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EVALUATION OF ANTIBACTERIAL ACTIVITY OF CYANOBACTERIA ISOLATED FROM FRESH WATER ECOSYSTEM OF TIRUCHIRAPPALLI DISTRICT, TAMILNADU, INDIA

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ABSTRACT: Cyanobacteria inhabit a wide range of habitats and have potential to produce an elaborate array of secondary metabolites with unusual structures and potent bioactivity. The aim of the present study was to identify antibacterial activity of cyanobacterial samples isolated from different ecological niches of Tiruchirappalli district. Secondary metabolites were extracted in chloroform: methanol (1:4) and tested for antimicrobial activity against seven pathogenic bacterial strains *(Staphylococcus aureus, Klebsiella pneumoniae, and Pseudomonas* sp*, Vibrio cholerae, E.coli, Shigella flexneri, Proteus mirabilis*). The Antimicrobial activity was determined by agar well diffusion method, in which the culture extracts of nine different cyanobacterial samples produced antibacterial activity and inhibited the growth by expressing various zone of inhibitions. The results indicated that the culture crude extract of *Calothrix membranacea* KLR 006 was shown with significant and antibacterial activity (12.mm) against S.aureus *E.coli.* crude extract was reported to the active fractions shown with significant antibacterial activity (12.33 mm) against *S.aureus* and *E.coli*. Crude extract was fractionated to the active fractions and identified for chemical constituents using the HPLC and GC-HRMS. The purified fraction of *Calothrix membranacea* KLR006 was assessed for its bioactivity inhibitory concentration assay against the selected bacterial pathogens. The presence of calophycin was evident by GC-HRMS profiling in the tested cyanobacterial strain.

KEYWORDS: *Calothrix membranacea*, antimicrobial activity, well diffusion assay, MIC assay, GC-HRMS.

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1.INTRODUCTION

The search for novel antimicrobial agents with clinically important pathogens is significant since many clinical pathogens such as *Mycobacterium tuberculosis*, *Enterococcus, Pseudomonas* sp. *Streptococcus pneumoniae* and *Staphylococcus aureus* are developing resistance to routinely used antimicrobials. Development of antibiotic resistance is a challengeable for the majority of the pathogens, which highlights the demand for new antibacterial product development. Microorganism is the best source of antibiotic. In the microbial population Cyanobacteria considered being one of the potential organisms, which constitute a versatile group of microorganisms, occur in diverse habitats ranging from alkaline hot springs to permanent snowfields in the poles which can be useful to mankind in various ways. Cyanobacteria, the photosynthetic prokarytoes are prolific source of natural products with a great choice for new drug developments in biotechnology and pharmaceutical industries. [1]. Cyanobacterial metabolites show an interesting and exciting range of biological activities ranging from antimicrobial, anticancer, antiviral, immunosuppressant, insecticidal, anti-inflammatory to proteinase-inhibiting activities which are striking targets of biomedical research [2,3], Many marine natural products possess novel functional groups and molecular structures compared to those from terrestrial sources. To date, 21,800 natural products have been described from marine organisms [4, 5]. A number of these natural products possess potent biological properties and currently are either in preclinical or clinical testing for the treatment of various human ailments [6]. The important compounds identified as antimicrobial are fatty acids, acrylic acid, halogenated aliphatic compounds, terpenes, sulphur containing hetero cyclic compounds, carbohydrates and phenols [7]. In the past few decades, several classes of aquatic cyanobacteria have been studied for their antibacterial and antifungal activity in the pharmaceutical industry. They have been proven useful for various applications, especially as new therapeutic agents for various diseases [8]. Secondary metabolites of cyanobacteria have toxic properties and are anti-bacterial, anti-fungal, anti-yeast and anti-cancer [9]. The properties of their secondary metabolites are not fully clear in nature. Secondary metabolites influence other organisms in the vicinity and are thought to be of phylogenetic importance. The properties of secondary metabolites in nature are not thoroughly distinguished. The ability to produce bioactive substances may be noticed not only as a defense mechanism but also as a good source of new bioactive compounds from a pharmaceutical point of view [10, 11], has studied bioactive allelochemical compounds from Os*cillatoria* species (Egyptian isolates). Many unique compounds of fresh water origin with various biological activities have been isolated and some of them are under investigation to develop new pharmaceuticals [12, 13, 14, 15]. A couple of biologically active compounds were identified among exometabolites, e.g. certain antibacterial diterpenoids in *Nostoc commune* [16], and antifungal peptides in *Tolypotrix byssoidea* [17]. Antimicrobial activity depends on both algal species and the solvents used for their extraction [18]. The antimicrobial activity of algae extracts is generally

