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Research Article

Antineoplastic activity of the extracts from a Cyanobacterium *Lyngbya bipunctata* Lemm.

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Abstract

A cyanobacterium *Lyngbya bipunctata* was isolated from the collected soil samples from different locations. Identification was carried out by using morphological variation and taxonomical approaches according to Desikachary (1959). The axenic culture of *Lyngbya bipunctata* obtained in the laboratory. For the biomass production, different culture media were used namely BG-11 medium (Rippka et al., 1979). The biomass was harvested by filtration through double layered muslin cloth and dried using air blower. *Lyngbya bipunctata* cultures were isolated by enriching soil samples in BG-11 medium (Kaushik 1987). Axenic cultures of *Lyngbya bipunctata* were obtained by repeated liquid transfer of small amounts of material followed by antibiotic treatment (streptomycin, chloramphenicol, and penicillin (10 mg ml^{-1}) (Kaushik, 1987). Unialgal cultures were purified by successive transfer from liquid to solid media. Isolates were grown axenically in 100 ml of BG-11 medium at 7.5 pH and 24°C temperature in 300 ml glass bottles. The procedure for extraction requires solvents of varying polarity, viz, (i) hexane (ii) Chloroform (iii) Methanol and (iv) Distilled Water. The powdered biomass of *Lyngbya bipunctata* samples were weighed and added to each solvent in the ratio 1:10 (w/v) for metabolite extraction. Three successive extract filtrations using Whatman paper were carried out and the subsequent filtrates were collected. Treatment of HeLa cells with Methanol, chloroform, hexane and aqueous extracts resulted in dose-dependent cell killing as evidenced by the continuous reduction in cell survival evaluated by MTT assays. The extracts derived from *Lyngbya bipunctata* were tested and showed antineoplastic activity but with varying proportion. The most effective activity was shown by aqueous extract of *Lyngbya bipunctata.*

Keywords: Antineoplstic activity, BG-11, *Lyngbya bipunctata*, HeLa cells, MTT assay.

INTRODUCTION

Cancer is commonly defined as an uncontrolled growth of cells, with loss of differentiation and commonly with metastasis to other tissues and organs. Cancer is malignant growth and its treatment involves surgery, radiation and chemotherapeutic drugs, singly or in combination. Majority of synthetic anticancer drugs act by interfering with cancerous cell growth, however these drugs commonly affect not only the cancerous cells but normal cells that reproduce quickly like the cancer cells. Thus, resistance of cancers to synthetic chemotherapeutic drugs and their adverse effects are of great concern in cancer treatment today.

Cancer is a devastating disease that affects millions of people every year. Natural plant-derived products are important in the treatment of cancer. In a study of anti-cancer drugs in the market from the 1940s-2002, only 36% of the drugs were purely synthetic while the remaining 64% were of natural origin (Newman et al., 2003).

The blue-green algae or cyanobacteria, have received growing attention as producers of a diverse array of toxic or otherwise biologically active compounds with potential applications in biomedicine, as well as implications for environmental health (Moore, 1996; Gerwick et al., 2001; Osborne et al., 2001; Mayer and Gustafson, 2003; Shimizu, 2003). Among the compounds with different biological activity, those with antineoplastic properties attract particular attention. There are an increasing number of reports on cyanobacteria as producers of anticancer substances (Patterson et al., 1994; Wagner et al., 1999; Zorica et al., 2008). The antineoplastic activity was demonstrated among the genera such as *Nostoc*, *Scytonema*, *Hapalosiphon*, *Lyngbya* and *Symploca*. A promising cyanobacterial antitumor agents, such as Cryptophycin, scytophycins and tolytoxin (Patterson et al., 1991, 1994) were identified

