIAL CYANOBACTERIA AGAINST REFERENCE HUMAN PATHOGENIC STRAINS AS PRESENTED BY INHIBITION ZONE DIAMETER (in mm) WITH ANTIMICROBIAL INDEX (IN PARENTHESES)

Species (Sources)	Active fractions with R_f value	Inhibition Zone (mm)/antimicrobial index (AI)						Standard antibiotics (Ciprofloxacin: 10mg/ml); (Carbazine: 10mg/ml) Inhibition Zone (mm)
		B. subtilis (MTCC 121)	*E. coli* (MTCC 723)	*P. aeruginosa* (MTCC 741)	*S. aureus* (MTCC 902)	*C. albicans* (MTCC 4748)	*E. flocossum* (MTCC 7880)	
Lyngbya	M_3	10.0 ± 1.0	15.0 ± 3.0	14.0 ± 1.0	14.0 ± 1.0	-	-	16.0; 28.0; 18.0; 26.0
kuetzingiana	(0.84)	(AI=62.5)	(AI=53.5)	(AI=77.7)	(AI=53.8)			
Nostoc linckia	M_2	-	26.0 ± 2.0	11.0 ± 1.0	14.0 ± 1.0	-	-	28.0; 18.0; 26.0
	(0.72)		(AI=92.8)	(AI=61.1)	(AI=53.8)			
Scytonema sp.	A_1	-	18.0 ± 1.0	15.0 ± 1.0	-	-	-	28.0; 18.0
	(0.96)		(AI=64.2)	(AI=83.3)				
Scytonema	M_3	10.0 ± 1.0	23.0 ± 1.0	18.0 ± 2.0	-	-	-	16.0; 28.0; 18.0
pseudoguyanensis	(0.86)	(AI=62.5)	(AI=82.1)	(AI=100)				
Scytonema	M_2	10.0 ± 1.0	20.0 ± 1.0	14.0 ± 1.0	-	-	-	16.0; 28.0; 18.0
pseudohofmani	(0.86)	(AI=62.5)	(AI=71.4)	(AI=77.7)				
Scytonema	A_2	-	20.0 ± 1.0	16.0 ± 0.0	20.0 ± 1.0	14.6 ± 1.0	11.3 ± 1.0	28.0; 18.0; 26.0; 24.0;
hyalinum	(0.96)		(AI=71.4)	(AI=88.8)	(AI=76.9)	(AI=60.8)	(AI=37.6)	30.0
Scytonema	A_2	-	24.8 ± 1.0	16.8 ± 1.0	-	-	-	28.0; 18.0
ocellatum	(0.96)		(AI=88.5)	(AI=93.3)				
Scytonema crispum	A_2	-	21.0 ± 2.0	12.3 ± 1.0	-	-	-	28.0; 18.0
	(0.96)		(AI=75)	(AI=68.5)				
Tolypothrix	C_{10}	-	11.0 ± 0.0	-	-	-	-	28.0
rechingeri	(0.84)		(AI=39.2)					
	A_2	-	11.3 ± 1.0	-	-	11.3 ± 2.0	23.3 ± 2.0	28.0, 24.0, 30.0
	(0.84)		(AI=40.3)			(AI=47)	(AI=77.6)	
	M_5	-	21.0 ± 0.0	-	22.0 ± 0.0	10.0 ± 0.0	-	28.0; 26.0; 24.0
	(0.84)		(AI=75)		(AI=84.6)	(AI=41.6)		
	H_9	-	10.6 ± 1.0	-	-	-	-	28.0
	(0.84)		(AI=37.8)					
Fischerella sp.	B_2	-	10.6 ± 1.0	-	-	-	-	28.0
	(0.86)		(AI=37.8)					
	A_2	-	Complete	-	-	16.3 ± 6.0	-	28.0; 24.0
	(0.96)		inhibition			(AI=68)		
	M_5	-	13.3 ± 2.0	-	-	-	-	28.0
	(0.84)		(AI=47.5)					

*Results are the means of diameter values ±standard deviation of three replicates. *R_f = Retention factor; A = Acetone; M = Methanol; C= Chloroform; B= Benzene and H= Hexane.

