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Antimycobacterial activity of cyanobacterial species isolated from the coastal regions of Tamil Nadu

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The extract of *Geitlerinema carotinosum* CNP 4003 showed promising antimicrobial activity (100 and 1000 µg/disc/well) against *E. coli* ATCC 35218, *S. aureus* ATCC 25923, and *M. smegmatis* with inhibition zones of 6, 8, and 11 mm, respectively. In addition, the extract also exhibited cytotoxic activity (IC$_{50}$ = 175 µg/ml) against mammalian (HepG2) liver cancer cell lines. Therefore, the crude extract was fractionated using column chromatography technique and the active fractions were identified. The active fractions tested against HepG2 cell line showed 95 % hemolytic activity at the concentration of 375 µg/ml with IC$_{50}$ as 63 µg/ml. Further, the compounds in the active fractions were analyzed and the results indicated the presence of indoles, terpenes, and peptides. The chemical composition of the active fraction was analyzed by using gas chromatography-mass spectrometry (GC-MS). The potential antimycobacterial strain G. *carotinosum* CNP 4003 was confirmed by molecular characterization and the DNA sequence was deposited in the gene bank.

[**Keywords**: Cytotoxicity, *Geitlerinema* sp., Hemolytic, Marine Cyanobacteria, *Mycobacterium smegmatis*]

Introduction

Tuberculosis (TB) is a precarious and communicable bacterial infection that causes considerable socioeconomic and public health problems in developing countries. Members of the *Mycobacterium* species such as *Mycobacterium bovis*, *M. africanum*, *M. tuberculosis*, *M. pinnipedii*, *M. canettii*, and *M. microti* are the main causative agents of TB causing significant health losses in human beings[1]. Around the world, *M. tuberculosis* is the predominant species to cause TB. *M. tuberculosis* is frequently developing resistance against all first- and second-line drugs like isoniazid, rifampicin, ethambutol, streptomycin, pyrazinamide, aminoglycosides, and fluoroquinolones[2]. Therefore, it is a vital requirement to find out novel medicines through valuable antimycobacterial action which is commercially viable, and eco-friendly. Various infectious diseases are known to be treated by natural products throughout the history of mankind[3].

Among the mycobacterium species, *M. smegmatis* is classified as saprophytic, relatively safe, and incapable of causing disease and doesn't usually cause disease in any humans[4]. Further, due to its properties such as non virulence, fast grower or fast doubling time and similarities with *M. tuberculosis* (> 2000 common homologs; unusual cell wall structure that can oxidize carbon monoxide aerobically), it has become the simple model for TB. In modern times, several new species belonging to this genus (*M. avium*, *M. leprea*, *M. bovis*, and *M. tuberculosis*) are identified as virulent that cause TB and leprosy. Therefore, non virulent bacteria of this type are necessary to tackle the mycobacerium associated health problems. In the majority cases, *M. smegmatis* is used as secure and a virulent alternative[5]. One of our earlier reports emphasized the efficacy of ethyl acetate extract (from the *Oscillatoria laetevirens* BDU 141071) activity against *M. smegmatis*[6].

Extracts from *Spirulina platensis* isolated from an Egyptian water station has been accounted to hold anti-bacterial properties. Methanolic and aqueous extracts from the powder of cyanobacterial culture were found to possess action beside *Streptococcus faecalis*, *Salmonella typhimurium* and *Escherichia coli* 100 % (0.3 mg/ml), 86.2 % (0.5 mg/ml) and 91.6 % (0.7 mg/ml) from MeOH and 72.6 % (0.1 mg/ml), 99.3 % (0.9 mg/ml) and 74.4 % (0.9 mg/ml) from aqueous extracts)[7]. Murugan[8] reported excellent activity of sodium phosphate buffer extracts of

S. platensis (C-phycocyanin) against *Klebsiella pneumonia*, *Bacillus subtilis*, *E. coli*, *Staphylococcus aureus* (11, 11, 6, and 12 mm of inhibition zone, respectively at 30 µg/disc). Also, aqueous extracts of *Oscillatoria* sp. showed activity against *Streptococcus mutants* (20 mm of zone at 750 µg/ml)[9]. Crude extracts of *Lyngbya aestuarii* and *Oscillatoria boryana* inhibited the growth of three of the pathogens, *E. coli*, *Enterobacter aerogenes* and *Salmonella typhi*[10].

The hexane extract of *Trichodesmium erythraeum* exhibited antifungal activity against *Trichophyton simii* (31.25 µg/ml) and *T. rubrum*, *T. mentagrophytes*, *Aspergillus flavus*, *A. niger*, *Scopulariopsis* sp, and *Botrytis cinerea* at 1000 µg/ml[11]. Ethyl acetate extract of *Phormidium valderianum* exhibited anticandidal activity at the concentration of 0.5 mg/disc against *Candida albicans*[12]. Frankmölle *et al.*[13] demonstrated a moderate antifungal action of crude ethanolic extracts of *Anabaena laxa* on *Aspergillus oryzae* and *C. albicans* (26 and 19 mm of inhibition zone at > 10 µg/ml). Further, the methanolic extracts of *L. aestuarii* showed a reasonable antifungal action to *C. albicans*[14].

