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EVALUATION OF ANTICANCER ACTIVITY AND PARTIAL CHARACTERIZATION OF FATTY ACID METABOLITE FROM MARINE CYANOBACTERIA

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ABSTRACT

Marine cyanobacteria samples were collected from saltpan, of southwest coast of Tamil Nadu. The collected marine cyanobacteria samples were purified by repeated serial dilution and plating techniques. Marine cyanobacteria were identified as *Spirulina sp, Oscillatoria sp, Microcystis sp* by light microscopic observation.In BG11 medium, ASN 111 Medium were used for mass cultivation of marine cyanobacteria. The growth curve shows that the marine cyanobacteria grew exponentially up to two week followed by stationary phase. In BG11medium, higher amount of dried marine cyanobacterial biomass were produced. Among the three marine cyanobacteria *Oscillatoria sp* could produce higher amount of dried biomass. Biochemical constituent of three cyanobacterial was measured. The total lipid component was separated by TLC and brown colour spots were appeared. FAME was confirmed by HPLC, FTIR, GAS chromatography techniques. Anticancer property of fatty acids from *Oscillatoria sp* was revealed against human lung cancer cells (A549) line. *Oscillatoria sp* possesses anticancer activity of about 85% against the cell line.

KEY WORDS: Anticancer activity, Chlorophyll, *Cyanobacteria*, *Microcystis*, *Oscillatoria*, TLC.

INTRODUCTION

Cancer is a class of diseases in which a cell, or a group of cells represents uncontrolled growth (i.e. division beyond the normal limits), invasion (i.e. intrusion on and distortion of adjacent tissues), and metastasis (spread from one part to another part in the body through lymph or blood). These three malignant properties of cancers differentiate them from benign tumors. Which are self-limited, and do not invade or metastasize while malignant tumors are not self-limited and metastasize. Most of cancers ocur form a tumor. Cancer is a human tragedy that affects people at all ages with the risk for most types increasing with age. It caused about 13% of all human deaths in 2007(7.6 million). Cancers are primarily an environmental disease with 90-95% of cases due tomodification in lifestyle and environmental factors and 5-10% due to genetics. Cancer is caused by both external factors (tobacco, chemicals, radiation and infectious organisms) and internal factors (inherited mutations, hormones, immune conditions and mutation that occurs from metabolism). Common environmental factors leading to cancer death include: tobacco (25-30%), diet and obesity (30-35%), infections (15-20%), radiation, stress, lack of physical activity and environmental pollutants. These environmental factors cause abnormalities in the genetic material of cells.

One of the most important treatments currently available for cancer and other diseases is chemotherapy which has limited effectiveness due to some serious lifethreatening side effects and development of drug resistance cancer cells. In spite of the increasing success of chemotherapy, especially in achieving initial responses, it often fails in terms of long-term results because of the development of drug resistance by the cancer cells. Curacin A 4 isolated from the Curaso strain marine Cyanobacterium *Lyngbya majuscula* by Gerwick's group in 1994 [1]. It is an important lead compound for a new type of anticancer drugs.

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It is an antimitotic agent (IC50 values in three cell line ranging from 7 to 200 nm) that inhibits microtubule assembly and binding of cholchicine to tubulin (Gerwick *et al*., 1994). Two linear cytotoxic pentapeptides; majusculamide D 5 and deoxymajusuculamide D 6 were isolated from a deepwater variety of the marine blue–green alga *Lyngby majuscule*. A highly interesting bioactive compounds have been isolated from blue–green algae including alkaloids (e.g. lyngbyatoxin) 7, polyketides (e.g. tolytoxin), cyclic peptide (e.g. microcystin), depsipeptide (e.g. majusculamide 8) etc. Many of these compounds showed a versatile biological activity [2]. Cryptophycin-l 9 from *Nostoc* species shows a fungicidal activity and rediscovered by Smith's group [3] as a microtubule depolymerizing agent. The compound and its analogues are very effective against solid tumors.

MATERIALS AND METHODS

Sample collection

The marine cyanobacterial samples mats were collected from saltpan sampling sites in South West coast of Tamil Nadu. The collection sites are situated salt pan region of Cape Comorin.