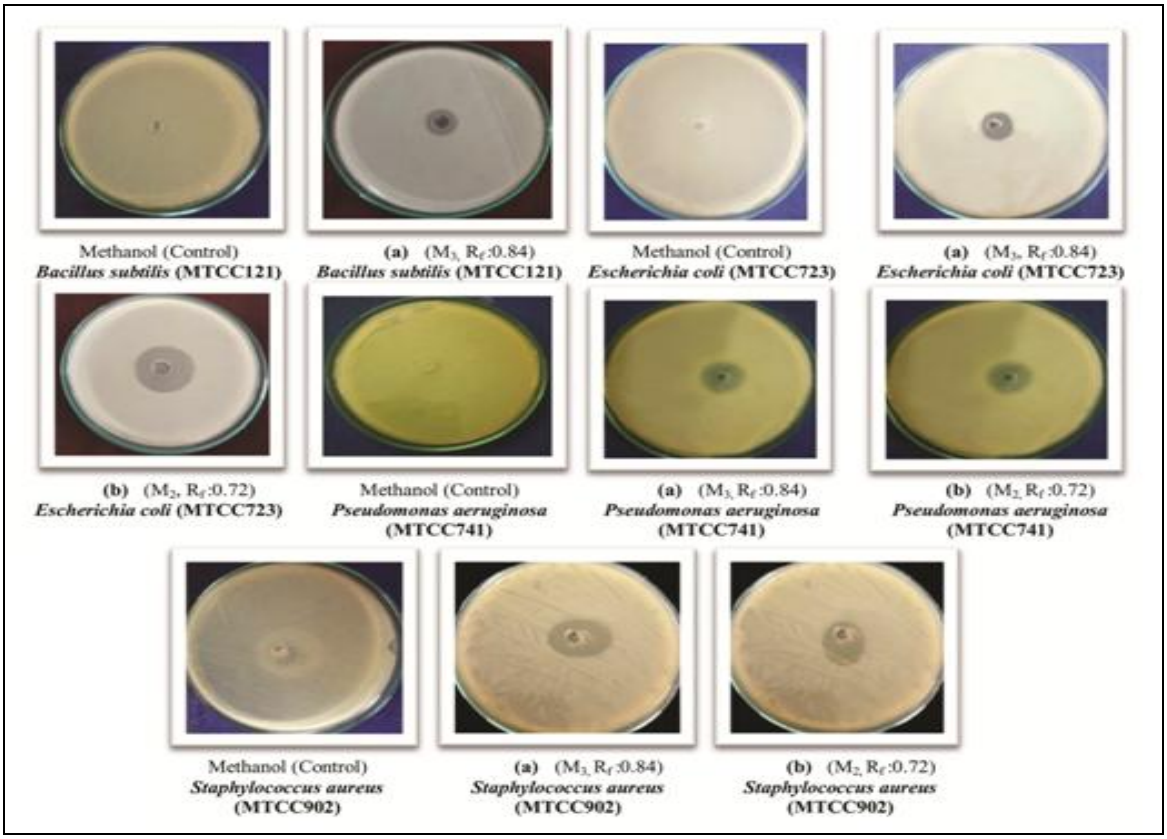

FIG. 1: ANTIBACTERIAL ACTIVITY OF ACTIVE FRACTIONS OF METHANOL EXTRACT OF (A) *LYNGBYA KUETZINGIANA* AND (B) *NOSTOC LINCKIA* AGAINST TARGET PATHOGENIC STRAINS

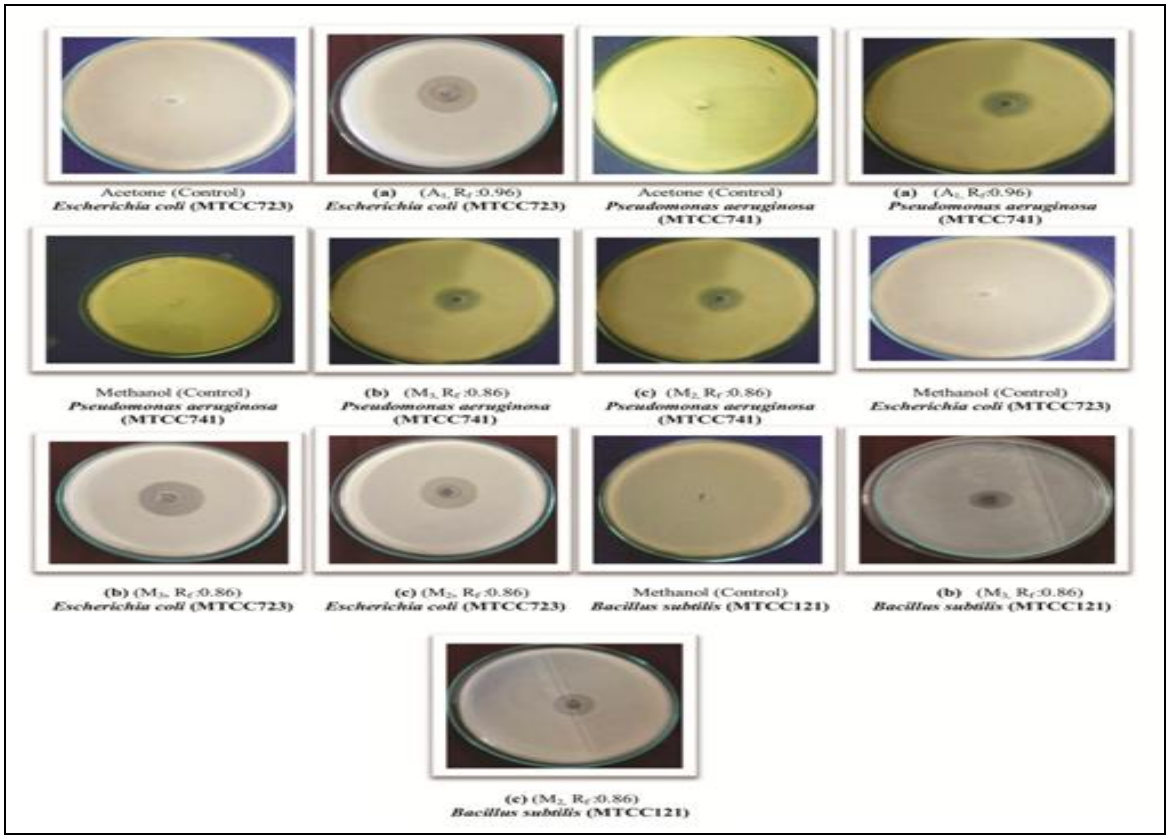

FIG. 2: ANTIMICROBIAL ACTIVITY OF ACTIVE FRACTIONS OF ACETONE/OR AND METHANOL EXTRACT OF (A) *SCYTONEMA* SP., (B) *S. PSEUDOGUYANENSIS* AND (C) *S. PSEUDOHOFMANI* AGAINST TARGET PATHOGENIC STRAINS