Freshwater, terrestrial and marine cyanobacteria (blue-green algae) have been identified as one of the promising groups of organisms to produce bioactive metabolites mainly of cytotoxins, enzyme inhibitors, antiparasitic, allelopathic, antimicrobial and AF compounds[15]. The exploration of cyanobacterial biodiversity in the marine environment and its associated bioactive metabolites diversity has resulted clinical development of new marine bioactive substances. Till date, several potent compounds isolated from different species of marine cyanobacteria having potent activity against bacteria, fungi, and mycobacterium (Table S1). In these work, antimicrobial activities of cyanobacterial extract was tested on pathogenic bacteria and fungi. Further, the extracts were tested for mammalian toxicity by *in vitro* test (red blood cell toxicity or HepG2 cells). Then various columns chromatographic techniques were used to partially purify the active extracts. Subsequently, thin-layer chromatography (TLC) and gas chromatography-mass spectrometry (GC-MS) were used for active fraction analysis. At last, the potential antimicrobial metabolite generating cyanobacteria was identified and described by 16S rRNA analysis.

Materials and Methods

Collection, isolation, identification, cultivation of cyanobacteria, and extract preparation

Marine cyanobacteria strains were isolated, cultivated in ASN-III medium and identified by using light microscope (Olympus CH20i) at 100X magnification[16]. Strains were kept at 27±2 °C, in a light (intensity of 36.45 µmol/m^2/s^1; illuminated by cold white fluorescent tubes) and dark cycle of 14 h and 10 h, respectively[17]. After the incubation, cells were harvested and extracts were obtained by the cold extraction method. The solvent extracts were centrifuged at 10000 rpm for 10 min and the recovered supernatant was subsequently dried and stored at −20 °C. Thus, obtained crude extracts of the cyanobacterial strains were dissolved in DMSO for antimicrobial assay.

Phylogenetic analysis of 16S rRNA gene and amplification by PCR

The polymerase chain reaction (PCR) was used for amplifying the 16S rRNA gene of the isolated DNA of *Geitlerinema carotinosum* (Geitler) Anagnostidis CNP 4003. CYA106F (5′ CGG ACG GGT GAG TAA CGC GTG A 3′) and CYA781 (R)b (5′ GAC TAC TGG GGT ATC TAA TCC CAT T 3′) primers were used as illustrated earlier by Maruthanayagam *et al.*[18]. The PCR quantities were detained 30 µl having 10 µl of Milli-Q water, one ml of each primer (10 pmol/ml), 16 µl of PCR mixture 2X Master Mix (Prime), and two µl (& 20 ng) of DNA. My gene thermocycler (Applied Biosystems, Foster City, CA) was used for PCR amplification with the following conditions: four min at 94 °C for initial denaturation; 35 cycles of denaturation at 94 °C for four min, annealing at 50 °C for one minute, and extension at 72 °C for 1.3 min; followed by seven minutes of final extension at 72 °C. Agarose gel electrophoresis (1.2 %) and RPC purification kit (Real Biotech Corporation (RBC), Bangiao City, Taiwan) were used to confirm the PCR products (589 bp DNA) and their purity, respectively. Reverse and forward primers were used for sequencing the gene. The BLAST system was used for analyzing the 16S rRNA gene sequences to identify their closest relatives. The public domain GenBank database was used for the gene sequence deposition. ClustalW multiple alignment algorithms were used for multiple alignments and created with reference to the selected GenBank sequences. In the analysis of alignment positions, sequences with one or more gaps or ambiguities were omitted. Neighborjoining method and the bootstrap method of phylogeny with 1500 replications were used for determining the evolutionary history using MEGA 5.0(ref. 19). The entire place enclosed gaps and missing data were removed.

Antimicrobial screening test

Antimicrobial activities of extracts were screened against the bacterial strains *S. aureus* ATCC 25923, *M. smegmatis* (National Institute of Research in Tuberculosis, Chennai, India), *Pseudomonas aeruginosa* ATCC 27853, *Klebsiella pneumoniae* ATCC 17106, *E. coli* ATCC 35218, and a fungal pathogen, *C. albicans* ATCC 10028. The nutrient agar and Sabouraud dextrose agar were used to maintain the bacterial and fungal cultures, respectively. About 0.85 % NaCl was used for suspending the microbial strains and the turbidity was adjusted to 10^8 CFU/ml, equivalent to 0.5 MacFarland standards according to NCCLS. Using the non-toxic cotton swab, the cell suspension was inoculated onto agar and incubated for 20 min at 37 °C). Each crude/fractionated extract was estimated mostly by the disc or well diffusion assay according to Kirby-Bauer method[20]. For the purpose, each those extracts were individually suspended in sterilized DMSO at the concentration of 100 µg or 1 mg and then loaded on to 6-mm Whatman No. 3 filter disc (at a concentration of 100 µg/disc) or well. DMSO was used as a control solvent. Thereafter, those extract spotted plates (were incubated (37 °C, 24 h) and the antimicrobial potential was assessed based on the zone of inhibition (millimeters).

