In vitro assessment of *Lyngbya* sp. and *Phormidium* sp. extracts for antibacterial and antioxidant properties.

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Abstract

The exploration of bioactive secondary metabolites for antimicrobial and antioxidant potency from selected cyanobacterial strains *Lyngbya* sp. and *Phormidium* sp. were evaluated. Among the gradient solvent extraction system employed, Methanol: Chlorofrom (70:30) exhibits higher antimicrobial potency. The fraction extract LP 6 from *Lyngbya* sp. proved to be the most prominent fraction showing inhibitory against the human pathogens *Salmonella typhi*, *Staphylococcus aureus* and with moderate inhibition against *Vibrio cholerae, Salmonella paratyphi* A. The PF 3 fraction extract of *Phormidium* sp. reported for maximum antibacterial potency towards the MRSA bacterium alone, while other tested pathogens were resistive. Of all the tested pathogens, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* being the most resistive to all the cyanobacterial extracts tested. LF 6 fraction showed the minimum inhibitory concentration (MIC) value of 31.2 µg/ ml against the ciprofloxacin resistant *Salmonella typhi,* while the PF 3 fraction shows lowest inhibitory value of 62.5 µg/ml against MRSA strain. Further, the LF 6 fraction reported moderate toxicity with a LC_{50} of 153.9 µg/ml, while the PF3 fraction exhibits highest toxicity of LC_{50} of 51.06 µg/ml against brine shrimp larvae. The active fractions were identified for its chemical constituents using the GC-MS and NIST library match. As cyanobacteria were well known for antioxidant feature, both the fraction extracts LF 6 and PF 3 showed inhibition of free radical formation in 2, 2-diphenyl- 1-picrylhydrazyl (DPPH) assay at the concentration of 66 µg/ml and 42 µg/ml, respectively. Our study clearly evident that fraction compounds of *Lyngbya* sp. and *Phormidium* sp. posses strong antimicrobial activity and significant antioxidant potential.

Keywords: *Lyngbya* sp., *Phormidium* sp., antioxidant activity, antibacterial activity, brine shrimp toxicity, Disc diffusion, MIC assay, GC-MS, DPPH assay

1. Introduction

The cyanobacteria, photosynthetic prokaryotes dates back to 3 – 4 billion years (Kaushik et al., 2009) and its medical, nutritive value were recognized about 1500 BC (Liu and Chen, 2003). In habituate, cyanobacteria were adopted to thrive in all conceivable environments includes limnic, marine water, terrestrial habitat, planktonic, benthic and even under highly environmental stressed conditions like thermal hot springs (Chorus and Bartram, 1999; Kehr et al., 2011) and they diverse in their morphology from unicellular to multicellular, single coccoidal to branched filamentous forms (Kaushik et al., 2009). They have the ability to adapt with huge change in the environmental factors like salinity, temperature and light intensity (Chorus and Bartram, 1999; Funari and Testai, 2008). The cyanobacterial metabolites are being the attractive source of novel classes of pharmacologically active compounds with wide range of pharmaceutical, medical and industrial applications (Abdel-Raouf et al., 2011). Many study revealed that both intracellular and extracellular metabolites exhibits antimicrobial activity (El Semary, 2012), immunosuppressant (Koehn et al., 1992), anticancer (Maruthanayagam et al., 2013), antiviral, antifungal, antibiotics (Patterson et al., 1994; Volk and Furkert, 2006) and anti-inflammatory to proteinase-inhibiting agents (Mayer and Gustafson, 2003; Rastogi and Sinha, 2009), even shown to be active against multi drug resistant *Mycobacterium tuberculosis* (Rao et al., 2007). In addition, the cyanobacteria were shown to promote plant growth by secreting auxins and cytokins (Gayathri et al., 2015) and best alternative energy source with its lipid content (Anahas and Muralitharan, 2015). Cyanobacterial strains belonging to the genus *Lyngbya, Oscillatoria, Symploca, Calothrix, Leptolyngbya, Dichothrix, Geitlerinema, Schizothrix, Aphanothece, Blennothrix, Synechocystis*, *Anabaena, Microcystis, Scytonema* and *Nostoc* are known to produce diverse class of metabolites, like terpenes, halogenated aliphatic compounds, poly-phenolic compounds heterocyclic compounds, acyclic compounds, linear decadepsipeptides, linear alkynoic lipopeptides, linear lipopeptides cyclic depsipeptides, cyclic undecapeptides, lipophylic cyclic peptides, cyclic hexapeptides, glycolipids, macrolactones, paracyclophanes and more such compounds, while all were acknowledged for its bioactivity (Nagarajan et al., 2012). Genera *Lyngbya* and *Symploca* species alone contributes to the majority of cyanobacterial metabolites (Tan, 2007). The polyketide synthase (PKS) and non-ribosomal polypeptide synthase (NRPS) are being the major genetic theme in the synthesis of cyanobacterial natural products with diverse structures. Though, the cyanobacterial secondary metabolites were showing prolific bioactive effect, they were very minimally

exploited (Thajuddin and Subramanian, 2010). The present study was aimed to assess the antimicrobial properties of *Lyngbya* sp. and *Phormidium* sp. extracts against selected human bacterial pathogens, toxic effect and its antioxidant property.