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assayed using various organic solvents which always provide a higher efficiency in extracting compounds for antimicrobial activity [19, 20]. Analytical methods play important roles in the discovery, development and manufacture of bioactive molecules [21]. India is one among the mega diversified country with abundant natural resources, the proper utilization of these resources is very much important.

2. MATERIALS AND METHODS

2.1 Cyanobacterial strains tested

Cyanobacterial samples were collected from various freshwater ponds and lakes in and around Tiruchirappalli district using forceps, knifes and plankton net (mesh size 42µm) and the species diversity was measured already [22]. Cyanobacterial specimens were identified based on morphological descriptions [23, 24, 25]. Axenic cyanobacterial cultures were obtained as described [26]. Cultures were incubated at 28 ±2ºc with a light intensity of 25-30 µmM photon $m^{-2}s^{-1}$ for 15 to 20 days. After growth axenic cyanobacterial biomass was harvested by centrifugation at 5,000 rpm for 20 min, freeze dried and used for screening and bioactive compound extraction.

2.2 Pathogenic bacterial strains

Seven different pathogenic bacterial strains representing both gram positive and gram negative groups were used in this study. The bacterial isolates includes gram-positive species of *Staphylococcus aureus, Methicillin-resistant Staphylococcus aureus* (MRSA) and gram-negative species of *Escherichia coli*, *Shigella flexneri, Pseudomonas aeruginosa*, *Proteus mirabilis* and *Vibrio cholerae*. All the bacterial strains were isolated from clinical specimens and identified based on Bergey's manual of systematic bacteriology [27]. The bacterial isolates were grown overnight at 37º C in nutrient broth before being used for antimicrobial assays.

2.3 Cyanobacterial extract preparation

2 gram of dried powder was extracted in 20 ml of chloroform, 80 ml of methanol (1:4) to get the compounds and by shaking overnight for complete extraction. The extracts were filtered and the filtrates were evaporated under reduced pressure at 37- 40º C for drying. Dried extracts were weighed and dissolved in 1 ml of dimethyl sulfoxide (DMSO) and it was preserved at 4º C until use for the antimicrobial bioassay study. 50 µg/ml concentration of crude cyanobacterial extract was taken.

2.4 High performance liquid chromatography analysis

The samples were analysed by using the HPLC (Varian Prostar, UK) with an RP 18 reverse phase column (YMC-Pack ODS-AQ, 250 x 10 cm) as stationary phase. The methanol: water liner gradient (20-100% over 60 min, then 100% MeOH for 10 min) as mobile phase maintained at a flow rate of 1mL min^{-1}under isocratic condition. The UV detection was set at the wavelength of 220 and 254 nm. Fraction of the single peak eluting from 2 to 5 min was collected and evaporated for dryness. The final residue was dissolved for further mass detection.

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2. 5 GC-HRMS analysis of active fraction of cyanobacterial extract

For the identification of the chemical constituents in the active fraction, GC- HRMS analysis was carried out. The active fraction was dissolved in MeOH and the mass analysis was performed using Joel, ACCU TOF GCV (Agilent 7890, Agilent Technologies, USA). GC coupled with high resolution mass spectrometer (GC-HRMS) was used by injecting the sample volume of 2 µl into 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 performed 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 in the active fractions were mass range analyzed from mass range at 10- 600 m/z using flame ionization detector (FID). The compounds were authenticated with NIST mass library databases (http://www.sisweb.com/software/ms/nist.htm) [28].