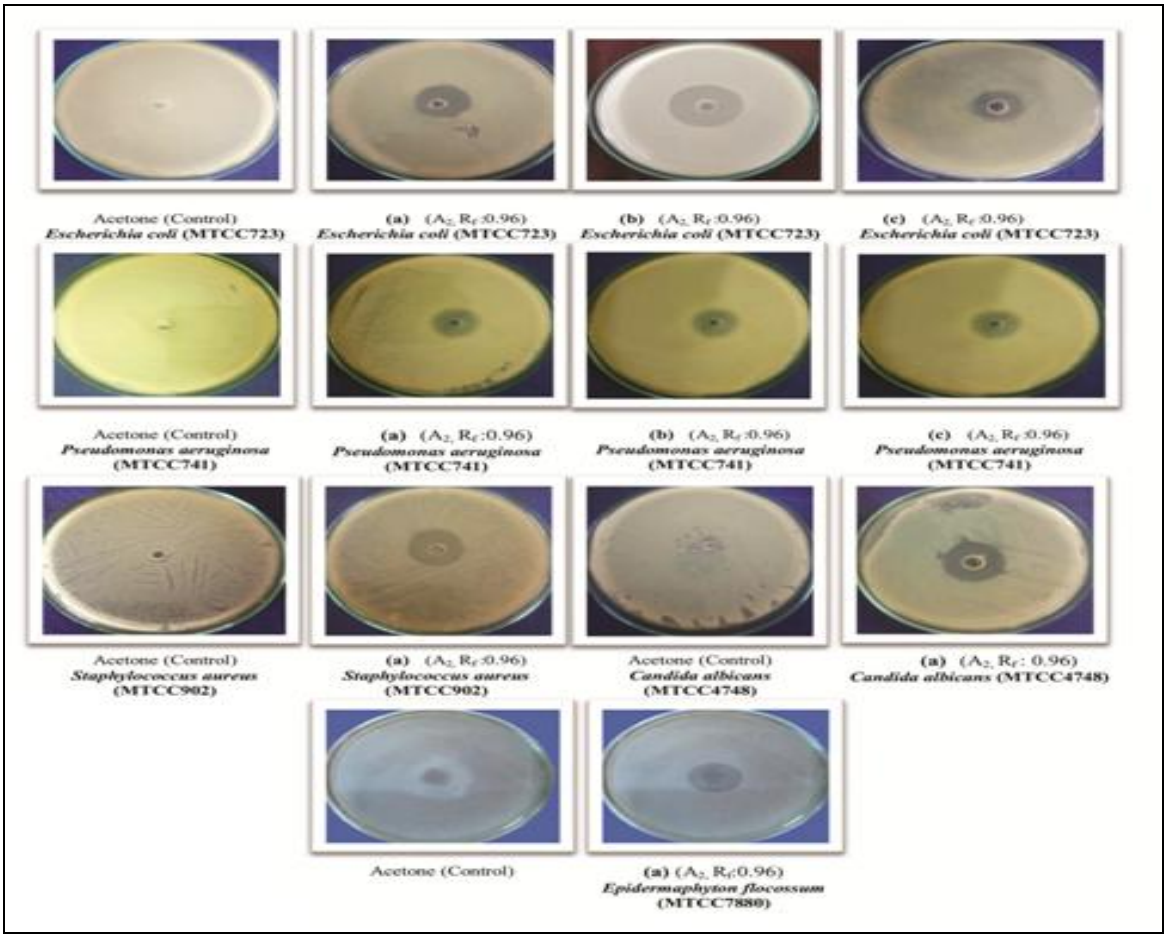

FIG. 3: ANTIMICROBIAL ACTIVITY OF ACTIVE FRACTIONS OF ACETONE EXTRACTS OF (A) *SCYTONEMA HYALINUM*, (B) *S. OCELLATUM* AND (C) *S. CRISPUM* AGAINST TARGET PATHOGENIC STRAINS