Silica gel column chromatography

The silica gel (100-200 mesh) was prepared and the active fraction was loaded, and separated by the solvent gradient. The collected fractions were spotted in the in house prepared TLC plates (0.25-mm) and kept in the developing chamber (30 % EtoAC: 70 % PE). After developing the plates, 5 % H_2SO_4: methanol solution was sprayed on it. Then the plates were placed in the iodine chamber and heated in hot air oven to remove the excess iodine vapor and finally to observe the spot in UV Transilluminator. As observed, they were similar in composition, as shown by thin-layer chromatography, and combined and dried. The active fraction with antimycobacterial activity against *M. smegmatis* was selected and used for further analysis.

Evaluation of minimal inhibitory concentration by broth macro dilution method

The minimal inhibitory concentration (MIC) was analyzed by using fraction 6 to determine the least concentration of the active fraction that can cause noticeable growth inhibition and bactericidal effects against the *M. smegmatis*. A fresh colony of *M. smegmatis* was inoculated in nutrient broth and grown with vigorous aeration for overnight at 37 ºC. Ten (10) µl of the mid-exponential culture (1×10^6 cells/ml) were mixed with the active fraction dissolved in methanol at concentrations of 50 – 3000 µg in one ml total volume of nutrient broth and incubated at 37 ºC for 24 h with gentle agitation. After incubation, the culture pellets were gained by centrifugation at 10000 rpm (REMI Cooling Centrifuge 24 BL) and re-suspended in 0.1 ml saline, and the whole suspension was swabbed onto the respective agar plates and allowed to grow for a further 24 – 48 h at 37 ºC and then the MIC and MBC were determined[21].

In vitro cytotoxic assay using marine cyanobacterial extracts

The HepG2 human liver cancer cell line obtained from the National Center for Cell Science (NCCS), Pune, India, was used to estimate the toxicity of cyanobacterial extract by using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) assay[22]. DMEM medium (Sigma-Aldrich, St. Louis, MO, USA) (10 % fetal bovine serum (Gibco) and 100 U/ml of penicillin and 100 µg/ml of streptomycin as antibiotics (Gibco)) was used for the cultivation of HepG2 cells in 96 well plates at 37 °C in a humidified atmosphere of 5 % CO_2 in a CO_2 incubator (Forma, Thermo Scientific, USA). Cells from passage 15 or less were used for the entire experimentation. Briefly, 96-well flat-bottom tissue-culture plates in the complete culture medium for cells [(5×10^3 cells (200 µl/well)] seeding. After overnight-seeding, the extracts were added and incubated for 24 hrs in the same CO_2 incubator. DMSO (10 %) added well was used as a positive control. About 20 µl of MTT solution [5 mg/ml in phosphate-buffered saline (PBS)] was added to each well after 24 and 48 h, covered with aluminum foil and incubated for 4 h at 37 °C. After the incubation, 100 µl of DMSO was added to each well to suspend the purple formazan product. The 96-well plate reader (Bio-Rad, Hercules, CA, USA) was used to measure the absorbance at 570 nm (measurement) and 630 nm (reference). Mean of the three replicates were used to calculate the percentage of inhibition as follows: Percentage of Inhibition = (Mean OD of untreated cells (control) - Mean OD of treated cells (treat))/ Mean absorbance of untreated cells (control).

Spector *et al.*[23] were described as the acridine orange and ethidium bromide staining. About 25 µl of AO and EB solution (3.8 µM of AO and 2.5 µM of EB in PBS) was added to the each cell suspension (5×10^5 cells) and observed under a fluorescent microscope (Carl Zeiss, Germany) with a UV filter (450 - 490 nm). Three hundred cells per sample were counted in triplicate for each dose point. In addition, the morphological alteration was photographed.

Hemolytic Assay

Sodium citrate-EDTA containing centrifuge tubes (15 ml capacity) were used to collect human blood samples. After the collection, blood was transferred to a 50 ml centrifuge tube. To the blood, 30 ml of calcium- and magnesium-free PBS was added and centrifugation at 4000 rpm for 10 min until supernatant turned clear and colorless. The supernatant was discarded and the procedure was repeated twice to obtain clean cells. These cells were kept in PBS (20 ml) and used within 2 weeks. To the 0.5 ml cell suspension in PBS, 5 ml of the test material from a stock solution was added. The number of compounds 93.75, 187.5, 375, 750, 1500, and 3000 µg (final erythrocyte concentration approx. 0.5×10^9/ml) were used for testing. The mixture was incubated for one hour at 37 ºC with mild shaking and then centrifuged at 4000 rpm for 10 min. The obtained supernatant was diluted 10-fold with PBS and the optical density was measured at 540 nm. PBS or 0.1 % Triton X-100 (w/v in water) was used for calculating the 0 and 100 % lysis and the tests were performed in duplicate[24].