Isolation and purification of cyanobacteria

The different cyanobacterial samples were isolated from the collected algal mats. A loopful of the algal mat was suspended in 5ml of sterilized distilled water, homogenized by vigorous shaking and 0.5 to 1.0 ml was surface plated on agar plates containing the suitable medium [4].

Culture media

There are three different medium were selected for cultivation of marine blue green algae. The three culture media such as MN111, ASN 111 and BG11.Marine broth (medium BG11 for marine cyanobacteria) were selected for growth of the isolated culture.

Preparation of the media

Medium ASN111 contained (g/l-1); NaNO3,0.75; K2HPO4.3H2O,0.02;MgSO4.7H2O,0.038; CaCl2.H2O, 0.018; Citric acid,0.003; ammonium citrate,0.003; Na2EDTA,0.0005; Na2CO3,0.02; Seawater,750 ml and trace metal solution 1ml at pH 7.3 and was used for the isolation of cyanobacteria . Medium BG-11 Contained (g/l-1):;NaNO3,0.75; K2HPO4.3H2O,0.02; MgSO4.7H2O, 0.038; CaCl2.H2O,0.018; Citric acid,0.003; ammonium citrate,0.003; Na2EDTA,0.0005; Na2CO3,0.02 and trace metal solution 1ml (including H3BO3 2.86 g, MnCl2.4H2O 1.81g, ZnSO4.7H2O 0.222 g Na2MoO4.2H2O 0.390 g CuSO4.5H2O 79 mg and CO(NO3)2.6H2O 49.4 mg/1 at pH 7.5). Medium MN-111Contained (g/l-1):KNO3 1;MgSO4.7H2O, 0.25:NaCl 0.1; Na2EDTA,0.25; FeSO4. 7H2O,0.002 and trace solution 1ml (including H3BO3 2.86 g, MnCl2.4H2O 1.81g, ZnSO4.7H2O 0.222 g Na2MoO4.2H2O 0.390 g CuSO4.5H2O 79 mg and CO(NO3)2.6H2O 49.4 mg/1 at pH 7.5). For ASN 111 media and BG11 marine broth preparation. The micro and macro nutrient's' except B12 solution was added to distilled H2O and brought its volume 29 to 900ml.Then autoclave the media for minutes at 15 psi pressure at 121oC. After sterilization cool the media and then aspectically transfer 100ml of sterilized media and then mixed it thoroughly. Then it was distributed into sterile tubes flasks. For the preparation of MN111 medium, all the ingredients were added to the distilled water and brought the volume to 1000ml.Then mixed it thoroughly. Then it was autoclaved for 10 minutes at 15 psi pressure at 121^0C. Then cool the medium to 45oC-50oC. Then add 1ml of trace metal mix A5 to the particular medium and mixed it thoroughly. Then it is distributed into the sterile tubes of flaks.

Estimation of carbohydrate [5]

1.0g of dried, preweighed marine microalgae and 10ml of distilled water wereused to prepare an extract. One ml of homogenized marine cyanobacteria suspension was taken in a test tube. (A blank containing 1 ml distilled water). 4 ml of anthrone reagent was added to each tube, shaked gently but thoroughly. The tubes were kept in a boiling water bath at 100^0C for 10 min. to develop the colour and the mouth of each tube was covered with aluminium foil to prevent evaporation. The tubes were cooled in running tap water. The absorbance was read by spectrophotometer at 620 nm against the blank and the percentage of carbohydrate was calculated from the known OD value of the working standard [6].

Separation of total lipids by thin layer chromatography

The extracted marine cyanobacteria total lipid was separated by thin layer chromatography technique. The stationary phase was prepared using silica gel and water at 1:2 ratios and applied to a glass plate, as a thin uniform layer by using a TLC spreader. Then the plates were air dried for 10-15 min. and then oven dried for 10-15 min at 100oC- 110oC. A line was drawn lightly with pencil about 1.5-2.0 cm from the bottom of the plate. The algal lipid samples were spotted in TLC plate using capillary tubes 1.5cm distance between them. The thin layer plate was placed gently into the tank containing the solvent hexane: ethyl acetate (9:1) and allowed to stand for about 60 min. As the 32 solvent front reaches about 1-2cm from the top of the plates, the plates were removed, solvent front was marked and allowed to air dried for 20 minutes. Then the plates were sprayed with 25% -50% concentrated sulphuric acid, in ethanol and incubated at 100oC for 10-15 min. Then the spots can detect using UV light.