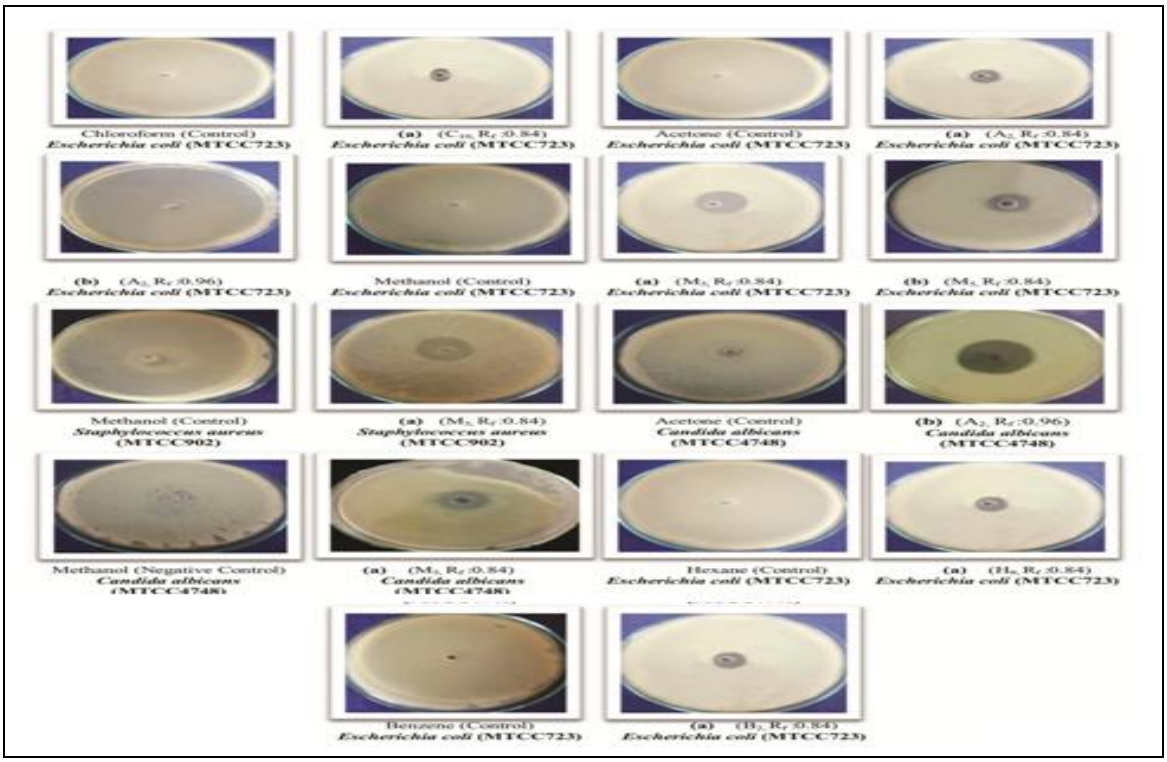

FIG. 4: ANTIMICROBIAL ACTIVITY OF THE ACTIVE FRACTIONS OF HEXANE/BENZENE/CHLOROFORM/ ACETONE AND OR METHANOL EXTRACTS OF (A) *TOLYPOTHRIX RECHINGERI* AND (B) *FISCHERELLA* SP. AGAINST TARGET PATHOGENIC STRAINS

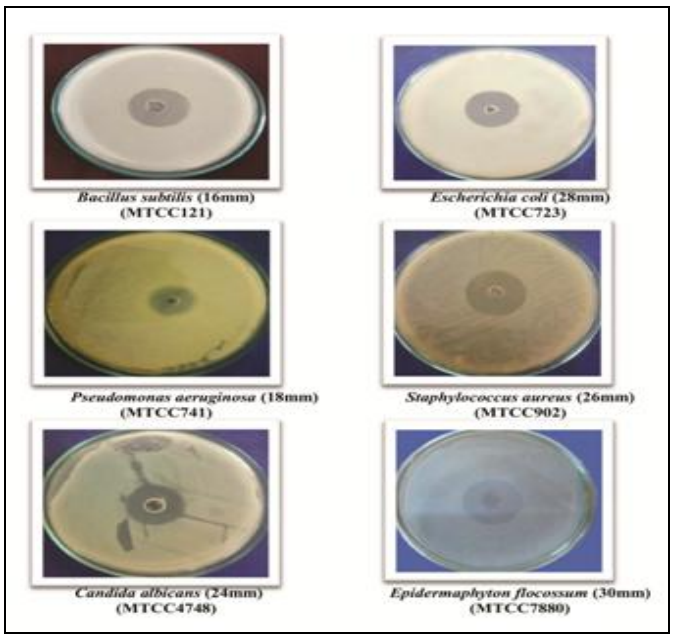

FIG. 5: ANTIMICROBIAL ACTIVITY OF THE STANDARD ANTIBIOTICS: CIPROFLOXACIN (10mg/ml) AND CARBAZINE (10mg/ml) AGAINST TARGET PATHOGENIC STRAINS

Minimum Inhibitory Concentration (MIC): The minimum inhibitory concentration (MIC) of the isolated compounds of different extracts exhibiting best antimicrobial activity of ten sub-aerial Cyanobacteria were investigated to determine the effectiveness. The MICs value of different compounds isolated from sub-aerial Cyanobacteria exhibited antibacterial /or antifungal activity ranged between 0.312-5mg/ml, are shown in **Table 3**. It was noted that the lower MIC (0.312mg/ml) was obtained only against *E. coli* MTCC723 treated with isolated compound (A_2) of *Fischerella* species whereas, the other compound isolated from species such as *Nostoc linckia*, *Scytonema ocellatum*, *S. hyalinum*, and *Tolypothrix rechingeri* showed MIC similar to standard antibiotics (0.625mg/ml) respectively. The compounds of methanol and acetone extracts of four strains, *Scytonema pseudoguyanensis*, *S. hyalinum*, *S. ocellatum* and *S. crispum* exhibited with similar MIC value (0.625mg/ml) respectively against *P. aeruginosa* MTCC741. However, moderate MIC value ranged from 1.25-5mg/ml was observed with *Lyngbya kuetzingiana*, *Nostoc linckia*, *Scytonema pseudoguyanensis*, *S. pseudohofmani*, and *S. ocellatum* extracts (methanol and acetone) against *E. coli* and *P. aeruginosa.*

Compounds (A_2) isolated respectively from *Fischerella* sp. and *Scytonema hyalinum* showed MIC value (0.625 mg/ml, 1.25mg/ml) respectively against *C. albicans* followed by *Tolypothrix rechingeri* (M_5, 1.25 mg/ml). However, the isolated compounds of other species also showed moderate activity ranged between 0.625-5mg/ml against *B. subtilis*, *E.coli*, *P. aeruginosa*, *S. aureus*, *C. albicans*, and *E. flocossum*, respectively. MICs values of bioactive compounds and their zone of inhibition were significantly correlated, i.e., compounds with higher zones of inhibition showed lower MIC values with $p \leq 0.05$.