2.6 Antibacterial assay by disc diffusion

The active cyanobacterial fractions were tested against clinical bacterial pathogens for antibacterial activity following standard CLSI method (CLSI, 2009). 30 µl of active fraction concentrates were applied twice to 6 mm whatman no.1 filter paper disc and placed on MHA plates with the bacterial pathogens. Plates were incubated at 37ºc for 24 hrs. DMSO was used as solvent control and the standard antibiotics ciprofloxacin (10 mcg) Streptomycin (25 mcg) and oxacillin (5 mcg) were used as positive control.

2.7 Determination of Minimum inhibitory concentration (MIC)

The eluted fractions were subjected to antimicrobial assay using minimal inhibitory concentration (MIC) according to the National Committee for Clinical Laboratory Standards guidelines. The tests were performed using standard 96 well U bottom micro-titer plates with standard controls. All the test wells of the micro-titter plates were added with 100 µl of Muller Hinton broth and seeded with 50 µl of 1×10^7 test pathogens. The cyanobacterial fractions 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 with DMSO (solvent) and DMSO is served as control, medium control to achieve final volume of 250 µl. The test plates were incubated at 37ºC for 24 hrs. All the wells were read in ELISA reader at 590 nm and the lowest concentration of active fraction that prevented the bacterial growth was considered to be the MIC [29, 30].

3. RESULTS AND DISCUSSION

The axenic culture of cyanobacterial strains isolated from the fresh water bodies were screened for their antimicrobial properties. Table 1 depicts the list of nine cyanobacterial strains which showed promising antibacterial activities among the other strains. All the freeze dried axenic fresh water cyanobacterial culture were repeatedly extracted with chloroform: methanol (1:4 v/v) and subjected to antimicrobial activity against *S. aureus*, MRSA, *E.coli*, *Shigella flexneri*, *V. cholerae*, *Proteus*

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mirabilis, and *Pseudomonas aeruginosa.* The active cyanobacterial crude was preliminarily screened for fraction elution using the silica-gel column chromatography with the gradient solvent system. Among the tested strains, the nine of the fresh water cyanobacterial isolates were showed antimicrobial activity against the test organisms as selectively, while the crude extracts from all other cyanobacterial isolates does not show any activity against the tested human pathogenic microorganisms. Similarly Vijayakumar et al., 2011 [31], studied on fresh water cyanobacterial strains and stated that only five species of fresh water cyanobacterium among the 46 investigated species had antimicrobial activity against the selected human pathogens which includes *Bacillus* sp., *S. aureus, Streptococcus mutans, E.coli, Micrococcus mutans, K. pneumoniae, Saccharomyces cerevisiae* and *Candida albicans*. In an another similar study, among 76 cyanobacterial isolates from the paddy soil, only 17 of the axenic cyanobacteria belong to the family Chroococcaceae, Oscillatoriaeae, Stigonemataceae showed potent antimicrobial activity against pathogenic microorganisms [32].The results of Issac, 1999 [33], were in agreement with our findings that gradient solvent extracts of cyanobacteria showed effective bioactivity against both gram positive and negative organisms. In our investigation, the crude extracts from *Lyngbya* sp TVL 013, *Goleotrichia* sp. KLR 0062, *Anabaena* sp. MNR 005, *Nostoc* sp. MTL 515, *Rivularia* sp. KLR 0061, *Oscillatoria* sp. VYL 102, *Oscillatoria tenuis* VYL 1021. *Calothrix membranacea* KLR 006. and *Phormidium* sp. TVL 0131. (C1 to C9) showed antibacterial activity against pathogenic strains such as *Staphylococcus aureus, E.coli, Shigella flexneri,* and *Vibrio Cholerae* and the results were depicted in the Table 1. The other cyanobacteria species (C10-C62) does not showed any observable antimicrobial activity against the selected bacterial and fungal pathogenic strains. by [34], reported that *Anabaena variabilis* showed antibacterial activity with 7.59 mm zone of inhibition against *E. coli,* 4.36 mm zone of inhibition against *Pseudomonas aeruginosa* and highest effective zone of inhibition of 8.56 mm against *Staphylococcus aureus.* Pandey and Pandey, 2002 [35], reported for the antibacterial activity of three cyanobacterial species *Microcystis aeruginosa, Lyngbya majascula,* and *Plectonema boryanum*. In this study, the crude methanolic extracts of *Calothirx membranacea* KLR 006. showed maximum zone of inhibition against *E. coli* (24 mm in diameter) (Fig: 1 B) followed by *S. aureus* (20 mm) (Fig: 1 A). The crude extract of *Calothrix membranacea* KLR 006. showed moderate inhibition against *Shigella flexneri* with zone of inhibition of 19 mm, while the *Vibrio cholerae* exhibited moderate inhibition of 15 mm. Similarly many of the investigation reported on the potency of methanol extracts of cyanobacetrium for highest degree of antibacterial activity. *Calothirx brevissima* have been reported that produce an antimicrobial substance [36]. Bhattacharyya et al., 2013 [37], also reported on the methanol extract of *Anabaena variabilis* and *Anabaena fertilisima* showed antimicrobial activity against *E. coli* (23.6±.8 mm and 14.6±1.1 mm). Many investigation reports that the methanolic extract of a cyanobacteria has been showed potential bioactivity in *in-vitro* antimicrobial assessment against