Extraction of chlorophyll

A known amount of marine cyanobacteria suspension was taken in a centrifuge tube. The algal cells were harvested by centrifugation at 5000 rpm for 10 minutes at

room temperature. The harvested algal cells were transferred into test tubes and a known volume of (10ml) 95% methanol was added. The contents of tubes were shaked well. The tubes were placed in a water bath at 60oC for 30 min. and the mouths of the tubes were covered with an aluminium foil. After 30 minutes of extraction the tubes were removed from the water bath and cooled to room temperature. The samples were centrifuged again at 3500 rpm for 15 min at 4ºC and the pigment was stored on refrigerator.

HPLC analysis

Chromatography was performed with FAME was analysed by schimadzu, LU- 10AT VP HPLC,available at science Instrumentation centre, Lady dock college Madurai. The analytical column was a Discovery C18 (5x4 mm I.D., 5 µm) . The mobile phase con-sisted of a mixture of solvent A (10 mM ammonium acetate, pH=5.5) and solvent B (10 mM ammonium acetate-acetonitrile, 80:20, v/v) as follows: 0% of B at 0 min, 100% of B at 45 min to 65 min using a linear gradient. Flow-rate was 0.8 ml/min and UV detection was performed at 238 nm.. The column was reequilibrated with 8 ml of the solvent A between runs. Each standard was run separately (AnTx-*a* 5 µg/ml, MC-LR 5 µg/ml, STX 40.5 pg/ml, 200 µl injection volume) and thereafter a mixture of all standards with the same concentra-tions in 200 µl was run again. 200 µl of each sample were injected for HPLC analysis. FAME and their concentrations in the samples were determined by comparing retention times and peak areas.

Anticancer activity of marine cyanobacteria Cytotoxic activity (MTT assay)

Cytotoxicity of OS extracts at various concentrations (12.5- 1000 µg/ml) was assessed using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) ((Sigma, USA) assay. Human lung cancer cells (A549) Human lung carcinoma cell lines obtained from National centre for cell sciences Pune (NCCS). The cells were maintained in DMEM (GIBCO, USA) supplemented with 10%FBS (GIBCO, USA), penicillin (100U/ml), Sigma, USA) and streptomycin (100µg/ml), Sigma, USA) in a humidified atmosphere of CO2 incubator at 37ºC.Assay plates were read using a Multiplate reader (SpectraMax® M3 – Molecular devices, USA). The absorbance at 570 nm was measured with a multiplate reader using wells without sample containing cells as blanks. All experiments were performed in triplicate. The effect of the samples on the proliferation of human lung cancer cells (A549) was expressed as the % of cell viability.

RESULTS

Mass cultivation of marine cyanobacteria

For this present investigation, the algal samples were collected from the Capecomorin coast of Tamil Nadu. The three different cyanobacteria such as., *Oscillatoria sp*, *Microcystis sp*, *Spirulina sp* were isolated from the mixed culture by the pure culture techniques.

Dried biomass analysis

The dried biomass of three marine cyanobacteria cultivated in three different cultivation medium was analysed. After 30 days of cultivation period, among the cyanobacteria Oscillatoria sp has obtained higher biomass of above 20±1.32 gl-1 whencultivated in ASN111 medium. Among the three cultivation medium ASN 111 supports higher biomass when compared with MN 111 and BG 11 marine broth.(Fig:2)

Estimation of carbohydrate

Oscillatoria sp (15.5%) contain higher amount of carbohydrate than *Microcystis sp* (13.2%) and *Spirulina sp* (11.1%). (Fig: 3)

Estimation of total lipids

Oscillatoria sp Contain higher amount of lipid (24%) than *Microcystis sp* (18.5) *Spirulina sp* contain lower amount of lipid component (16%). (Fig: 4)