TABLE 3: MINIMUM INHIBITORY CONCENTRATIONS (MIC) OF ACTIVE FRACTION OF SUB-AERIAL CYANOBACTERIA AGAINST TEST PATHOGENS

Species (Sources)	Active fractions with R_f value	MIC values (mg/ml)					
		B. subtilis (MTCC 121)	*E. coli* (MTCC 723)	*P. aeruginosa* (MTCC 741)	*S. aureus* (MTCC 902)	*C. albicans* (MTCC 4748)	*E. flocossum* (MTCC 7880)
Lyngbya kuetzingiana	M_3 (0.84)	5.0	2.5	2.5	2.5	-	-
Nostoc linckia	M_2 (0.72)	-	0.625	5.0	5.0	-	-
Scytonema sp.	A_1 (0.96)	-	2.5	1.25	-	-	-
Scytonema pseudoguyanensis	M_3 (0.86)	5.0	1.25	0.625	-	-	-
Scytonema pseudohofmani	M_2 (0.86)	5.0	1.25	1.25	-	-	-
Scytonema hyalinum	A_2 (0.96)	-	0.625	0.625	2.5	1.25	5.0
Scytonema ocellatum	A_2 (0.96)	-	0.625	0.625	-	-	-
Scytonema crispum	A_2 (0.96)	-	0.625	0.625	-	-	-
Tolypothrix rechingeri	C_{10} (0.84)	-	5.0	-	-	-	-
	A_2	-	5.0	-	-	5.0	0.625

	(0.84) M_5	-	0.625	-	2.5	1.25	-
Fischerella sp.	(0.84) H_9	-	5.0	-	-	-	-
	(0.84) B_2	-	5.0	-	-	-	-
	(0.86) A_2	-	0.312	-	-	0.625	-
	(0.96) M_5	-	2.5	-	-	-	-
Ciprofloxacin (10mg/ml)	(0.84) mg/ml	2.5	0.625	0.625	2.5	-	-
Carbazine (10mg/ml)	mg/ml	-	-	-	-	0.312	0.625

*R_f = Retention factor; A = Acetone; M = Methanol; C= Chloroform; B= Benzene and H= Hexane; numerals with the extracts indicate the no of active fraction of the extract.

Characterization and Identification of Bioactive Compound: Based on highest activity index, the antimicrobial active purified compounds (acetone/ or methanol) of each extract of the isolates were further characterized by FTIR and LC-MS, and different functional groups identified in these compounds are presented in **Table 4**, **Fig. 6(a-g)**.

TABLE 4: FT-IR ANALYSIS OF ACTIVE FRACTIONS OF THE TWO DIFFERENT ORGANIC EXTRACTS OF POTENTIAL SUB-AERIAL CYANOBACTERIA SPECIES

Functional groups	Types of vibrations	Characteristics absorption (cm^{-1})						
		Lyngbya kuetzingiana **Methanol extract (M_3, R_f - 0.84)**	***Nostoc linckia*** **Methanol extract (M_2, R_f - 0.84)**	***Scytonema* sp. Acetone extract and *S. pseudoguyanensis* & *S. pseudohofmani* Methanol extract (A_1, M_2, M_3, R_f - 0.96, 0.86, 0.86)**	***Scytonema hyalinum*** **Acetone extract (A_2, R_f - 0.96)**	***Scytonema ocellatum*** **&** **S. *crispum*** **Acetone extract (A_2, R_f - 0.96)**	***Tolypothrix rechingeri*** **Methanol extract (M_5, R_f - 0.84)**	***Fischerella*** **sp. Acetone extract (A_2, R_f - 0.96)**
Hydroxyl Amide A	O-H (stretch) N-H (stretch)	3317.3	3350.78	3366.5	-	-	3314.3	3534.7
Alkanes	$-CH_2$ (stretch)	-	2976, 2896	-	2958.5, 2924.1, 2859.7	2958.5, 2924.1, 2859.7	2944.3, 2832.6	-
	$-CH_3$ (bending)	1371.1	1384	1384	1378.9	1378.9	1449.2	-
Alkenes	=C-H (stretch)	-	-	-	724.52	724.52	-	3003.8
	=C-H (bending)	-	-	-	-	-	-	-
Alkynes	-C=C- (stretch)	2140.3	-	-	-	-	-	-
	C-H (bend)	-	-	-	1459.23	1459.23	616.7	
Amine II	C-N (stretch)	-	-	1508.33	-	-	-	1220.5
	N-H (bending)	-		-				903.0
Aromatic rings	C=C (stretch)			-			-	-
	C-C (stretch)							1420.3
	C-H (stretch)		-	-	-	-	-	-
Alcohol,	C= O (stretch)	1635	-	1704.7	-	-	-	1707.8
Carboxylic acids, esters	C- O (stretch)	-	1044	1044	1037.77	1037.77	1022.1	1092.1
Nitro compound	N-O (stretch)		1508.33					1358.8
Alkyl halides	C-Br (stretch)	-	532	-	-	-	1113.7	529
Phosphodiester	>P=O	-	-	1229.6	-	-	-	-
Iron	Fe	-	-	-	-	-	-	420

The isolated compound (M_3) from methanol extract of *Lyngbya kuetzingiana* showed peak at 3317.3cm^{-1}, 2140.3cm^{-1}, 1635.10cm^{-1}, 1371.1cm^{-1} due to the presence of O-H, -C=C, C=O, $-CH_3$ as functional properties **Fig. 6a** whereas compound (M_2) of methanol extract of *Nostoc linckia* showed peaks at 3350.78cm^{-1}, 2976cm^{-1}, 2896cm^{-1}, 1508.33cm^{-1}, 1384cm^{-1}, 1044cm^{-1}, 532.5cm^{-1} contains O-H, =C-H, C-H, C=C, C-O group **Fig. 6b**. In addition to these, the isolated compounds (A_1, M_3, and M_2) from acetone/or methanol extract of three species, *Scytonema* sp., *S. pseudoguyanensis*, and *S. pseudohofmani*, respectively, from different sites showed similar peaks at 3366.5cm^{-1}, 1704.7cm^{-1}, 1384cm^{-1}, 1229.6cm^{-1}, 532.5cm^{-1} indicated the presence of -N-H, OH group, C=O, C-H, C-O and