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Bacillus cereus, Pseudomonas aeruginosa, Proteus mirabilis, Escherichia coli, Aspergillus flavus, *Aspergillus niger* and *Rhizopus nigricans* using agar cup diffusion method [38]. The present study showed that most of the antibacterial activity was observed (Table.1) in crude and fraction of *Calothirx membranacea* KLR 006. and *Oscillatoria* sp. VYL 102. and the results were in consistent with other earlier reports [39].

Table No. 1 **Assessment of antimicrobial activity by well diffusion assay method**

S. No	**Cyanobacterial Genera**	***S.aureus***	***MRSA***	***E.coli***	***Shigella flexneri***	***V.Cholerae***	***Proteus Mirabilis***	***Pseudomonas aeruginosa***
C1	*Lyngbya* sp. TVL 013	10	7 mm	NA	8 mm	11	NA	NA
C2	*Goleotrichia* sp. KLR 062	7	NA	7 mm	8 mm	6	NA	NA
C3	*Anabaena* sp. MNR 005	5	NA	9 mm	9 mm	7 mm	NA	NA
C4	*Nostoc* sp. MTL515	11	NA	12 mm	9 mm	12 mm	NA	NA
C5	*Rivularia* sp. KLR0061	9	9 mm	NA	15 mm	NA	NA	NA
C6	*Calothrix membranacea* KLR 006	20 mm	NA	24 mm	19 mm	15 mm	NA	NA
C7	*Oscillatoria* sp. VYL 102	7	NA	8 mm	7 mm	7 mm	NA	NA
C8	*Oscillatoria tenuis* VYL 1021	12 mm	10 mm	14 mm	8 mm	NA	NA	NA
C9	*Phormidium* sp. TVL 0131	9 mm	7 mm	NA	8 mm	NA	NA	NA
C	Control	NA	NA	NA	NA	NA	NA	NA

Assessment of antimicrobial activity by well diffusion assay method for cyanobacterial crude Fig 1: (MeOH: $CHCl_3$, 1:4 v/v) extract against human bacterial pathogens.