2. Materials and Methods

2.1. Collection and purification of cyanobacterial strains:

The cyanobacterial samples *Lyngbya* sp. and *Phormidium* sp. were collected from fresh water sites of Mathur (Lat 10° 68' 81" N Long 78° 73' 35" E), Tiruchirappalli, Tamilnadu, India. The pure cultures were obtained through by streak plate method in BG-11 agar plates incorporated with antibiotics, germanium dioxide and cycloheximide to get rid of bacterial and eukaryotes contamination (Rippka et al., 1979). The cultures were incubated with constant light intensity using cool white fluorescent tubes (40- 50 µE m^{-2} s^{-1}) at 25 ± 2 ºC with continuous aeration and light/dark cycle for 14/10 hrs. Simultaneously, the purity of the cultures were repeatedly tested through regular microscopic observation with subsequent exclusion of contaminants by plating it on LB agar (Volk and Furkert, 2006). After 21 days, all the axenic cyanobacterial biomass was harvested by centrifugation at 5000 rpm for 20 min, washed with isotonic solution to remove antibiotic traces, freeze dried and used for bioactive compound extraction.

2.2. Morphological characterization

The culture morphology of the tested cyanobacterial strains were identified with bright field microscope (Optika, Italy) and its taxonomic identification was done based on the descriptions of Desikachary (1959) and Geitler (1932).

2.3. Molecular characterization

The tested cyanobacterial strains were further confirmed through molecular methods by isolating genomic DNA and performing PCR amplification of the 16S rRNA gene as described previously (Thajuddin et al., 2010). Total genomic DNA extraction was carried out from the cyanobacterial strains by the following methods and used as a template in PCR reaction. Eight days old culture was pelleted by centrifugation, the medium was decanted, and the pellet was resuspended in 500 µl of 50 mM Tris-HCl (pH 8.0) – 5mM EDTA (pH 8.0) -50 mM NaCl. Lysozyme was added to obtain a final concentration of 1 mg/ml, and the solution was incubated at 55 °C for 10 min or until the solution cleared (complete cell lysis). The solution was chilled on ice and extracted with an equal volume of phenol-chloroform-isomylalcohol (25:24:1). The organic extraction was repeated, and the supernatant was added to an equal volume of 4 M ammonium acetate. Total genomic DNA was precipitated by the addition of 2 volumes of isopropanol followed by centrifugation for 10 min at room temperature. The pellet was washed with 70% ethanol, dissolved in TE buffer (10 mM Tris- HCl, 1mM EDTA, pH 8.0) and stored at -20 °C. DNA concentration and purity was measured on a Du 800 Spectrophotometer (Beckman coulter, USA).

Fragment of the 16S rRNA gene was amplified using the primer pairs A2F (5'-AGAGTTTGATCCTGGCTCAG -3') and S17R (5'- GGCTACCTTGTTACGAC -3') (Iteman et al., 2002). PCR were performed in a total volume of 50 µl containing 1 X PCR buffer, 2.5 mM MgCl$_2$ 250 µm of each deoxynucleotide triphosphate, 1µl of each primer and 25µl of Taq DNA polymerase. Thermal cycling was carried out using veriti 96-well thermal cycler (Applied Biosystems, USA). PCR conditions for the 16S rRNA gene amplification as follows: after an initial denaturation at 92 °C for 2 min; 30 cycles of 92 °C for 20s, 58 °C for 30s and 72 °C for 60s and a final extension step at 72 °C for 7 min. PCR products were analyzed by electrophoresis in 1.2% agarose gel in 1X buffer stained with ethidium bromide and photographed under UV transillumination using gel documentation system (BioRad gel Doc XR, USA).

2.4. Pathogenic Bacterial strains tested

Both the gram-positive and gram-negative bacterial strains including *Staphylococcus aureus*, Methicillin resistant *Staphylococcus aureus* (MRSA), *Salmonella typhi*, *Salmonella paratyphi* A, *Klebsiella pneumoniae*, *Vibrio cholerae* and *Pseudomonas aeruginosa* were isolated from clinical samples and maintained in nutrient agar slant at 4 º C before being used as test strains. All the bacterial strains were identified by morphological characterization, motility test, biochemical tests, culturing in selective, differential medium and antibiogram test in accordance with the Bergey's manual of determinative and systematic bacteriology bacteriology (Buchanan, 1974; Williams et al., 1989).

2.5. Biological screening

2.5.1. Cyanobacterial extracts preparation by column chromatography

Briefly, one gram of freeze dried axenic cyanobacterial biomass was extracted with solvent mixture of methanol: chloroform (1:1 v/v) and stored at 4 $^{\circ}$ C for overnight. The centrifugation was carried at 10000 g for 30 minutes at cold condition. Taking the extracts leaving the sediment behind twice re-extracted in the same solvent system and the extracts were concentrated to dryness in flash rotary evaporator at 60 $^{\circ}$ C. The concentrate were applied on silica gel column G100 and G 200 and subjected for extraction with solvent gradient system. The fraction columns were developed with solvent gradient which includes Hexane (Hex), Dichloromethane (DCM), Ethyl acetate (EtoAc), and Chloroform ($CHCl_3$) and Methanol (MeOH). All the fractions were subjected for antimicrobial activity testing and the active fraction was evaluated for further toxicity assay, GC-HRMS analysis and antioxidant activity (Maruthanayagam et al., 2013).

2.5.2. GC-HRMS analysis of active cyanobacterial extract

GC- HRMS analysis was performed for the identification of the chemical constituents for the active fraction. To perform, the active fraction was dissolved at a concentration of 1mg/1ml in chromatography grade MeOH and the mass analysis was performed using Joel, ACCU TOF GCV (Agilent 7890, Agilent Technologies, USA). GC coupled with high resolution mass spectrometer according to standard settings. The GC separation was achieved by injecting the sample volume of 2 µl in to the injector with holding temperature of 220 °C. The capillary column of 30 m length × diameter of 0.32 mm with 0.25 µm thickness was used. The gas chromatography run was using carrier gas as Helium with constant flow of 2 ml/min. The initial oven temperature was started at 80 °C for 2 min followed with an increase of 5-6 °C per minute to reach final temperature of 280 °C. The compounds of active fractions were mass range analyzed at 10- 600 *m/z* using Flame Ionization detector (FID). The identity of the compounds was authenticated with NIST mass library databases (http://www.sisweb.com/software/ms/nist.htm) (Krall et al., 2009).