alkyl halide group **Fig. 6c**, but the compound (A_2) of acetone extract of *Scytonema hyalinum* exhibited broad absorption bands at 2958.5cm^{-1}, 2924.1cm^{-1} and 2859.7cm^{-1}, at 1459.2cm^{-1}, 1378.9cm^{-1}, 1037.7cm^{-1} and at 724.5cm^{-1} revealed the presence the functional properties like CH_2, C-N or C=C, C-O, –C=H functional groups **Fig. 6d**. The compound (A_2) isolated separately from two species, *Scytonema ocellatum* and *S. crispum* showed similarity in peaks at 3366cm^{-1}, 2945cm^{-1}, 2832cm^{-1}, 1704cm^{-1}, 1420cm^{-1}, 1364cm^{-1}, 1229cm^{-1}, 1093cm^{-1}, 1022cm^{-1} and 532cm^{-1} confirmed the presence of O-H, N-H, CH_2, C=O, C-O , C=C, –CH_3, P=O and alkyl halides **Fig. 6e**. However, compound (M_5) of methanol extract of *Tolypothrix rechingeri* showed peaks at 3314.3cm^{-1}, 2944.3cm^{-1} and 2832.6cm^{-1} in the presence of O-H, N-H, and at 1449.2cm^{-1}, 1113.7cm^{-1}, 1022.1cm^{-1} indicated the presence of -CH_3, C-O, C-O-C groups and at 616.7cm^{-1} of alkyl halides **Fig. 6f**. The compound (A_2) of acetone extract of *Fischerella* sp. showed peaks at 3534cm^{-1}, 3003.8cm^{-1}, 1707.8cm^{-1}, 1420.3cm$^{-1,}$ and 1358.8cm^{-1} due to the presence of NH_2, OH, NH, CH_2, C=O, C-O, N-O group and at 1220.5cm^{-1}, 1092.1cm^{-1}, 903cm^{-1}, 529cm^{-1} and at 420cm^{-1} due to the presence of phosphodiester, N-H, alkyl halides and Fe group **Fig. 6g**.

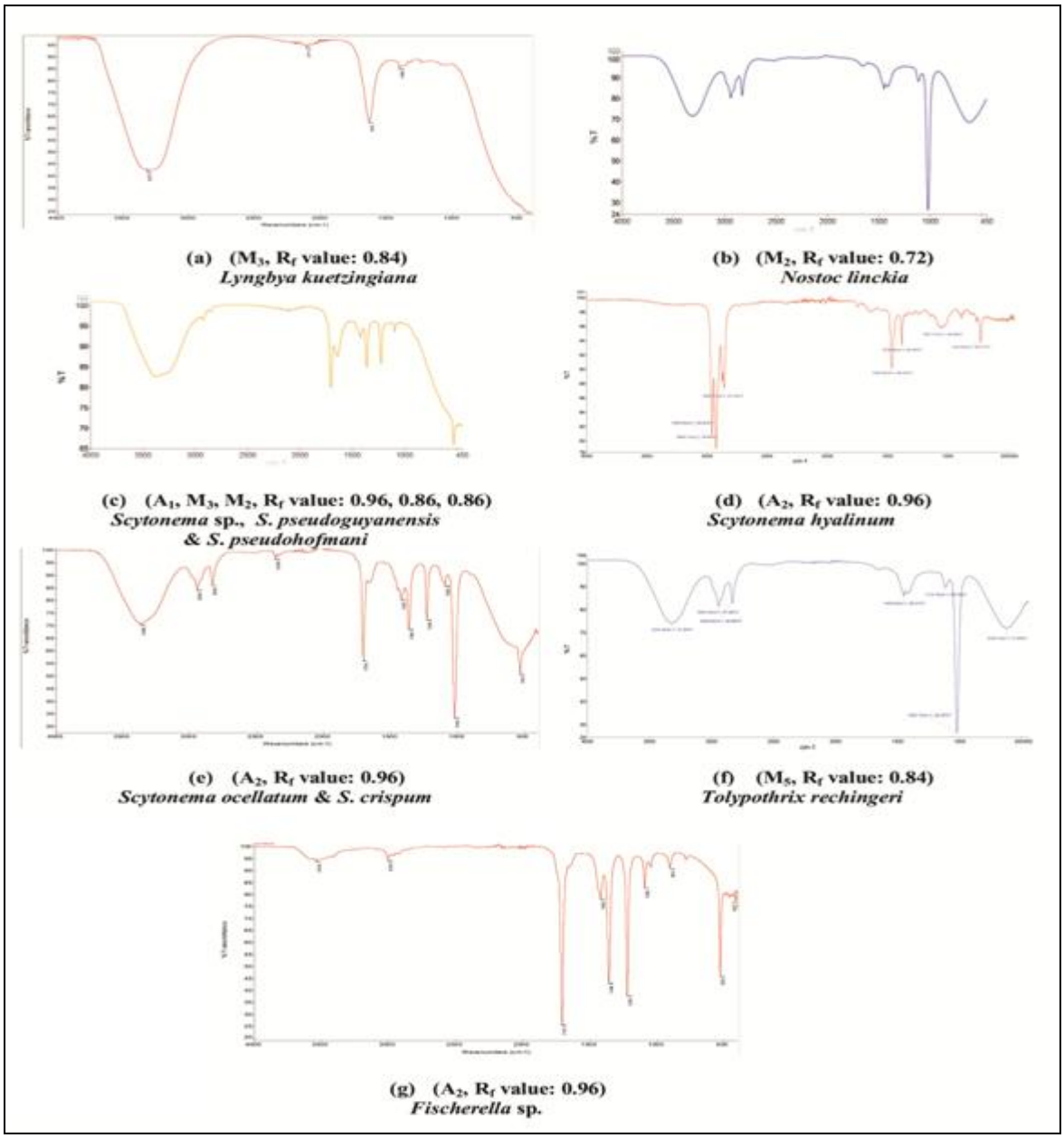

FIG. 6: FT-IR SPECTRUM OF THE FUNCTIONAL GROUPS OF ACTIVE FRACTIONS OF THE TEN SUB-AERIAL CYANOBACTERIA. A= ACETONE EXTRACT AND M=METHANOL EXTRACT