Phytochemical analysis

TLC study and developing a solvent system

Precoated silica gel G 60 F254 was purchased from Merck, Germany and the solvents used were of analytical grade. A solution of the active fraction at a concentration of 5 mg/100 µl was prepared in ethyl acetate. Using a capillary tube, the active fraction was loaded in the Silica gel 60F254 TLC plate and developed with solvent system Acetonitrile: Heptane: Chloroform (1:4.5:4.5 V/V/V) for satisfactory separation of the compounds. After the development of plates, they were air-dried and the numbers of spots were noted. Spots were observed by spraying with various spraying reagents as follows: Ehrlich's reagent (0.1 g of p-dimethyl aminobenzaldehyde in five ml of hydrochloric acid and methanol; plates heated for 20 min at 50 °C) for sulfonamides, amines, indoles, and ergot alkaloids, Dragendorff's reagent (0.011 g and 0.018 g dissolved in acetic acid and makeup into 10 ml of distilled water) for alkaloids, ninhydrin (0.1 g of ninhydrin dissolved in 9.5 ml of pyridine and 0.5 ml of acetic acid, heated to 110 °C until reddish spots) spray for peptides, phenol (Folin-Ciocalteu) reagent for phenolic compounds, 10 % sodium hydroxide reagent for flavonoids, anisaldehyde-sulphuric acid (0.75 g of anisaldehyde dissolved in 12.5 ml of ethanol and 0.125 ml of concentrated sulphuric acid) for sterols and terpenes, and ferric chloride reagent (5 % in methanol) and potassium hydroxide reagent (10 % in methanol) for Anthraquinone.

GC-MS analysis of active fraction

The coupled GC-MS was used for analyzing the active fraction (1 mg/1 ml ethyl acetate). THERMO GC-TRACE ULTRA VER: 5.0 analyzer with a flame ionization detector was used for the separation of compounds and analysis. DB 35-MS capillary standard non-polar column (length = 30 m, diameter = 0.25 mm, film thickness = 0.25 µm) was used for separation in GC-MS. The carrier gas was helium and the total GC run time was 40.50 min. 70 °C was kept as the initial oven temperature and it was ramped at 10 °C/min until it reached 250 °C. The injector temperature was 250 °C. Ethyl acetate was used as a solvent to separate the compounds in the extracts and the split ratio 1:10. (http://www.sisweb.com/software/ms/nist.htm). The closest match with the highest probability in the NIST Mass Spectral Library was recorded and interpreted.

Results

A total of 12 filamentous forms of marine cyanobacteria (*Aphanocapsa littoralis* Hansgirg CNP 2022, *Phormidium abronema* Skuja CNP 2012, *Oscillatoria annae v*an Goor CNP 1020, *Anabaena circinalis* Var. crassa Ghose CNP 2061, *Synechococcus pevalekii* Nag. CNP 7001, *G. pseudacutissimum* Geitler) Anagnostidis CNP 1019, *Oscillatoria princeps* Vaucher ex Gomont CNP 1014, *Anabaena constricta* (Szaffer) Geitler CNP 5007, *Phormidium valderianum* (Delp.) Gomont CNP 4011, *Oscillatoria salina* Biswas CNP 1006, *G. carotinosum* (Geitler) Anagnostidis CNP 4003 and *O. boryana Bory* ex Gomont BDU 91451) belonging to the genera *Phormidium*, *Oscillatoria*, *Aphanocapsa*, *Synechococcus*, *Anabaena*, and *Geitlerinema* were taken for the initial screening. Classical methods based on morphology were used for identifying the strains (Desikachari, 1959) and *G. carotinosum* CNP 4003 (Fig. 1a) was further confirmed by phylogenetic analysis (Fig. 1c).