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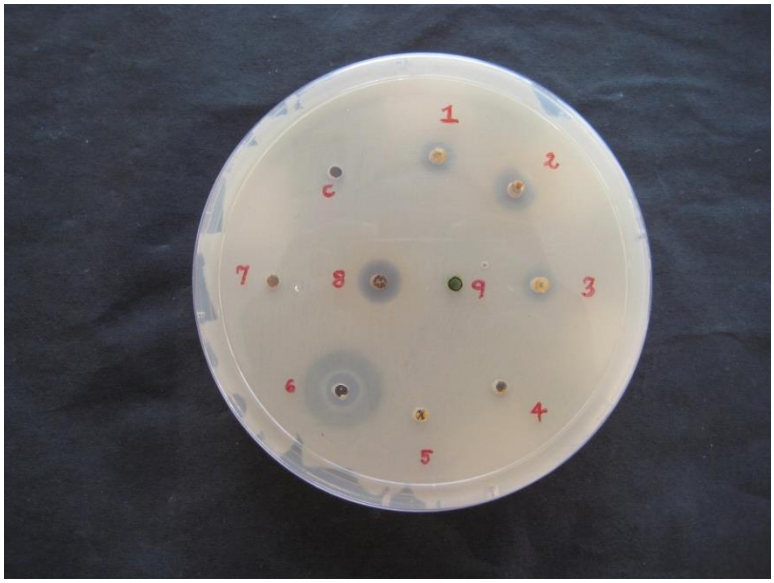

Fig 1 A), Zone of inhibition of C1-C9 cyanobacterial crude extracts against *Staphylococcus aureus.*

C1= Lyngbya sp. TVL013, *C2= Goleotrichia* sp. KLR 002, *C3= Anabaena* sp. MNR005, *C4= Nostoc* sp. MTL 515, *C5= Rivularia* sp. KLR 0061, *C6 = Calothrix membranacea* KLR 006, *C7= Oscillatoria* sp VYL 102., *C8= Oscillatoria tenuis*VYL1021, *C9 = Phormidium* sp. TVL 0131

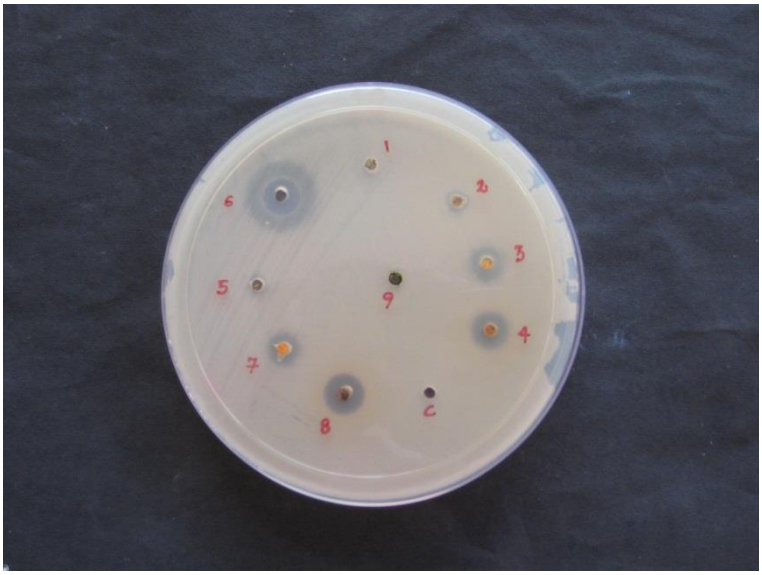

Fig 1B), Zone of inhibition of C1-C9 cyanobacterial crude extract against *E. coli.*

C1= Lyngbya sp. TVL013, *C2= Goleotrichia* sp. KLR 002, *C3= Anabaena* sp. MNR005, *C4= Nostoc* sp. MTL 515, *C5= Rivularia* sp. KLR 0061, *C6 = Calothrix membranacea* KLR 006, *C7= Oscillatoria* sp. VYL 102, *C8= Oscillatoria tenuis*VYL1021, *C9 = Phormidium* sp. TVL 0131

The silica gel (100-200 Mesh size) purified subfraction F1-F11 of *Calothirx membranacea* KLR 006 was assessed for its bioactivity by minimum inhibitory concentration assay against the selected bacterial pathogens. About 11 fractions F1-F11 were yielded on elutions, among all, the fraction F4 showed noticeable antimicrobial and antifungal activity. The F4 fraction of *Calothirx membranacea* KLR 006 showed highest MIC of 500 µg/ml against *Vibrio cholerae,* while the lowest MIC of 125