LC-MS analyses were performed with purified compound of the crude extracts (acetone and methanol) of the target species. Chemical components of the analyzed active compounds are presented in **Table 5**. In our data, for each sub-aerial Cyanobacteria species, purified compounds chemical components was recorded such on 1,3-dihydroxycyclohexane, $C_6H_{12}O_6$: Mw-116g/mol [(compound-M_3), *Lyngbya kuetzingiana*]; 4-(4-hydroxylphenyl) butanoic acid, $C_{10}H_{12}O_3$:Mw-178g/mol [(compound-M_2), *Nostoc linckia*]; Octa-hydro-1H-indole-2-carboxylic acid, $C_9H_{15}NO_2$: Mw-169g/mol [(compound-A_1, M_3, M_2), *Scytonema* sp., *S. pseudoguyanensis* and *S. pseudohofmani*]; Ergost-5-en-3-ol, $C_{28}H_{38}O_4$: Mw- 704g/mol [(compound-A_2), *Scytonema hyalinum*]; 7, 11-dihydroxysolasodine, $C_{27}H_{43}NO_4$: Mw-413g/mol [(compound-A_2), *Scytonema ocellatum* and *S. crispum*]; 2, 4-Bis (2-methyl-2-propanyl) phenol - phosphorous acid, $C_{42}H_{69}O_6P$: Mw-700g/mol [(compound-M_5), *Tolypothrix rechingeri*] and Pentaphenyl ferrocene carboxamide, $C_{41}H_{31}FeNO$: Mw-610g/mol [(compound-A_2), *Fischerella* sp.]. The resultant major peaks of the active fractions of extracts were surveyed by the use of the available database PubChem, provided by the National Centre for Biotechnology Information (NCBI) at http://pubchem.ncbi. Nlm.nih.gov. Classification, biomedical features, and biological assay activity was obtained for some of the resultant fractions, and data are represented in **Table 5**.

TABLE 5: MASS SPECTRA COMPONENT IN ACTIVE FRACTIONS OF SUB-AERIAL CYANOBACTERIA SPECIES

Sub-aerial Cyanobacteria species	LC-MS spectra compound	Chemical Class	Compound ID in PubChem (CID)	Antimicrobial effects
Lyngbya kuetzingiana	1,3-dihydroxycyclohexane $C_6H_{12}O_6$:Mw-116g/mol	Fatty alcohol	10433	No reported activity
Nostoc linckia	4-(4-hydroxylphenyl)butanoic acid $C_{10}H_{12}O_3$:Mw-178g/mol	Fatty acids	279983	No reported activity
Scytonema sp.	Octahydro-1H-indole-2-carboxylic	Fatty	3274680	No reported activity
S. pseudoguyanensis	acid	acids/Carboxylic acid		No reported activity
S. pseudohofmani	$C_9H_{15}NO_2$:Mw-169g/mol			
Scytonema hyalinum	Ergost-5-en-3-ol $C_{28}H_{38}O_4$:Mw-704g/mol	Steroidal alkaloids	6428659	Reported activity [27]
Scytonema ocellatum	7, 11-dihydroxysolasodine	Steroidal alkaloids	575210	No reported activity
S. crispum	$C_{27}H_{43}NO_4$:Mw-413g/mol			
Tolypothrix rechingeri	2, 4-Bis (2-methyl-2-propanyl) phenol - phosphorous acid $C_{42}H_{69}O_6P$:Mw-700g/mol	Phenolic compound	71374778	No reported activity
Fischerella sp.	Pentaphenyl ferrocene carboxamide $C_{41}H_{31}FeNO$:Mw-610g/mol	Heterocyclic alkaloids	71310582	No reported activity

DISCUSSION: The present investigation was carried out to evaluate the potential of ten sub-aerial Cyanobacteria with antimicrobial activities against pathogenic bacterial and fungal strains. During the study, secondary metabolites like alkaloids, phenols, flavonoids, glycosides, quinones, and steroids were observed to be present in the different crude extracts of the isolates studied. The presence of major secondary metabolites were observed in the acetone and methanol extracts as compared to the hexane, benzene, and chloroform extracts. Many reports indicated that natural alkaloids, phenolic compounds, and flavonoids are highly effective against a wide spectrum of pathogens. It was also reported that Cyanobacteria existing with secondary metabolites like alkaloids, phenols, flavonoids, quinones, glycosides, terpenoids, tannins, steroids, and saponin could be used in the development of antimicrobials, against resistant strains of bacteria, fungi, and mycobacterium, as base molecules use in drug development [19].

Further, while studying the antimicrobial activity of different extracts (and their compounds) of the ten sub-aerial Cyanobacteria the authors found that the purified compounds isolated from three species *i.e. Fischerella* sp., *Scytonema hyalinum* followed by *Tolypothrix rechingeri* showed significant antimicrobial activities against target reference human pathogenic strains. The compounds from other seven isolates, *Lyngbya kuetzingiana*, *Nostoc linckia*, *Scytonema* sp., *S. pseudoguyanensis*, *S. pseudohofmani*, *S. ocellatum* and *S. crispum* also exhibited antibacterial activities against *Bacillus subtilis*, *E. coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*, but no activity was found against *C. albicans* and *E. flocossum.*

During the study we observed that the most purified compounds exhibited better antibacterial activities against *E. coli* and *P. aeruginosa* than *Bacillus subtilis* and *Staphylococcus aureus*. In contrast to our findings it was reported that Gram-positive bacteria seemed to be more sensitive to the Cyanobacteria than Gram-negative bacteria. Among the isolates *Fischerella* sp. was observed to be more effective against the test pathogens [20].