2.5.3. Antibacterial activity by disc diffusion method

The antibacterial activity of tested cyanobacterial extracts were performed with CLSI (formerly NCCLS) standard using disc diffusion and minimum inhibitory concentration method (Shalini et al., 2014). Briefly, all the individual fraction concentrates were dried aseptically, weighed and dissolved in 10% DMSO. About 30 µl of fraction concentrates were applied twice after consecutive drying to 6mm Whatman filter paper No. 1 disc to get final concentration of 0.5, 0.25, 0.125 and 0.062 mg/disc and placed aseptically on the Muller Hinton agar plates (20ml) seeded with test organism (0.5 Mc Farland turbidometry). All the test and standard control plates were incubated at 37 °C for 24 hrs and the zone of inhibition was measured. The DMSO was used as solvent control and ciprofloxacin (10 mcg), streptomycin (25 mcg) and oxacillin (5 mcg) were used as antibiotic standards. All the experiments were performed in triplicates and the average with standard deviation was calculated (Duraipandiyan et al., 2006; CLSI, 2009).

2.5.4. Minimum inhibitory concentration assay

All the active fraction extracts were screened for their MIC values by following National Committee for Clinical Laboratory Standards guidelines. The micro titer plates were added with 50 µl of Muller Hinton broth and seeded with 50 µl test pathogens. The serial dilution of cyanobacterial fraction (LF 6 and PF 3) were prepared from stock to get final concentrations of 250 µg/ml, 125 µg/ml, 62.5 µg/ml, 31.2 µg/ml, 15.6 µg/ml, 7.8 µg/ml and 3.9 µg/ml by two fold dilution along with DMSO (solvent) control, medium control to achieve final volume of 200 µl. The test plates were incubated for 24 hrs and examined for lowest concentration of active fraction that prevented the bacterial growth was considered to be the MIC (Skov et al., 2000; Prasannabalaji et al., 2012).

2.5.5. Minimum bactericidal concentration

Simultaneously, the MBC was calculated by plating the MIC test broth culture which not showing any visible growth in MIC assay in MHA plates, after incubation at 37 ºC of 18-24 hrs representing that the highest diluted MIC concentration which inhibits any visible colony formation on MHA plates was considered.

2.5.6. Artemia salina toxicity bio-assay

The toxicity of active fraction extracts of cyanobacteria was investigated by Brine shrimp lethality bioassay. The commercially available Brine shrimps cysts (*Artemia salina*) were hatched in a glass vessel filled with sterile seawater under constant aeration and light for 24hrs. Followed with removal of floating unhatched cysts and selecting hatched active *nauplii* of 2nd and 3rd instar stages. For toxicity assessment, the initial extract stock was prepared by

dissolving 0.01 g fraction extract in 1ml of DMSO. About 0.9 ml of the brine solution was mixed to 0.1 ml of the stock to give 1000 ppm solution and subsequent dilution gives final concentrations of 100 ppm and 10 ppm of extracts. To evaluate the lethal concentration, 10 nauplii were pipetted into glass culture tubes and exposed to all the four different concentration of tested cyanobacterial extracts. Each of the tests was done in triplicates along with DMSO control and brine solution control. All the culture tubes were maintained at room temperature for 24 hrs under fluorescent light. The number of surviving larvae counted and calculated for percentage of mortality as described earlier (Reish and Oshida calculation, 1987).

% mortality = No. of dead nauplii/ Initial no. of live nauplii   × 100

The cyanobacterial extract required to kill 50 % of shrimps population with in 24 hrs (LC_{50}) was calculated by the means of surviving nauplii's using the finneys probit analysis (Krishnaraju et al., 2005).

2.5.7. Antioxidant activity by DPPH assay

The free radical scavenging activity of cyanobacterial extract was determined by its reducing ability using the DPPH solution. For the experimental procedure, about 100 µM of DPPH (5 ml) stock solution was prepared in MeOH solution and stored in dark. About 0.5 ml of 500 µM DPPH solution in MeOH was mixed with 0.5ml (50 µg/ml) of cyanobacterial fraction concentrate to get final concentrate of 25 µg/ml of fraction concentrate in 250 µM DPPH solution. The reaction mixture was incubated in dark for 40 minutes and absorbance was registered. Similarly, the aliquots of 400 µg/ml, 200 µg/ml, 100 µg/ml, 50 µg/ml and 25 µg/ml test sample was prepared and assayed. The ascorbic acid and vitamin E were used as standard control. All the test and standard samples were measured at 517 nm by UV–vis Spectrophotometer (Shimadzu, Kyoto, Japan) in triplicates. The percentage of inhibition of absorbance was used to calculate the radical scavenging potency (antioxidant activity) (Chu et al., 2010; Geethalakshmi and Sarada, 2013).

Percentage of inhibition of absorbance (%) = (OD_{517} (DPPH+MeOH) -OD_{517} (sample))/OD_{517} (DPPH + MeOH) ×100%.