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µg/ml was recorded for *E.coli* and *Staphylococcus aureus.* Further, the entropathogenic *Shigella flexneri* showed moderate sensitivity with a MIC of 250 µg/ml concentration. While the strains MRSA, *Salmonella typhi, Proteus mirabilis* and *Pseudomonas aeruginosa* does not show any observable (Table .2) inhibition in MIC assay. (Sundaramanickam et al., 2015) [40], reported the MIC activity of extract showed excellent antibacterial activity against pathogens variance range and from 0.312-2.5 mg/ml. *Lyngbya* sp. showed MIC value of 0.312 mg/ml against *E. coli,* 0.625 mg/ml against *B. subtilis*, 1.25 mg/ml against *P. aeruginosa* and 0.625 mg/ml against *S. aureus*. *Claothrix*sp. and *Nostoc*sp. extract showed moderate MIC activity against bacterial and fungal pathogens and no inhibition was observed against *P. aeruginosa,* This is in agreement with over study, that *Calothrix membranacea* LKR 006 showed antimicrobial activity against *E. coli, S. aureus, and V. cholerae* (Helen Diana et al., 2014) [41], reported that *Spirulina major*, *Oscillatoria salina* and *Plectonema terebrans* extract showed antimicrobial activity against *Escherichia coli, Streptococcus aureus, Salmonella typhi, Klebsiella pneumoniae, Streptococcus faecalis* and *Bacillus subtilis*. Screening of fresh water and marine cyanobacterial extracts from culture collection showed potent antibacterial, antifungal, antiviral and immunomodulatory activities particular in *O. late-virions* which showed potent anti-candidal activity. Furthermore, the active fraction F4 were subjected for Reverse phase HPLC fraction purification and analysed for GC-HRMS analysis.

Table 2: Minimum inhibitory concentration assay for active fraction of *Calothirx membranacea* KLR006fractions F4 and F5

S.No	Test Organisms	Minimum inhibitory concentration (MIC) (µg/ml)	
		Fraction	Fraction
		F 4	F 5
1	*E. coli*	125 µg/ml	250 µg/ml
2	*MRSA*	NA	NA
3	*Salmonella typhi*	NA	NA
4	*Proteus vulgaris*	NA	NA
5	*Vibrio cholera*	500 µg/ml	500 µg/ml
6	*Pseudomonas aeruginosa*	NA	NA
7	*Staphylococcus aureus*	125 µg/ml	250 µg/ml
8	*Shigellaflexneri*	250 µg/ml	500 µg/ml