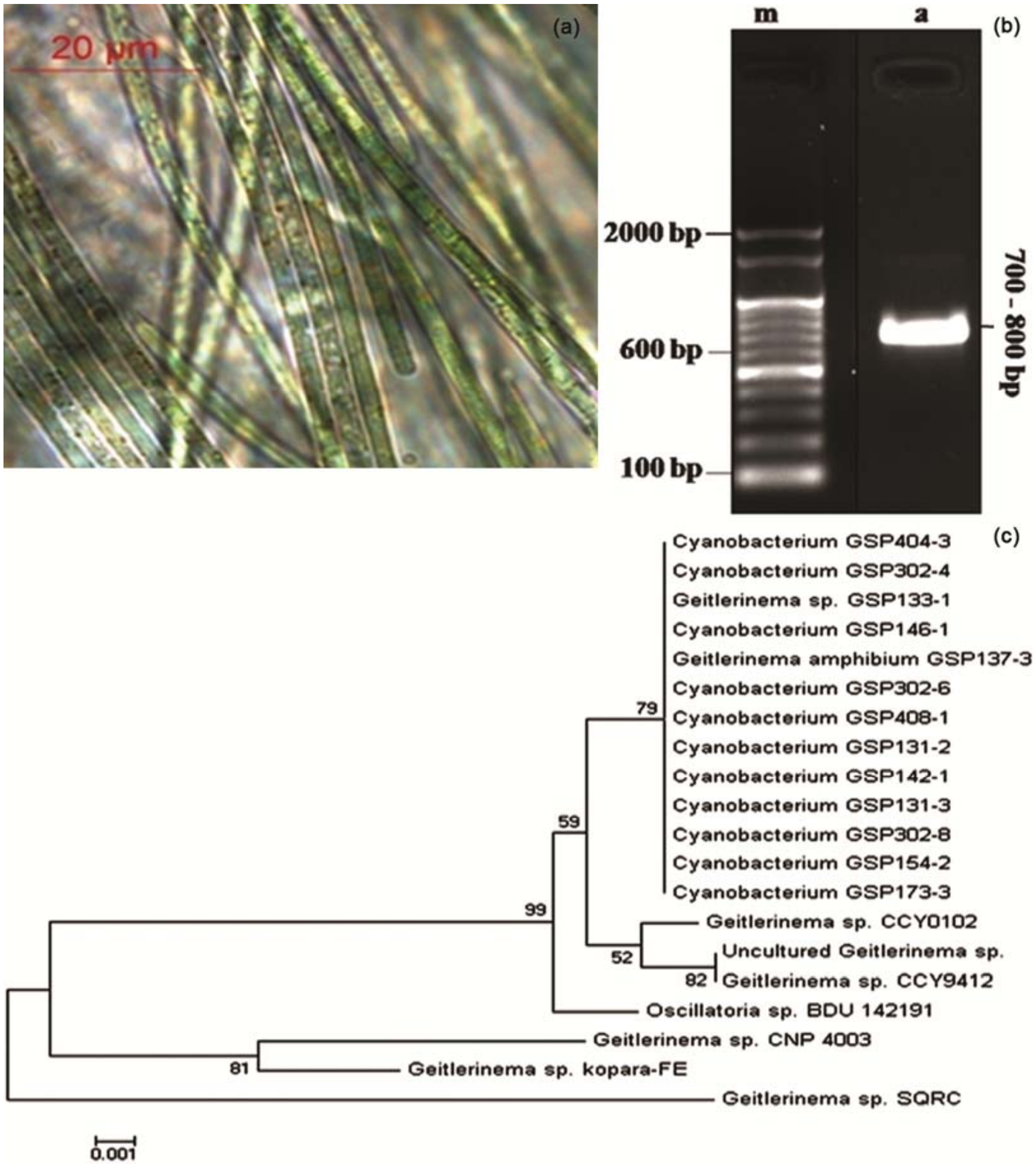

Fig. 1 — Morphology and molecular identification of *G. carotinosum* CNP 4003: (A) Microscopic image of *G. carotinosum* CNP 4003, (B) amplification product of *G. carotinosum* CNP 4003 16S rRNA (*a - G. carotinosum* CNP 4003, *m -100 bp marker*), and (C) phylogenetic tree

Therefore, classification by 16S rRNA gene sequencing was reserved to find out the genus for certain cases. The PCR amplification of the 16S sequence of *G. carotinosum* CNP 4003 (Fig. 1b) resulted in 589 bp DNA and it was used for the BLAST search. The DNA isolated from this cyanobacterium exhibited sequence resemblance with the DNA of another cyanobacterium (accession number AJ621834) and the sequence was deposited in the GenBank database with accession number KC404068.

In this study, organic crude extracts from the above listed cyanobacteria were screened (Table S2) for their antifungal and antibacterial activity against *Candida*, Gram-negative and Gram-positive strains of bacteria. Preliminary testing for the antimicrobial study of extracts was performed by the disc diffusion and agar well method. The best antibacterial activity was observed in the extract of *G. carotinosum* CNP 4003 at 100 µg /disc and 1000 µg/well against *S. aureus* ATCC 25923, *E. coli* ATCC 35218, and *M. smegmatis* with inhibition zones of 6, 8, and 11 mm, correspondingly (Fig. S1). The extract of *G. carotinosum* CNP 4003 showed the highest inhibition level against *M. smegmatis*. The cytotoxicity result (Fig. 2) revealed that the potent extract of *G. carotinosum* CNP 4003 was moderately cytotoxic to mammalian cell line HepG2 (IC_{50} 175 µg/ml) with antimycobacterial activity against *M. smegmatis* at 1 mg/well. Furthermore, this extract was fractionated by silica gel chromatography and antimycobacterial activity was checked for 9 pooled fractions of C17 (F1-F9). Of all the tested fractions, fraction 6 showed the maximum inhibition activity at 250 µg from per disc concentration with 8 mm of inhibition zone (Fig. S2). This fraction was chosen to determine the MIC. Fraction 6 was serially diluted 50 to 3000 µg and MIC value was determined (Fig. S3). Further, the treated cells were collected and plated for viable count analysis. The results showed that the MBC and MIC of the fraction 6 was 800 and 1500 µg as tested on the *M. smegmatis* strain. Very low hemolytic activity, i.e. 63 % (Fig. 3) was observed at 93.75 µg/ml, whereas the highest hemolysis (95 %) was observed at the concentration of 375 µg/ml. Further, the active fraction F6 was relatively cytotoxic to liver cell line HepG2 (IC_{50} 63 µg/ml). Qualitative analysis of the active fraction was given in Table 1 and Figure 4. The results clearly showed that active fraction may consist of indoles or terpenes or peptides. Finally, GC-MS were used for analyzing the chemical composition of the active fraction. A high percentage of the probability for five chemical compounds was noticed in the active fraction with peaks at 3.29, 7.98, 10.10, 12.69, 15.60, and 30.44 RT. In the commercially available databases, the spectra of twenty-five peaks did not match, signifying the newness of these molecules (Table 2).