3. Results

The axenic cyanobacterial strains isolated from the fresh water bodies were identified as *Lyngbya* sp. and *Phormidium* sp. based on the morphological characteristic features (Fig.1).
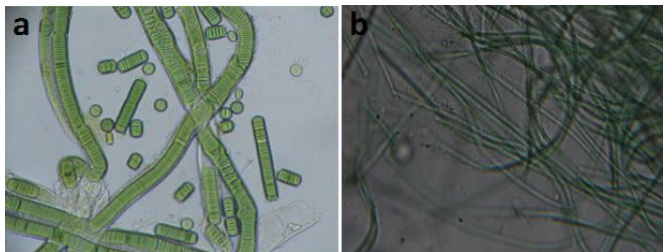

Fig 1. Photomicrographs illustrating the morphological features of filamentous non-heterocystous cyanobacteria a) *Lyngbya* sp. and b) *Phormidium* sp. tested in this study

Lyngbya sp.

Filaments single or forming a brown or dull blue-green thallus, sometimes having false branches, nearly straight or coiled, sometimes calcium incrustations; sheath at first thin, later thick, yellow brown, lamellated, only sometimes brownish on the inside and colourless outside; cells 8-24 µ broad, 2.7-5.6 µ long, not constricted at the cross-walls often granulated, contents sometimes with gas-vacuoles; end cells flat with thickened membrane, slightly attenuated.

Phormidium sp.

Thallus pale blue-green, thin, membranous, expanded; trichome straight or slightly bent, densely entangled, slightly constricted at the cross–walls, attenuated at the ends, 1-2 µ broad, pale blue-green; sheath thin, diffluent; cell up to 3 times longer than broad, 2.5-5 µ long, septa not granulated, cross-walls not commonly visible; end-cell acute-conical, calyptra absent.

The 16S rRNA gene amplification profiles of *Lyngbya* sp. and *Phormidium* sp. were electrophoresed along with molecular weight marker and are depicted Fig.2. The solvent extraction system yields 1 g of crude concentrate which on further subjecting with gradient solvent system extraction in silica gel chromatography yields 10 different fractions.
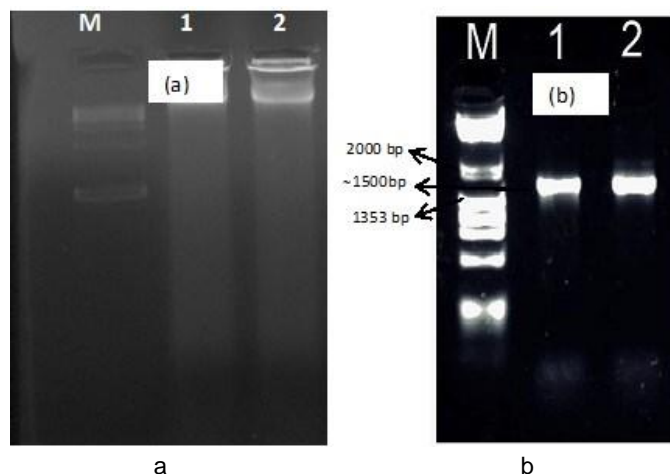
Fig.2. a) Genomic DNA and b) PCR amplification of 16S rRNA gene for filamentous non-heterocystous cyanobacterial strains. Lane M-Molecular weight marker (Finnzymes), Lane 1-*Lyngbya* sp. and Lane 2-*Phormidium* sp.

3.1. Antibacterial activity by disc diffusion method

      Comparatively, the cyanobacetria *Lyngbya* sp. fraction 6 (LF 6) of MeoH: $CHCl_3$ (70:30) and *Phormidium* sp. fraction 3 (PF 3) of MeoH: $CHCl_3$ (70:30) showed highest degree of antibacterial activity than all the other fraction elutes. Among the two cyanobacetrial extracts, the *Lyngbya* sp. (LF6) exhibits major antibacterial activity towards *Salmonella typhi, Staphylococcus aureus,* moderately active against *Vibrio cholerae*, minimally to *Salmonalla paratyphi* A and MRSA at highest concentration. While, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* bacterial pathogens were found to be highly resistant to all the fraction extracts. The results clearly depicts that LF 6 showed the highest zone of inhibition of 24 mm against *Salmonella typhi* and followed by 21 mm against *Staphylococcus aureus*. Similarly, the inhibition rate with 19 mm diameter of zone was observed against *Vibrio cholerae*. On the other hand, the fraction LF6 exhibits minimal activity with a zone of inhibition of 11 mm towards MRSA strains and 12 mm towards *Salmonella paratyphi A*. Among all the tested strains, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* exhibits higher degree of resistance with no zone of inhibition. The assessment of *Phormidium* sp. fraction 3 (PF 3) of MeoH: $CHCl_3$ (70:30) demonstrate highest zone of inhibition of 16 mm against MRSA*,* 12 mm minimal activity to *Staphylococcus aureus* and 11 mm against *Vibrio cholerae* at higher concentration tested *.* Based on the results, the *Salmonella typhi, Salmonella paratyphi A, Klebsiella pneumonia* and *Pseudomonas aeruginosa* found to be highly resistive, while the other strains were moderately sensitive to PF3 fraction. The degree of inhibition was directly proportional to the concentration of the extract. The results registered that *Lyngbya* sp. fraction (LF 6) were highly active against enteroinvasive *Salmonella typhi* , pyogenic *Staphylococcus aureus* and moderately to *Vibrio cholerae* while *Phormidium* sp. fraction (PF 3) were active against MRSA and minimally active to *Staphylococcus aureus* and *Vibrio cholerae.*

Table 1. Antimicrobial disc diffusion assay of active fraction LF 6 and PF 3 from the tested cyanobacterial strains *Lyngbya* sp. and *Phormidium* sp. on selected human pathogenic bacterial strains.