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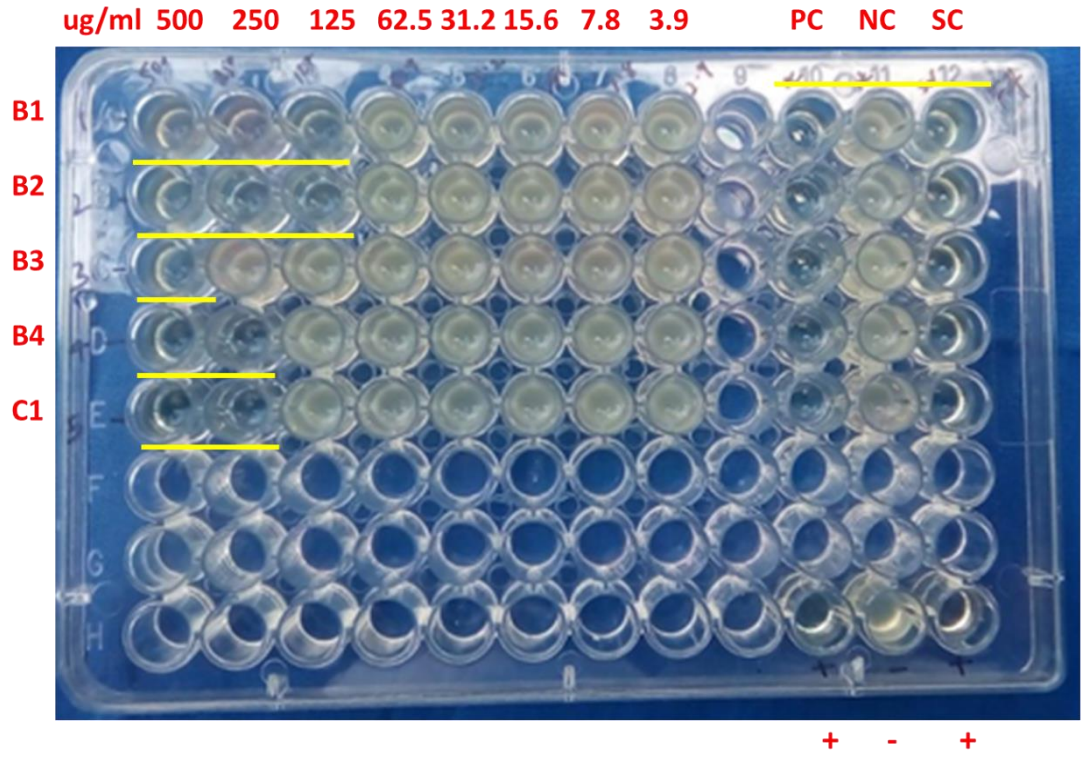

Fig No. 2 Minimum inhibitory concentration assay of the MeOH : $CHCl_3$ (7:3) F4 fraction against selected -human pathogenic bacterial strains.

a) MIC assay of *Calothirx membranacea* KLR 006 silica gel (MeOH : $CHCl_3$ (7:3)) fraction F4 Concentration: 500 µg/ml, 250 µg/ml, 125 µg/ml, 62.5 µg/ml, 31.2 µg/ml, 15.6 µg/ml, 7.8 µg/ml &3.9 µg/ml.

Bacterial strains: B1, *Staphylococccus aureus*; B2 -*E. coli*; B3- *V. cholerae;* B4- *Shigella flexneri.* (control): PC= positive control; NC= Negative control; SC- Standard antibiotic.

Since high antimicrobial activity and cytotoxic bioactivity of *Calothrix membranacea* KLR 006 MeoH:$CHCl_3$ extract fractions F4 was purified by HPLC and analysed using GC-HRMS (Fig : 3). In this study the active compound were eluted at 4.4 min in isocratic 95% acetonitrile using Reverse Phase HPLC. Further, the chemical composition of the active fractions was analysed through GC-HRMS. The data analysis showed that the chemical structure of the active fraction based in the maximum hit percentage with the closest comparison with NIST library structure. Similarly, , (Patra et al., 2013) [42], reported that the active compounds were eluted from green seaweed *Enteromorpha linza* at 5.1 min respectively in isocratic 95% a acetonitrile solvent by RP-HPLC, showing the identity as gamma-linolenic acid (GLA) in GC-HRMS analysis. Studied the fungicidal activity of HPLC purified fraction eluted from *Calothrix elenkini* at retention time 4.43 and the identity of the compound was revealed as calophycin through GC-MS library assessment, matching with our bioactive compound F4.The NIST GC-MS chemical library determine the identity of the most peak by direct comparison, the higher percentage of (%) chemical compounds matching with NIST