Separation of total lipids by TLC

The extracted marine cyanobacteria total lipid was separated by thin layer chromatography. Brown colour spots were appeared on thin layer plate, which indicates the presence of lipid component in marine cyanobacteria and the RF value wascalculated and recorded in the table;1, (Fig. 5)

Estimation of chlorophyll

The extracted chlorophyll pigment was estimated. Oscillatoria sp. Contain higher amount of total chlorophyll 'a' (18.9 mg/100g) than Spirulina sp. (15.6 mg/100g), Microcystis sp (6.9 mg/100g). Among chlorophyll pigments estimated chlorophyll 'b' which was higher than chlorophyll 'a' (Fig:4). FAME production from marine cyanobacteria HPLC From marine cyanobacteria the oil was extracted. After transesterification the FAME was separated from sediment layer by using separating funnel. As a result FAME (Plate; 4). The FAME was analyzed by HPLC, FTIR and GAS Chromatography techniques and compared with the previous findings.

Anticancer activity

The results of the present study clearly showed that the given sample of Oscillatoria sp showed anti-cancer activity against human lung cancer cells (A549). The presence of the bioactive compounds present in the FAME extract of these samples may 41posses the anti-cancer activity against the human lung cancer cells (A549).The sample showed varying inhibition of viability (IC50) Oscillatoria sp.with12.5µg/ml. This was shown in fig 7 and 8. Trypsinize was used as the standard solvent. No inhibition was seen with the cell control and the viability of

the cells was marked as 100% in which the crude extract was not added. To treat the diseases like cancer the world is looking for biological sources, as the already existing chemotherapeutic agent may cause side effects like fatigue, irritation of oesophagus that can cause difficuty in swallowing and inflammation of lungs. It may also cause vomiting, neutropenia, anemia, another infectious complicatios. Penicillin ((100U/ml), streptomycin ((100µg/ml), bacterial derived protein showed anticancer activity against human lung cancer *in vitro*. The cyanobacteria Oscillatoria spp. are a group of organisms which plays a major role in photoproduction of biofuels, ammonia, various metabolites, vitamins, toxins, therapeutic substances, aqua or animal feed. Cyanobacteria are also used as energy source and biofertilizers.

From MTT assay it is clear that the sample possess anti-cancer activity. Further research is necessary for successful separation, purification and characterization of bioactive compounds using chromatographic methods and spectroscopic techniques.

Table 1. Rf value for total lipid of marine cyanobacteria by TLC

S.NO	Marine cyanobacteria	Rf value (cm)
1	Oscillatoria sp	0.46
2	*Microcystis sp*	0.41
3	*Spirulina sp*	0.38