S.No	Test Organisms	Different concentration of MeOH:CHCl$_3$ active Fraction								Antibiotic control		
		LF 6 *				PF 3 *				Cip[+] (10µg)	Oxa[+] (5µg)	Strp[+] (25 µg)
		0.5	0.25	0.125	0.062	0.5	0.25	0.125	0.062			
1.	*Staphylococcus aureus*	21	18	16	12	12	10	9	8.5	27	29	28
2.	*MRSA*	11	9.5	8	7	16	13.5	11	9.5	21	12	18
3.	*Salmonella typhi*	24	21	18	15	NA	NA	NA	NA	15	17	19
4.	*Salmonella paratyphi*	12	11	9.5	8.5	NA	NA	NA	NA	28	26	24
5.	*Vibrio cholerae*	19	16	14	11	11	9.5	7	NA	24	26	23
6.	*Klebsiellapneumoniae*	NA	NA	NA	NA	NA	NA	NA	NA	31	28	19
7.	*Pseudomonas aeruginosa*	NA	NA	NA	NA	NA	NA	NA	NA	26	25	22

*= mg/disc; NA = No activity; Cip+- Ciprofloxacin; Oxa[+] - Oxacillin; Strp[+]- Streptomycin.

3.2. Minimum inhibitory concentration assay

The active fraction was subjected to minimum inhibition concentration assay and showed potent bacteriostatic and bacteriocidal property and the results were correlated to the zone of inhibition by disc diffusion. On screening the MIC activity, the lowest MIC value of 31.2 µg/ml against *Salmonella typhi* followed with 62.5 µg/ml concentration against *Staphylococcus aureus* and with *Vibrio cholerae*being 125 µg/ml(MIC=MBC) was observed for the *Lyngbya* sp. LF6 fraction. On the other hand, the minimum inhibition concentration of bioactive fraction PF 3 was shown to be active at 62.5 µg/ml against MRSA strains alone, being much lowest value than LF6 fraction (250 µg/ml). In MBC screening, all the MIC tests samples showed the values equals to MIC values (i.e. MIC=MBC) in LF6 fraction while PF3 fraction MBC in *Staphylococcus aureus* was observed at higher concentration of 250 µg/ml which comparatively higher to MIC values. The results depicted in the Table 2 clearly indicates that, bioactive fraction LF 6 can be considered as strong inhibitor of *Salmonella typhi*, *Staphylococcus aureus* and moderately to *Vibrio cholerae*, while fraction PF3 as active inhibitor of MRSA strains.

Table 2. Antibacterial activity of *Lyngbya* sp. and *Phormidium* sp. active fraction extracts of LF 6 and PF 3 showing minimum inhibitory concentration (MIC) assay against human bacterial pathogens.

S.No	Test Organisms	Minimum inhibitory concentration (MIC) (µg/ml)				
		Fraction LF 6	Fraction PF 3	*Standard antibiotic control*		
				(Cip[+])	(Oxa+)	(Strp[+])
1.	*Staphylococcus aureus*	62.5 **µg/ml**	250 **µg/ml**	≤ 1	≤ 1	≤ 1
2.	*MRSA*	250 **µg/ml**	62.5 **µg/ml**	≤ 1	≤ 6	≤ 3
3.	*Salmonella typhi*	31.2 **µg/ml**	NA	≤ 5	≤ 1	≤ 3
4.	*Salmonella paratyphi*	250 **µg/ml**	NA	≤ 2	≤ 1	≤ 2
5.	*Vibrio cholerae*	125 **µg/ml**	250 **µg/ml**	≤ 1	≤ 1	≤ 1
6.	*Klebsiellapneumoniae*	NA	NA	≤ 2	≤ 1	≤ 2
7.	*Pseudomonas aeruginosa*	NA	NA	≤ 2	≤ 1	≤ 1

Standard control antibiotics: Cip+- Ciprofloxacin; Oxa[+] - Oxacillin; Strp[+]- Streptomycin.

Assessment of *cyanobacterial* extracts for antibacterial and antioxidant properties.

Table 3. Toxicity assay of *Lyngbya* sp. and *Phormidium* sp. fraction extracts using Brine shrimp larvae (*Artemia salina*).

S.No	Fraction Extracts	Total no. of Live nauplii exposed	Total no of nauplii mortality			Percentage of mortality (%) at different concentration			LC_{50} µg mL^{-1}
			1000 ppm	100 ppm	10 ppm	1000 ppm	100 ppm	10 ppm	
1	LF 6	30	25	12	4	83	40	13	**153.9**
2	PF 3	30	27	16	9	90	54	**30**	51.06

3.3. GC-MS profiling

Due to the high antimicrobial activity of *Lyngbya* sp. fraction 6 (LF 6) of MeoH: $CHCl_3$ (70:30) the GC-MS profiling was performed (Fig.4) only for this fraction. The data analysis depicts that the chemical structure of active fraction based on the maximum hit percentage with the closest comparison of structure available NIST library structures. The results were tabulated (Table 4) for retention time, area %, molecular weight and structure obtained. About seven different compounds were identified in the active fraction LF6 includes 9,12-octadecadienoic acid, heptanoic acid 9-decen-1-yl ester, tetradecanoic acid 12-methyl ester, 4-methoxymethyoxy-hex-5-ynylidene-cyclohexane, Cyclopentane undecanoic acid methyl ester , 17- Pentatriacontene and Propionic acid, 2-(3-acetoxy-4,4,14-trimethylandrost-8-en-17).