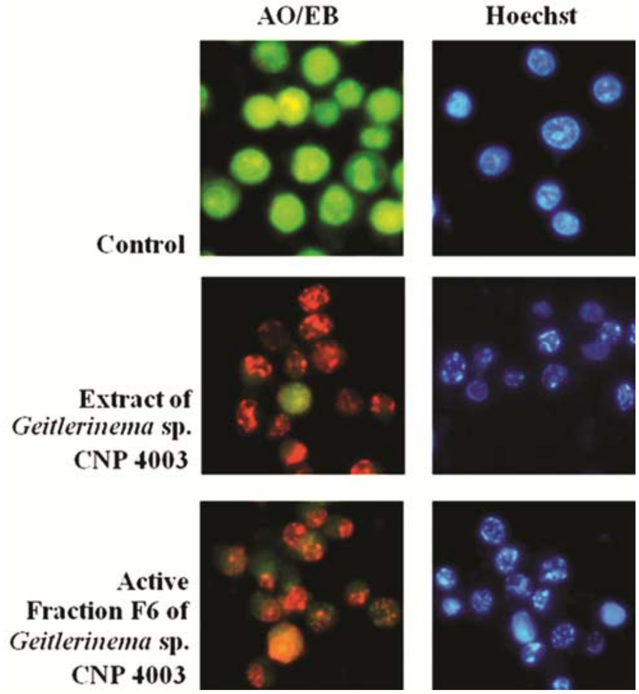

Fig. 2 — Cytotoxic activity of active fraction and extract of *G. carotinosum* CNP 4003: Photomicrographs showing the effect on untreated (Control), extract of *G. carotinosum* CNP 4003 (IC_{50} = 175 µg/ml) and active Fraction F6 of *G. carotinosum* CNP 4003 (IC_{50} = 63 µg/ml)

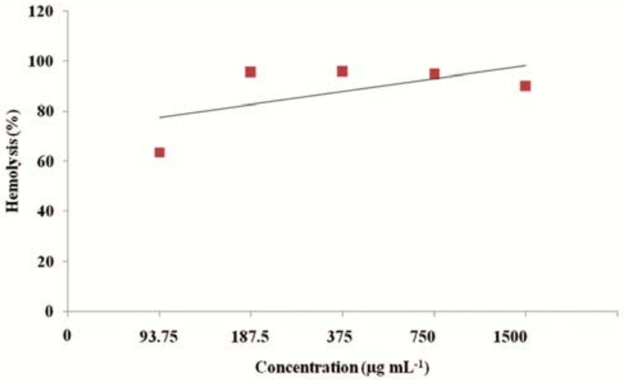

Fig. 3 — Hemolytic activity of active fraction

Discussion

The purpose of this study was to identify the active cyanobacterium among the marine isolates against pathogenic microbes and to select potential candidate for future research. In a similar study against *Mycobacterium,* the ethyl acetate extract of *Aerococcus* sp. was found to be active against *M. smegematis* with an inhibition zone of 8±0.1 mm[25]. Mariita *et al.*[26] reported antimycobacterial activity of MeOH extract of *Entada abysinnica* (2.0 and 1.0 mg/ml) showed antimycobacterial activity against *M. tuberculosis*, *M. fortuitum*, and *M. smegmatis*. The cell-free extract of *Nostoc muscorum* Ag (U2301) and *Fischerella ambiguia* (Näg) Gom (U 1903) exhibited significant antimicrobial

Table 1 — Constituent analysis of active fraction of *G. carotinosum* CNP 4003

Chemical Constituent (class)	Indicating Reagent	Results
Sulfonamides, amines, indoles, and ergot alkaloids	Ehrlich's reagent	+
Alkaloids	Dragendorff's reagent	−
Peptides	Ninhydrin	+
Phenolics	Phenol reagent	+
Flavonoids	10% NaOH	−
Sterol	Phosphomolybdate	+
Anthraquinone	10% sodium hydroxide	−
Tannins	Ferric chloride reagent	−
Terpenes	Anisaldehyde-perchlorate	+