as a microtubule de-polymerizing agents and also as a factor that induces apoptosis in human prostate cancer cells. *Hapalosiphon*, *Microcoleus*, *Scytonema*, *Tolypothrix* have been found to be toxic but as yet no toxin has been isolated and characterized from these genera (Scott, 1991; Skulberg et al., 1992). The oral supplementation of *Spirulina fusiforme* resulted in regression of subject with homogenous leukolakia (Mathew et al., 1995). *Phormidium tenue* contain several diacylglycerol that inhibit chemically induced tumor in mice (Tokuda et al., 1996). Filamentous sea cyanobacteria have been reported to prevent cancer growth, neurodegenerative and infectious diseases (Tan, 2013). Crude extracts of six cyanobacteria (Phormidium sp., Geitlerinema sp., Arthrospira sp., Phormidium sp. Phromidium sp. and Leptolyngbya sp. demonstrated concentrationdependent inhibitions of Homo Sapiens Kidney Carcinoma and Homo Sapiens Colon Colorectral Adenocarcinoma (Srivastava et al., 2015). A 14-membered glycosidic macrolide, lyngbouilloside (Frayman et al., 2013), was isolated from the marine cyanobacterium *Lyngbya bouillonii*, harvested from Papua New Guinea. It displays a modest cytotoxicity against neuroblastoma cells with an IC_{50} value of 17 µM (Tan et al., 2002). Another 14-membered macrolide, koshikalide (Shishido et al., 2015), was isolated from the marine cyanobacterium *Lyngbya* sp., collected from Mie Prefecture, Japan, and shows slight cytotoxicity against HeLa S_3 cells with an IC_{50} value of 42 µg/mL (Iwasaki et al., 2010). In the present study antineoplastic activity of the extracts of a Cyanobacterium Lyngbya *bipunctata* has been tested.

MATERIALS AND METHODS

Isolation of culture

Lyngbya bipunctata cultures were isolated by enriching soil samples in BG-11 medium (Kaushik 1987). Axenic cultures of filamentous *Lyngbya bipunctata* were obtained by repeated liquid transfer of small amounts of material followed by antibiotic treatment (streptomycin, chloramphenicol, and penicillin (10 mg ml^{-1}) (Kaushik, 1987). Unialgal cultures were purified by successive transfer from liquid to solid media. Isolates were grown axenically in 100 ml of BG-11 medium (Rippka et al., 1979) at pH 7.5 and 24°C temperature in 300 ml glass bottles.

Chemicals and media

DMSO, hexane, Chloroform, Methanol from SRL Ltd.; DMEM growth medium, FBS, 100X Antibiotic solution from Hi Media; Taxol (30mg/5ml); MTT (Hi Media, India) were used.

Cell lines

Human cervix epitheloid carcinoma, epithelial morphology (HeLa), was procured from National Centre of Cell Sciences (NCCS), Pune.

Processing and extraction

The procedure for extraction requires solvents of varying polarity, viz, (i) hexane (ii) Chloroform (iii) Methanol and (iv) Distilled Water. The powdered *Lyngbya bipunctata* biomass samples were weighed and added to each solvent in the ratio 1:10 (w/v) for metabolite extraction. Three successive extract filtrations using Whatman paper were carried out and the subsequent filtrates were collected. The aqueous extract samples were stored as such at 4°C till required. The organic solvent filtrates, on the other hand, were subjected to evaporation at room temperature enabling the loss of the volatile solvent to obtain the solid plant extract residues. These residues were weighed and dissolved in 20% aqueous DMSO so as to make 2.5mg/ml final concentrations crude plant extracts. These extracts were also stored at 4°C.

Cell culturing and maintenance

Both the cell lines were cultured in DMEM growth medium supplemented with 10% FBS and 0.1% Antibiotic solution in 75cm^2 tissue culture flasks. They were maintained at 37°C and 5% $CO_{2\ in}$ incubator. Confluent flasks were passaged appropriately to maintain steady propagation of the cells.

Cytotoxicity assay

Cells were plated in 96 well plate in a density of $2x10^4$ cells per well. The extracts were dried, reconstituted in 0.25% DMSO and distilled water. The cultures were incubated for 24, 48 and 72, hours (3-4 cells generation in control culture).

MTT Assay

The MTT [3-(4, 5- dimethylthiazol – 2-yl) -2, 5- diphenyl tetrazolium bromide] assay was adopted to determine the cell inhibition of the various extracts on cancerous cells *in vitro*. The adherent Hela monolayers formed in the 90cm^2 tissue culture flasks were uniformly scraped off the surface using a sterile cell scraper. The HeLa cell suspension thus forms was centrifuged at 5,000 rpm for 5 minutes, and the supernatant discarded while the cell pellet was re-suspended in medium. The cell count was taken using the Neubar chamber and in both cases 10^5 cells were seeded into 200 µl growth medium (DMEM +10% FBS+0.1% Antibiotic solution) contained in microtitre plates (96-well plates). In each case, 3 concentrations of the extracts in the range of 80 µg to 150 µg were added to the cells in triplicates. Taxol served as the positive control in the experiment. These plates were incubated for 24 hours at 37°C after which 20µl MTT solution was added to each well and mixed uniformly. The plates were re-incubated for 4 hours at 37°C allowing the reduction reaction to occur. Finally, the absorbance of the test and control wells was measured calorimetrically at 540 nm and 620 nm using the ELISA plate reader. The percentage cell viability was expressed as: (At*100)/Ac [%], where At is the absorbance of test sample and Ac is absorbance of the control. The IC_{50} were interpolated from the growth curve following 24 h of incubation.