Table 4. GC-MS profiling of *Lyngbya* sp. fraction extract (LF 6)

S. No	Compound name	Retention time	Area (m^2)	Total %	Molar mass (g.mol^{-1})	Structure
1.	9,12-octadecadienoic acid	16.3	981,884	34.7	280	$C_{18}H_{32}O_2$
2.	Tetradecanoic acid 12-methyl ester	18.9	244,316	8.6	256	$C_{16}H_{32}O_2$
3.	Cyclopentaneundecanoic acid	19.3	167,608	5.9	268	$C_{17}H_{32}O_2$
4.	Propionic acid, 2-(3-acetoxy-4,4,14-trimethylandrost-8-en-17)	19.5	269,103	9.6	430	$C_{27}H_{42}O_4$
5.	Heptanoic acid 9- decen-1-yl ester	24.2	474,368	16.7	253	$C_{17}H_{32}O_2$
6.	17- Pentatriacontene	27.6	391,752	13.8	490	$C_{35}H_{70}$
7.	4-methoxymethyoxy-hex-5-ynylidene-cyclohexane	29.6	299,772	10.6	230	$C_{14}H_{22}O_2$

3.4. Brine shrimp lethality assay

The toxicity assessment of fraction extracts were performed on brine shrimp nauplii (*A. salina*) exhibiting maximum viability of highest % in *Lyngbya* sp. cyanobacterial fraction and lowest viability in *Phormidium* sp. fraction extracts were shown in Table 5. Among the two cyanobacterium, the *Phormidium* sp. fraction PF 3 showed highest lethality of 90% (1000 ppm) followed with 30 % of mortality at the least concentration 10 ppm which comparatively higher than the *Lyngbya* sp.(LF 6). The active fraction PF3 of *Phormidium* sp.was exhibiting LC_{50} value of 51.06 µg/ml which represents the higher toxic potency. While the *Lyngbya* sp. fraction LF 6 observed minimum 13 % of mortality at 10 ppm. Further, the mortality rate was increased with 40 % at 100 ppm and 83 % at 1000 ppm representing the degree of increased toxicity with increasing concentration. The result obtained indicates that decreased viability at higher concentration with LC_{50} value of 153.9 µg/ ml (LF 6) exhibits moderate toxicity nature of the compound.

3.5. Antioxidant assay

In the antioxidant assay, the DPPH radical scavenging activities of MeOH solvent extracts of LF 6 and PF 3 were shown (Fig. 3). The MeOH fraction extract of *Lyngbya* sp. LF 6 exhibits highest inhibition activity of 66 % at 400 µg/ml, followed with minimal inhibition of 48% at 25 µg/ml. While, the *Phormidium* sp. PF 3 showed moderate to lesser DPPH free radicals scavenging activity with 42 % of inhibition at 400 µg/ml and least of 29 % at 25 µg/ml. On

comparing with standard, all the tested extracts, the MeOH extract of *Lyngbya* sp.LF 6 has significant DPPH radical scavenging activity. Despite, the *Phormidium* sp. fraction PF3 exhibits partial activity for antioxidant property which was comparatively very minimal to *Lyngbya*sp. The study revealed that DPPH radical scavenging activity were significantly augmented with increasing concentrations of the extract and concludes that *Lyngbya* sp.LF6 could be potential applicant for free radical scavenging (Fig. 3).
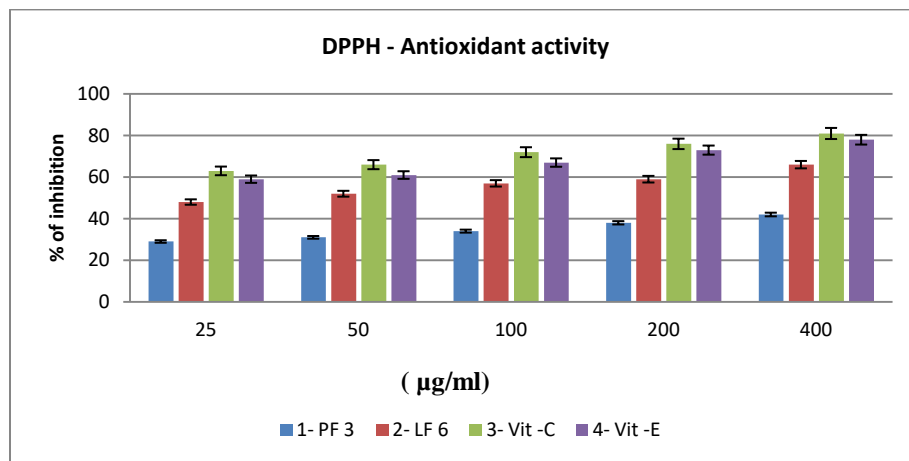

Fig 3. Antioxidantpotentialof *Lyngbya* sp. and *Phormidium* sp. fraction extracts using DPPH - (1,1 diphenyl-2-picryl hydrazyl radical) assay (PF 3 (*Phormidium* sp. fraction 3); LF 6(*Lyngbya* sp. fraction 6); Vit-C (vitamin –C standard); Vit-E (Vitamin-E standard))
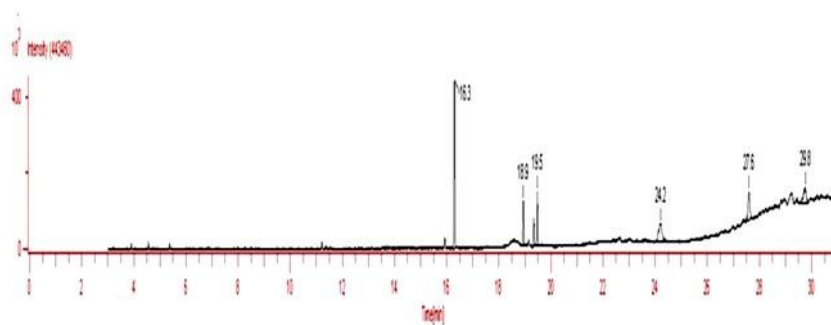