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library and identified as calophycin and gamma linolenic acid. The peaks matching with the corresponding hits based on NIST library search with compound name and molecular mass. The HPLC purified fraction F4 were confirmed for its identity by GC-HRMS at t_R = 17.07 min with fragmentation of base peak starts at 261*m/z*$[M]^+$ followed with the fragmentation ions at 235.9, 191.21, 153.6, 135.97, 108.8, 80.6, 81.13, 67.26, and 56.01 *m/z* were identical to gamma linolenic acid. The commercially available GLA showed antimicrobial activity for *P. intermedia* and *P. gingivalis* showed with no difference in MIC value. The MIC values of GLA were 78.12 µg/mL against *C. albicans*, 78.12 µg/mL against *A. actinomycetemcomitans*, 9.76 µg/mL against *F. nucleatum* sub sp. *vincenti*, and 625 µg/mL against *S. mutans*. GLA showed protective effect against periodontitis due to anti-inflammatory property in human trials Rosenstein et al., 2003), [43], and it was reported in breast milk [44], which inhibits oral pathogens and have display anti-inflammatory property.Similarly, (Moon et al., 1992) [45], showed that *Calothrix fusca* produce calophycin, a cyclic decapeptide compound with 1248 Da molecular weight, and showed fungicidal activity against *Aspergillus, Candida,* and *Penicillium,* even at very low concentrations.

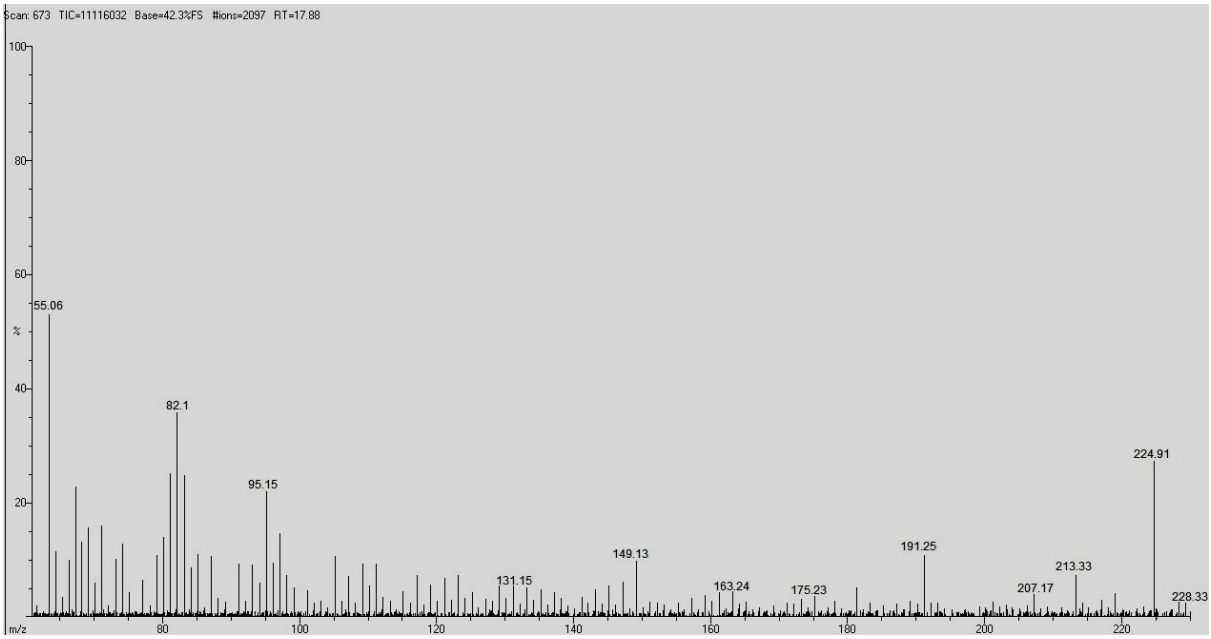

Fig: 3 a) Gas chromatography spectrum of fraction F4– showing calophycin

4. CONCLUSION

Successive extraction of bioactive metabolites with methanloic chloroform of extraction of cyanobacterial strain was reported. The extracts were active against gram positive and gram negative. Pathogenic bacterial strains Antimicrobial activity depends on both Cyanobacterial species and also on efficiency of extraction principles. In the present study is concluded that the *Calothrix membranacea* KLR 006 produced calophycin which showed potent antibacterial activity. This study has suggested that, further metabolites from fresh water cyanobacteria are of special interest in the development of new pharmaceutical compounds of commercial importance.

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CONFLICT OF INTEREST

Authors declared there is no conflict of interest.

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