+ indicates presence, − indicates absence

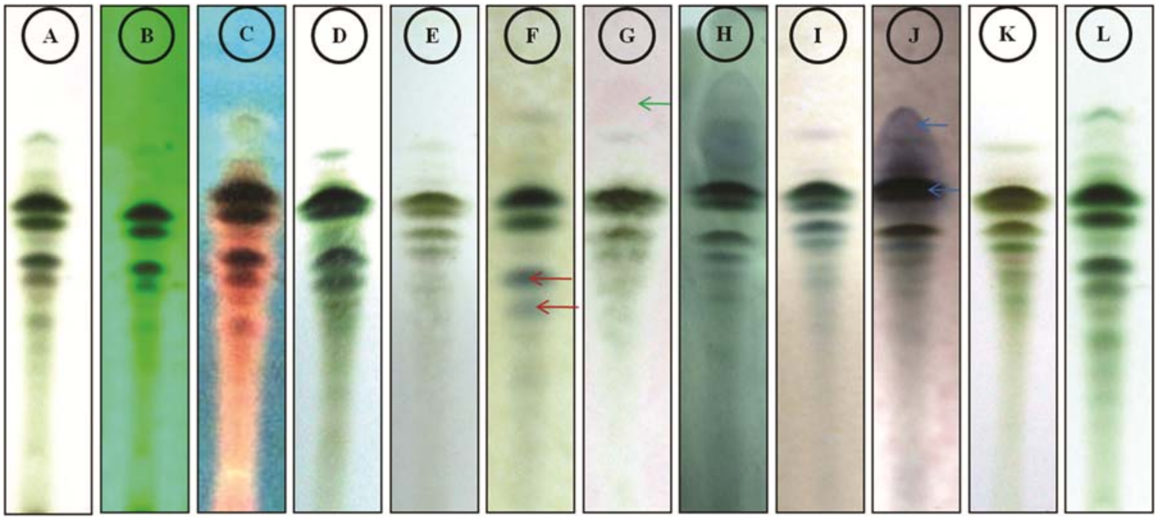

Fig. 4 — Qualitative identification of compound classes in the active fraction of *G. carotinosum CNP 4003*: Extract was loaded on silica gel plates and developed with acetonitrile:heptane:chloroform (1:4.5:4.5 v/v/v) solvent mixture. After development, the plate was observed directly (Panel A) or under UV light at 254 nm (Panel B) or 366 nm (Panel C). Other plates were sprayed (and developed subsequently as outlined in Methods) with Anisaldehyde–H_2SO_4 (Panel D), 10 % KOH (Panel E), Ehrlich's (Panel F), ninhydrin (Panel G), Folin–Ciocalteu (Panel H), Ferric chloride (Panel I), anisaldehyde-$HClO_4$ (Panel J), 10 % NaOH (Panel K) and Dragendorff's reagent (Panel L). Red arrow indicates presence of indoles (purple spot). Green arrow indicates presence of peptides (pink spot). Blue arrow indicates presence of terpenes (dark blue spot).

Table 2 — Composition of active fraction of *G. carotinosum* CNP 4003 as revealed by gas chromatography mass spectrometry (GC-MS)

Peak retention time (min)	% of area	Close match	% of similarity
3.29	75.66	Acetic acid, ethyl ester	93.50
5.30	3.29	UI	-
7.98	0.30	1,3-Bis(4-chlorobenzyl)-5,6-dihydrobenzo[f]quinazoline	53.44
10.10	0.32	Cycloheptasiloxane, tetradecamethyl	97.50
12.69	0.23	Hexadecamethylcyclooctasiloxane	94.52
14.54	0.13	UI	-
14.99	0.41	UI	-
15.60	0.18	Cyclononasiloxane, octadecamethyl	83.79
18.35	0.25	UI	-
18.81	0.27	UI	-
19.81	3.07	Neophytadiene	38.91
20.44	0.85	UI	-
20.81	1.77	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	35.21
22.88	0.41	UI	-
23.32	0.11	UI	-
23.95	8.63	Hexadecanoic acid	36.86
25.05	0.21	UI	-
26.12	0.56	UI	-
26.70	0.23	UI	-
27.98	0.17	UI	-
29.42	0.12	UI	-
29.95	0.18	UI	-
30.44	1.15	5,10-Dihexyl-5,10-diihydroindolo[3,2-b]indole-2,7-dicarbaldehyde	58.52
31.20	0.13	UI	-
32.44	0.16	UI	-
33.03	0.34	UI	-
33.73	0.11	UI	-
34.44	0.34	UI	-
38.23	0.15	UI	-
40.25	0.27	UI	

The data represents the relative area of the peaks in %. Peaks were identified by the NIST Mass Spectral library. Compounds that have a probability lower than 75 % to the closest match with the library are marked as unidentified (UI).

activity against *M. tuberculosis*[27]. Extract of the cultured cyanobacterium *Eucapsis* sp. (UTEX 1519) exhibited the most potent activity (MIC 9.7 µg/ml) against *M. tuberculosis*[28]. Malyngolide isolated from *L. majuscula* showed potential antibacterial activity against *M. smegmatis*[29]. The active fraction of *Hapalosiphon* sp. displayed a minimum inhibitory concentration at 125-2000 µg/ml against strains of *M. Tuberculosis*[30]. Isolation of fischambiguine B from the cultured cyanobacterium, *Fischerella ambigua* (UTEX 1903) showed activity against *M. tuberculosis* with a MIC value of 2 µM and did not exhibit cytotoxicity on Vero cell line[31]. Brunsvicamide B from *Tychonema* sp. isolated from the pond of a sugar factory potentially inhibited *M. tuberculosis* enzyme MptpB with an IC_{50} value of 7.3 µM[32]. Also *Fischerella* sp. isolated from Neem tree bark exhibited antimycobacterial activity against *M. tuberculosis*[33].