Fig 4.GC-MS profiling spectrum of *Lyngbya* sp. LF6 fraction extract

4. Discussion

Cyanobacteria are the potential source of novel classes of pharmacologically important bioactive compounds. Due to the indiscriminate use of antibiotics for the treatment of various human diseases, there comes more resistant group of human pathogens which spread all over the world. Particularly, the resistant forms are the serious threat in developing countries including India and China due to overwhelming population. This pandemic urgency made interesting importance towards finding for innovative class of antimicrobial drugs from cyanobacterial origin. In early report, the recovery of compounds from cyanobacteria shows antimicrobial, immunosuppressant, anticancer, anti-inflammatory, antiviral and antibiotic agents (El Semary, 2012; Mayer and Gustafson 2003, Rastogi and Sinha 2009). Based on the morphotaxonomic features including morphology, colour, size, filament nature, trichomes, cross-walls and constrictions, confirm the isolates belong to *Lyngbya* sp. and *Phormidium* sp. isolated in this study. The PCR amplified product showed band at ~1500 bp confirm for cyanobacterial 16 s rRNA gene. Among the two cyanobacterial species tested, *Lyngbya* sp.fraction extract showed maximum antibacterial activity compared to *Phormidium* sp. With the different gradient solvent system, the MeOH: CHCl$_3$(70:30) solvent extract of *Lyngbya* sp.fraction 6 (LF 6) showed highest antibacterial activity against selected gram positive and gram negative organism, while the *Phormidium* sp.PF 3 were comparatively active against MRSA organism. The LF 6 observed for significant activity against enteric invasive bacterium *Salmonella typhi* with maximum inhibition of 24 mm followed with coagulase positive gram positive *Staphylococcus aureus* with a zone of inhibition of 21 mm. While, the other

moderately inhibited bacteria were *Vibrio cholera* and least with *Salmonella paratyphi* A and MRSA strains. The antibacterial assessment of *Phormidium* sp.PF 3 showed comparatively reduced bioactivity against all the tested human pathogens with the exception toward MRSA bacterium, showing highest inhibition zone of 16 mm, which is comparatively higher than LF 6 fraction and moderately effective on comparing to NCCLS standards (CLSI, 2009). El Semary (2012) reported that lipophilic bioactive compound extracted using chloroform/methanol from *Leptolyngbya* can be used as potent antimicrobial and antioxidant agents. Previous reports support that secondary endometabolites of *Anabaena* sp. contributes for the inhibition of microbial growth (Campbell, 1985; Abdel-Raoufet al., 2011). Similarly, the antimicrobial activity of marine cyanobacteria *Synechocystis* sp. and *Synechococcus* sp. exhibits inhibition towards gram positive bacteria, suggesting the bacterial sensitivity to cyanobacterial compounds greatly depend on the permeability which influenced by the cell membrane composition of the bacteria (Dixon et al., 2004). On the other hand, the authors reports that the interaction of liphophilic compounds with bacterial cell wall leads to membrane integrity breakdown, altering membrane bound enzyme function followed with increased permeability and destruction of bacterium by PMF (proton motive force) (El Semary, 2012; Sikkema et al., 1995). The result suggests that the difference in bioactivity against human pathogens and antioxidant property of cyanobacterial extracts were greatly related to different compounds extracted in different polar gradient solvents. In the early stage of investigation, the disc diffusion assay will qualitatively assess the effective antibacterial fraction and MIC assay reveals the quantitative bactericidal property of different compounds. The MIC data reported that *Lyngbya* sp.LF 6 fraction shows significant bactericidal property against *Salmonella typhi* at concentration of 31.2 µg/ml, 62.5 µg/ml against *Staphylococcus aureus* and 125 µg/ml for *Vibrio cholerae* exhibiting its potency. While the obtained MIC value at 250 µg/ml for *Salmonella paratyphi* and *MRSA* human pathogens suggest enhanced antibacterial activity with PF 3 fraction. On the other hand, PF 3 fraction showed lower MIC value against MRSA bacterium suggesting for its bactericidal significance to MRSA strain alone, while all the other tested pathogens were comparatively resistive. The tested *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* strains are resistive of all cyanobacterial fraction extracts. Determining the MIC of our extracts was apparent for effective bactericidal property which reasonably correlates with recent reports. Kaushik et al. (2009) reported for effective antimicrobial activity against for cyanobacterial extracts against *Staphylococcus aureus*, *E.coli*, *Pseudomonas aeruginosa* and *Salmonella typhi* with the MIC lowest value of 256 µg/ml and 512 µg/ml. In a similar study, Pramanik et al. (2011) demonstrated the MIC value of *Lyngbya, Phormidium,*, *Oscillatoria* and *Synechocystis* crude extracts which exhibited bacterial inhibition at 250 µg/ml against *S. aureus, E. coli, B. subtilis* and 500 µg/ml against *P. aeruginosa*. The investigation of ethyl acetate extracts of *Anabaena* sp. revealed strong antibacterial activity with lowest MIC of 55.30 µg/ ml against pathogenic *Aeromonas* species (Abdel-Raouf and Ibraheem 2008). The screening of phytochemicals revealed that tannins, alkaloids, phenol, saponins and flavonoid substance reported to have antimicrobial effect (Kulik, 1995). Further, cyanobacterial bioactive compounds not only necessary for regular functioning in cellular metabolism but also do defensive mechanism to protect from environmental stress conditions (Scholz and Liebezeit 2006). It's well reported that cyanobacterial extracts from *Lyngbya majuscula, Phormidium* sp., *Schizothrix* sp., *Nostoc* sp., *Oscillatoria* sp. and *Limnothrix* sp., (Burja et al., 2001; Kreitlow et al., 1999) synthesizes potentially active antimicrobial compounds.