Figure 1. Mass cultivation of Marine Cyanobacteria

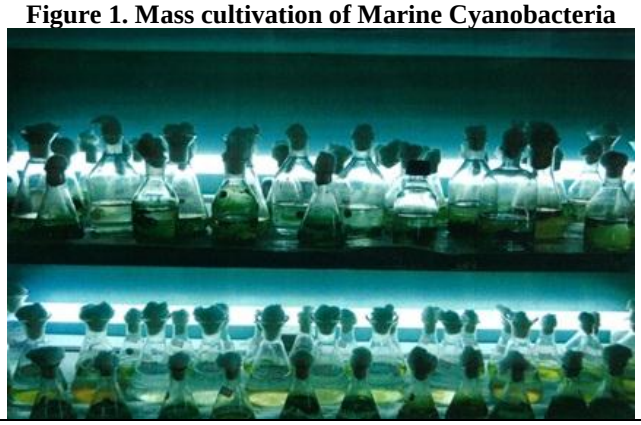


Figure 2. Biomass of Marine cyanobacteria in different cultivation media

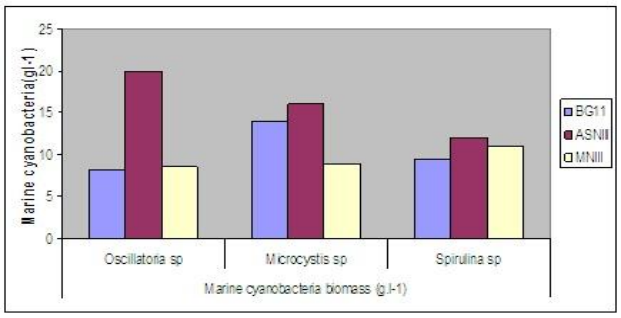


Figure 3. Estimation of Carbohydrate in Marine cyanobactedria

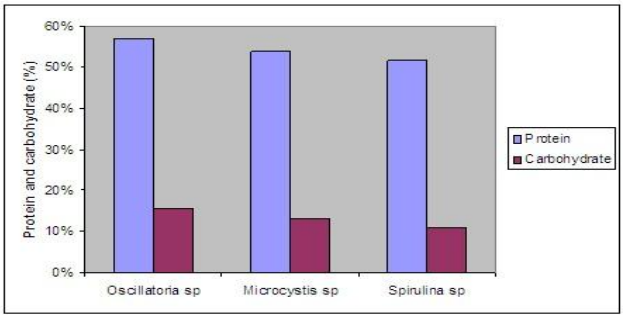


Figure 4. Estimation of total lipid content of marine cyanobacteria

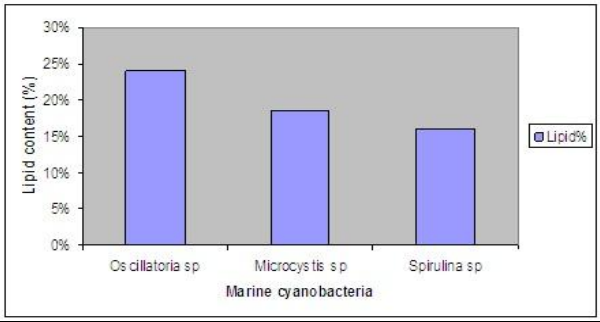


Figure 5. Lipid separation by TLC

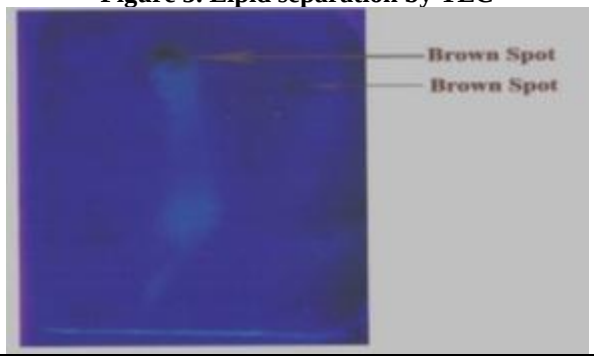


Figure 6. Chlorophyll 'a' and Chlorophyll 'b' Marine cyanobacteria

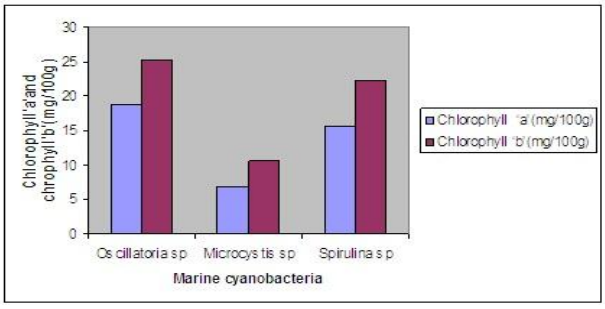


Figure 7. Anticancer activity of *Oscillatoria sp* in human lung cancer cells

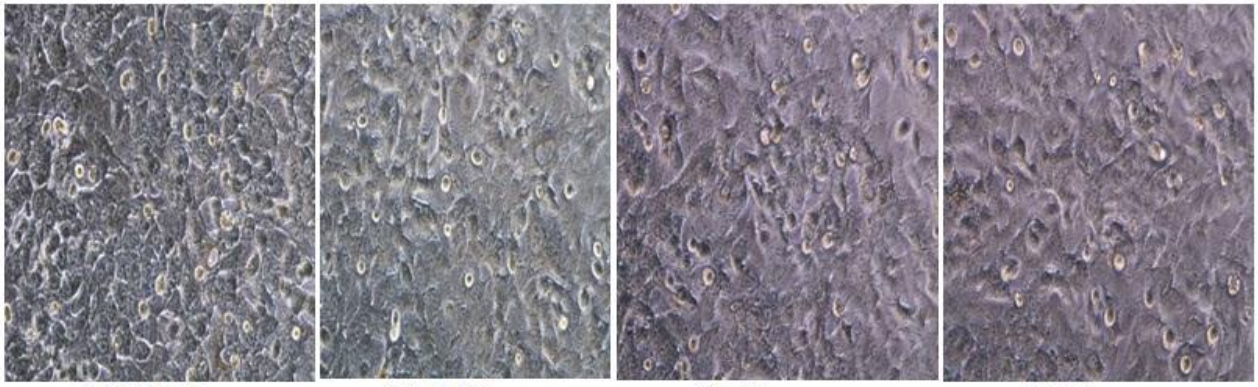
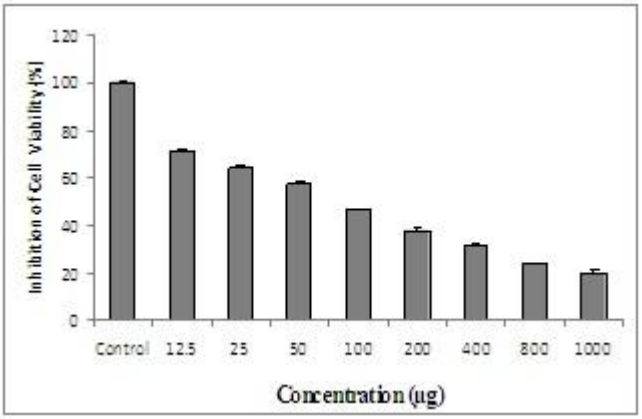


Figure 8. Anticancer activity of marine cyanobacterial extract aginst A549 cells



DISCUSSION

In this present investigation the cyanobacteria *Oscillatoria* spp which possess potent anticancerous activity. The results from the MTT assay widen the scope of the study and pave a way for further research analysis. Resorcinolic acid a natural amphiphilic phenol having bioactivity have been demonstrated in several algae and cyanobacteria. As it is obtained from a novel natural resource steps for drug development can be taken into consideration [7]. The cyanobacteria *Oscillatoria spp.* has many species exhibiting its biodiversity,with most of the species actively possessing bioactive compounds [8]. With the obtained results we will progress for the structural activity in relationship of the bioactive compounds infuture.