Among the 12 strains of cyanobacteria tested for antimicrobial activity, *G. carotinosum* CNP 4003 showed better antimicrobial activity than the other strains. Extract of *G. carotinosum* CNP 4003 showed distinct antimicrobial activity against *S. aureus* ATCC 25923, *E. coli* ATCC 35218, and *M. smegmatis*. Results of the hemolytic and cytotoxic activity of extract, and active fraction against human erythrocytes and HepG2 cell line showed its viability as a future drug candidate particularly for internal use. Bioactive molecules from marine cyanobacteria are well-known for their toxic effects against animals and some of the eukaryotic microbes. It is also reported that these structurally diverse antimicrobial compounds produced by cyanobacteria are discrete substances and differ from the cyanotoxins. Considering these properties, we have evaluated the cytotoxicity of the antimicrobial active extract against human erythrocytes and HepG2 cancer cells by hemolytic and MTT assay. This result demonstrated that the active fraction showed good antimycobacterial activity and high toxicity to human red blood cells and cancer cell lines. The assay indicated that the presence of toxic activity could be understood to the presence of cytotoxic activity for the active fraction.

Compounds from cyanobacteria have been accounted for having different modes of antibacterial actions. Assessment of cyanobacteria peculiarly growing on black band disease of corals revealed the production of antibacterial compounds in them and also indicated that their activity depends mainly on ecological conditions[34]. Preliminary screening of organic and aqueous extracts of cyanobacteria, Anabaena strains, isolated from terrestrial environment exhibited antibacterial and cytotoxic activity[35]. Asthana *et al.*[36] explored biochemical and antibacterial properties of Antarctic strain, *Nostoc* sp., and stated that antibacterial activity was specially connected with the habitat. Extracts from cyanobacterial mats of hot springs inhibited the growth of *Klebsiella pneumoniae*, *Salmonella enteric*, *Micrococcus luteus*, *Shigella sonnei*, *Bacillus* sp., *Amphora coffeaeformis* and QS of the reporter strain *Chromobacterium violaceum* CV017 and *Agrobacterium tumefaciens* NTL4[37]. Pramanik *et al.*[38] reported the antimicrobial activity of cyanobacteria isolated from Sundarbans mangrove forest against *P. aeruginosa*, *E. coli*, *B. subtilis*, and *S. aureus* multiple drug-resistant clinical isolates (MIC 0.25-0.5 mg/ml). Furthermore, these extracts were shown to be nontoxic to human colon adenocarcinoma cell line at the same MIC value. Biomass extract of antibacterial activity of *Geitlerinema* sp. was recorded by Caicedo *et al.*[39] and they further reported 100 % growth inhibition of *Bacillus subtilis* by the crude extract eluted with MeOH and isopropanol.

Jaiswal *et al.*[40] and Kim & Kim[41] reported the antifungal activity of extracts from *Microcystis aeruginosa* and *Nostoc commune* FA-103 against the growth of phytopathogenic fungus, *Fusarium oxysporum* f. sp. *lycopersici* and *Rhizoctonia solani*. Another study demonstrated the antimicrobial potency of *Nostoc* strain ATCC 53789 against *Sclerotinia sclerotiorum*, *Rosellinia* sp, *Penicillium expansum*, and *Armillaria* sp at 0.25 g/l[42]. In an investigation by Pawar & Puranik[43], methanolic extract of *Oscillatoria limosa* NMU-31 displayed a good antifungal activity against *Aspergillus flavus* with an inhibition zone of 28 mm diameter. Extracts obtained from *Geitlerinema* strain Flo1 by using Amberlite XAD-1180 resin exhibited activity against marine yeast, *Rhodosporidium sphaerocarpum*[44]. All these studies exposed the hidden novel antimycobacterial pharmacophores in marine cyanobacteria. Thus, it's understood that the primary extracts of various species of marine cyanobacteria displayed promising antimycobacterial activity against *Mycobacterium smegmatis*. The purification of these crude extracts by different chromatographic techniques and structure elucidation by various spectroscopic analyses, such as IR, NMR, and MS is in progress.

Conclusion

The results of the present study justified the promising antimycobacterial activity of the tested cyanobacterial crude extract. Further research is required to isolate, and identify the active principle(s) from cyanobacteria, and to evaluate their mode of action(s). The entire work specified at this point emphasize that the isolated cyanobacteria from the marine environment can be a potent antimicrobial agent.

Supplementary Data

Supplementary data associated with this article is available in the electronic form at http://nopr.niscair.res.in/jinfo/ijms/IJMS_49(07)1165-1174_SupplData.pdf

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Conflict of Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

Author Contributions

MS: Conceptualization, Project administration, Writing - review & editing; VM: Formal analysis, Investigation, Methodology, and Writing - original draft; MN: Formal analysis, Investigation, and Methodology.

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