The cyanobacterial compounds were reviewed for toxic bioactive nature by brine shrimp lethality assessment, since it is the more reliable and rapidly evaluated inexpensive method. Both fresh and marine cyanobacterial compounds were well studied for diverse pharmacologically active product with potent toxic activity. The fraction extract of *Lyngbya* sp. and *Phormidium* sp. were screened for biological activity with *Artemia salina* nauplii. The difference in the toxicity of fractions could be attributed to the presence of different phytochemicals and phytogeographical property. In close agreement with our results, Tripathi et al. (2010) found that the cyanobacterial compound Hantupeptin A from *Lyngbya majuscula* exhibited 100 % mortality at 10 ppm and 100ppm. Similarly, assessment by Maruthanayagam et al., (2013) showed that *Pseudoscytonema* sp.and *Oscillatoria* sp. showed weak toxicity, while the *Geitlerinema* sp. and *O. boryana* extracts exhibited stronger toxicity against *Artemia salina* with LC_{50} of 32, 61 µg mL^{-1}. Further, the compounds with LC_{50} values with < 10 µg mL^{-1} were considered as very actively toxic, being lower than 1000 µg mL^{-1} (<1000 µg/ml) in brine shrimp lethality bioassay was considered as toxic, LC_{50} values within 100 - 500 µg mL^{-1} were considered moderately toxic and LC_{50} values greater than 1000 µg/ml (>1000 µg/ml) was non-toxic (Babajide et al., 2010; Meyer et al., 1982). Further, the attempt was made to identify the active fraction of LF 6 by GC-MS chromatographic profiling revealed about seven compounds. The spectrum showing seven peaks were identified for its compound nature with NIST library within the maximum run period of thirty minutes. From the results, the fatty acids particularly long carbon chain with saturated fatty acid, polyphenols, propionic acid, methyl ester group of compounds were majorly constituted. The major hit at 16.3min, 19.5 min, 24.2 min and 27.6 min were

identified as 9,12- octadecadienonic acid, propionic acid, heptanoic acid 9- decen-1-yl ester   and 17 pentatriacontene groups respectively. Previously reported compound assessment by GC-MS profiling for phenol, fatty acid, oleic, linolenic acids in the *Phormidium* sp. extracts obtained with different polar solvents (Rodríguez-Meizosoet al., 2008). The chromatographic assessment of cyanobacteria *Phormidium fovelarum* showed that the content of indole alkaloid which was attributed to the pharmacological effects including antibacterial and anti-fungal property (Volk and Furkert, 2006). In a similar study, the polyphenols and fatty acids exhibits bactericidal property against both gram positive and gram negative bacteria (Nakai et al., 2005; Zakaria et al., 2011). In agreement with our result, Khairy and El-Kassas (2010) reported in GC/MS profiling for the presence of heptadecane and 7 methyl heptadecane compounds in *A. variabilis* extract as major constituent exhibiting high cytotoxicity and antimicrobial property. Our results indicated that fatty acid, linoleic acid and phenolic compounds could be attributed to antimicrobial and antioxidant property of the LF 6 fraction of *Lyngbya* sp.

This study further focused on evaluating the radical scavenging activity of active fractions using DPPH assay. Though, the number of assays has been used to evaluate antioxidant activity, the measurement of radical scavenging by DPPH and ABTS *in vitro* assaying were highly trustworthy. In our study, *Lyngbya* sp. and *Phormidium* sp.cyanobacterial fractions were shown for radical scavenging activity with increased concentration of more reducing power. The results depicted that *Lyngbya* sp.fraction extract LF6 were more potent in scavenging of DPPH radical at an inhibition rate of 66 % (400 µg/ml) while the *Phormidium* sp. exhibits comparative lower radical scavenging activity with 42 % (400 µg/ml). The differences in the antioxidant activity could be likely due to different scavenging mechanism of the fractions. Further, the compounds stability, shelf life, solubility, stereo selectivity and the hydrogen donating ability of extracts play a vital role in scavenging of free radicals (Benedetti et al., 2004, Conforti et al., 2005). The *Spirulina* extract and vitamin C gave higher antioxidant activity due to the constituent of phenolic compounds (Chu et al., 2010). Similarly, the water soluble phycobiliproteins – phycocyanin from cyanobacteria were multiple times more efficient than vitamin C and protects the cells against apoptosis by attenuating the free radicals and reactive oxygen species (ROS) formation (Chopra and Bishnoi, 2008; Li et al., 2009). This study reveals that the MeOH:CHCl$_3$ LF 6 extract has potent protective effect against DPPH induced free radical, also the higher activity of vitamins opts for the synergistic application could be of greater effectiveness as a drug.

5. Conclusion

The present study confirms that MeOH: CHCl$_3$ (70:30) fraction extracts were potentially active as bactericidal and bacteriostatic agents. The cyanobacterial extract of *Lyngbya* sp. confirms for potent antimicrobial activity against selected human pathogens including drug resistant *Salmonella typhi*. Similarly, *Phormidium* sp. fraction extract showed significant activity against MRSA pathogenic strain. Further, the LF 6 also exhibits effective antioxidant activity in DPPH radical scavenging assays which comparatively higher than PF 3 fraction. Though, the brine shrimp lethality being moderately effective for *Lyngbya* sp. LF 6fraction, still proven for its active toxic potency. Our results emphasize that the fraction extract (LF 6) of *Lyngbya* sp. could be the potential source of antibiotic compound against selected human pathogens with imperative antioxidant properties.

Acknowledgements

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