Cyanobacterial metabolites are also now being explored as important sources of pharmacologically active compounds useful in diagnostics or pigments as fluorescent probes and as nutraceuticals and as food/feed supplements. In recent times, the most significant discovery from cyanobacteria is the isolation of borophycin, a boroncontaining metabolite, isolated from marine cyanobacterial strains of *Nostoc linckia* and *Nostoc spongiaeforme* [9]. The compound exhibits potent cytotoxicity against human epidermoid carcinoma and human colorectal adenocarcinoma cell lines [10]. Cyanobacteria have gained much attention as a rich source of bioactive compounds and have been considered as one of the most promising groups of organisms to produce them [11,12]. Investigation of a Fijian collection of *Lyngbya majuscule* was shown in 2002 to produce a highly unusual neurotoxic dimeric lipopeptide, named somocystinamide A, containing two distinctive N-methyl enamide groups Nogle *et al* [13]. A subsequent screening program revealed that cancer cells with an active caspase 8 system were exquisitely sensitive to the apoptosis-inducing effect of somocystinamide A Wrasidlo *et al* [14]. The agent was also shown to inhibit neural tube formation in endothelial cells and to associate with lipid rafts. It was proposed that somocystinamide A functions through activation of the death-inducing signaling complex in cell membranes, thus sequentially activating caspases 8 and 3 to induce the extrinsic pathway of apoptotic cell death Suyama *et al* [15].