These finding somehow has an agreement with previous studies that indicated methanol extracts of *Lyngbya kuetzingiana* and *Nostoc linckia* had moderate antibacterial activities [21, 22]. While studying the MIC of different isolated, purified compounds of the isolates, through the broth dilution method, it was observed that the MIC values ranged between 0.312-5mg/ml **Table 3**.

Antimicrobial effects of the cyanobacteria extracts against pathogens were detected for the presence of various secondary metabolites [23]. The antimicrobial activity of the sub-aerial Cyanobacteria extract could be due to the presence of different chemicals that may include fatty acids, alkaloids, flavonoids, phenolic compounds, quinones, and steroids. These agreements are comparable with the present study's findings detected through direct bioautography with R_f value 0.72, 0.84, 0.86, and 0.96, which showed the presence of the phenolic compound, alkaloids, and steroids in the purified compounds of ten sub-aerial Cyanobacteria. In contrast to our observations, many studies have reported the presence of terpenoids, tannins, and saponin in the extracts of Cyanobacteria [24].

The FTIR spectrum confirmed the presence of alcohol, alkanes, alkenes, amines, ester, aromatic, alkyl halides, aldehydes, carboxylic acids, and phenolic and nitro compounds in all tested purified compounds isolated from crude extracts (acetone/or methanol) of Cyanobacteria and were predicted through the presence of the functional groups like O-H, N-H, =C-H, C-N, C-C, C=O, C-O, N-O, >P=O stretching and iron group. The antimicrobial activity of the compounds could be due to the presence of these secondary metabolites [25].

In the current investigation, the strong antimicrobial activity of the different purified compounds of acetone/or methanol extracts of each species was subjected for identification by LC-MS. The mass spectra of the compound were investigated with those similar in the PubChem database and some of our chemical components are reported to have a known importance in pharmaceutical fields **Table 5**. In this study, purified compound-M_3 of methanol extract of *Lyngbya kuetzingiana* contained 1, 3-dihydroxycyclohexane as alkyl compound and fatty alcohols, whereas other two compounds as carboxylic acids, 4-(4-hydroxylphenyl) butanoic acid [compound-M_2 of methanol extract of *Nostoc linckia*] and Octahydro-1H-indole-2-carboxylic acid [acetone/or methanol compounds-A_1, M_3, M_2 of three different species, *Scytonema* sp., *S. pseudoguyanensis*, *S. pseudohofmani*]. In the present study, fatty alcohol and carboxylic acid like compounds produced from *Lyngbya kuetzingiana*, *Nostoc linckia*, *Scytonema* sp., *S. Pseudo-guyanensis*, and *S. pseudohofmani* showed antibacterial activity with higher and MIC values against *E. coli* and *P. aeruginosa* **Table 2-3**.

Beside this, aromatic hydrocarbon like 2, 4-Bis (2-methyl-2-propanyl) phenol - phosphorous acid detected in the compound-M_5 of methanol extracts of *Tolypothrix rechingeri* also revealed better antimicrobial activities. This connection revealed that phenolic compound has broad application as antibacterial, antifungal, and antioxidant properties [26]. In present study, *Tolypothrix rechingeri* produced a compound having the highest antimicrobial action against *P. aeruginosa*, *S. aureus*, and *C. albicans*. In addition to these, two compounds, Ergost-5-en-3-ol from *Scytonema hyalinum* and 7,11-dihydroxysolasodine produced by two different species of *Scytonema ocellatum* and *Scytonema crispum* known as steroidal alkaloids, found to be present in the compound (A_2) of acetone extract respectively. In our study, steroids like compound ergost-5-en-3-ol and 7,11-dihydroxysolasodine found in two different *Scytonema* sp. showed good antibacterial activities against *E. coli* and *P. aeruginosa*. The heterocyclic alkaloids compound, amino (cyclopenta-2,4-dien-1-ylidene) methanolate also known as pentaphenyl ferrocene carboxamide was identified in the compound-A_2 isolated from acetone extract of *Fischerella* sp. showed excellent antimicrobial activity against *E. coli* and *C. albicans* with MIC value (0.312mg/ml and 0.625mg/mol). It is pertinent to mention that the observation of the

antimicrobial activity of these compounds isolated from sub-aerial Cyanobacteria against human pathogens is of its kind.

CONCLUSION: Sub-aerial Cyanobacteria that occupy extreme environments also known as extremophiles, are substantial resources for natural bioactive substances which could be exploited in the pharmacological industry. In this present study, we investigated the antimicrobial activity of some purified compounds of the crude extracts of sub-aerial Cyanobacteria, which showed significant results against human reference pathogens. The present investigation indicated that sub-aerial Cyanobacteria remain an interesting alternative source for new antimicrobial metabolites with better inhibition activity and also suggest that *Scytonema hyalinum*, *Tolypothrix rechingeri*, *Fischerella* sp. and could be potential sources for secondary metabolites with strong antibacterial and antifungal activities. The findings recorded in this investigation are of their kind. Though it is firsthand information regarding the isolation of extremophilic sub-aerial Cyanobacteria (from old monuments, temples, caves, building facades, etc. in Odisha, more specifically in the cities of Bhubaneswar and Puri), with the production of different antibacterial and antifungal compounds, further, studies are desired to find its way for use in pharmaceutical industries, for development of newer antimicrobials against costly harmful antibiotics and chemotherapeutics, in order to enjoy the benefits and/or the fruits of this investigation.

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