A new cyclic depsipeptide, lagunamide C, was isolated from the marine cyanobacterium, *L. majuscula*, collected from Pulau Hantu Besar, Singapore. Lagunamide C represents a new subclass of aurilide-related compounds by having a ring expansion due to the additional methylene carbon in the polyketide-derived moiety. Compound 1 was tested against a panel of five cancer cell lines, including

P388, A549, PC3, HCT8, and SK-OV cell, with IC50 values ranging from 2.1 nM to 24.4 nM. It also possesses significant antimalarial property. The amount of marine cyanobaceria biochemical components can vary according to environmental conditions and the age of the culture [14].

Among the three marine cyanobateria *Oscillatoria* spp. contain higher amount of protein, carbohydrate, chlorophyll. *Oscillatoria* spp contain higher amount of lipid when compared with other two species. The three marine cyanobaceria showed maximum production of biochemical components. Patil *et al* [17] studied fatty acid composition of several unicellular algae including *Isochrysis, Pavlova, Phaeodactylum, Porphyridium, Chroococcus, Synechococcus* and *Tribonema* and also recorded C16:0, C18:1, C18:2x6 and C18:3x3 as the dominant fatty acids.

From the present study it revealed that among the twenty one studied taxa of Sundarban area, chlorophycean genera like *Rhizoclonium riparium, R. africanum, Pithophora cleveana, Spirogyra orientalis, Cladophora crystallina* containing more MUFAs (16:1, 18:1) with a support of 16:0 FA, would be more suitable for as FAME production. Among the cyanobacterial taxa, fatty acid composition of freshwater genus *Synechocystis*, together with brackish water species *Lyngbya majuscula* and marine taxa *Phormidium valderianum* and *Phormidium tenue* with favorable fatty acid composition have also been projected as good feed stock for FAME in the present study. Therefore filamentous cyanobacteria with high growth rate can be a suitable alternate for FAME production in Indian scenario. FTIR spectroscopy has been widely used to provide information on a range of vibrationally active functional groups (including O–H, N–H, C=O, =C–H, –CH2, –CH3, C–O–C and >P=O) in biological specimens [18]. The HPLC chromatogram of a standard mixture including AnTx-a, STX and MCLR. Nostoc extracts and growth media showed distinct HPLC profiles. Both extracts as well as growth medium from *Nostoc linckia* showed peaks with retention time close to the neurotoxin AnTx-a (6.59-7.69 min). This peak was not found in *Nostoc punctiforme* growth medium. There was a peak in both extract and growth medium prepared from *Nostoc linckia* with retention time 15.01–15.32 min. A considerable peak with retention time 34.21 min was detected in the *Nostoc punctiforme* extract but not in the *Nostoc linckia* extract, which can explain the stimulatory effect on fish cells. HPLC chromatograms showed the presence of *microcystins* (retention time = 47.83 min) in *Nostoc linckia* extract.The invitro acitivities on HeLa cells of 12 cyanobacteria and seven microalgae from two culture collections were evaluated and compared.

CONCLUSION

Cyanobacteria are very essential source of novel bioactive natural compounds. Several cyanobacterial seconadary metabolites have been shown to have significant pharmaceutical potentials ranging from antiviral antiHIV antibacterial antifungal and antitumor activities. Cyanobacteria in the role for producing bioactive compounds .Hence cyanobacteria prove to a boon for medical research. The mechanism of selective cytotoxicity needs also to be clarified. Similarly detailed chemical studies for isolation, purification and identification of the bioactive molecules followed by the pharmacological investigations and toxicological. Picoplanktonic marine cyanobacteria of genera *Cyanobium*, *Synechocystis*, *Synechococcus* and filamentous forms of the genera, *Nodosilinea*, *Lyngbya*, *Pseudoanabaena* and *Romeria*, isolated from the Portuguese coast, revealed high potential as a source of anticancer compounds In conclusion, the most promising strains for further study are the thermal cyanobacteria *Synechocystis sp*. and especially *Gloeocapsa sp*.Which showed strong tumor growth inhibitory activity and produced wide range of cytotoxic components. Further evaluation of their antitumor properties on a more extensive panel of cell lines seems reasonable.

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