RESULTS AND DISCUSSION

To identify the potential anti-cancer phytochemicals, the hexane, chloroform, Methanol and aqueous extract of *Lyngbya bipunctata* were tested against Hela cells. Antineoplastic activity of the *Lyngbya bipunctata* extracts was assessed by MTT assay. The exposure of HeLa cells to 80–150 µg/ml of methanol and aqueous extract resulted in dose-dependent cell killing after the treatment of the cells for 24, 48 and 72 h.

It is observed that the trend of extract inhibition vary with different solvents. Aqueous extract of *Lyngbya bipunctata* showed more potent anticancerous activity. The surviving fraction of HeLa cells declined with increasing concentrations of aqueous extracts of *Lyngbya bipunctata* and the lowest surviving rate was observed below 80µg / ml concentration. IC_{50} was 52 µg/ml for aqueous extract. Methanol, chloroform and hexane extracts showed good cell killing effect. The cell viability was weakly affected after 72 h of exposure. Thus the results suggest that the extracts of biomass of *Lyngbya bipunctata* obtained using the different solvents showed the antineoplastic activity but in variable proportion. Among this the most promising are aqueous extract of *Lyngbya bipunctata***.** It must be appreciated that such superior results were obtained from crude extracts, and as such show promise in as antineoplastic natural products.

Lyngbya bipunctata extracts tested showed good scope for further investigation and analysis of their antineoplastic effects. These extracts must be purified and their bioactive compound must be quantitatively characterized. Further, the effect of these extracts must be tested on other cancer cell lines *in vitro* and *in vivo* too. Most importantly, of the pathway of antineoplastic action of the active agents must be determined.

With the technical advancement in diagnostic techniques and the increasing health awareness among people, more cases of cancer are coming to light than before. Cancer is a disease which evokes widespread fear among people. It is treated with surgery, chemotherapy and/or radiotherapy. However, there is not yet a definitive treatment for this disease, especially in its advanced stages. Therefore, it is essential to screen new medicines which are less toxic, highly effective and provide better management of the disease. In the present study, the methanol, chloroform, hexane and aqueous extracts of *Lyngbya bipunctata* against HeLa cells have been evaluated.

The maximum effect was observed at 150 µg/ml and thereafter the cell killing effect remained almost identical. Various drugs of plant origin, such as *Vinca* alkaloids, epipodophyllotoxins and taxol, have been reported to induce dose-dependent cell killing. Taxol, which has been used as a concurrent control in this study, reduced the cell survival in a dose dependent manner. Our results show that aqueous extract of *Lyngbya bipunctata* is as cytotoxic as or even more cytotoxic than Taxol at equimolar concentrations. A detected stimulatory effect at lower concentration of the extracts is common and well-known phenomenon for the toxic compounds. Interestingly, treatment of Hela cells with low concentration of *Lyngbya bipunctata* had a cytotoxic effect, while higher concentration had stimulatory effect. Certain strains of filamentous cyanobacteria, particularly those belonging to the genus *Lyngbya*, have shown to be an exceptional source of unique bioactive secondary metabolites (Gerwick et al., 2001; Tan, 2007). However, the majority of the investigations on biologically active compounds from *Lyngbya* have focused only on marine species (Moore, 1996; Gerwick et al., 2001; Thacker & Paul, 2004).

CONCLUSION

Two new cyclic desipeptides, hantupeptins A and B show activity against MOLT-4 (leukemia cancer cell line) and MCF-7 (breast cancer cell line) (Tripathi et al., 2010). Curacin A, the most potent molecule was isolated from the organic extract of the marine cyanobacterium, *Lyngbya majuscule* (Gerwick et al., 1994). Lyngbyabellins A and E with potent actin polymerization activity are reported from *L. majuscule* (Luesch et al., 2000; Han et al., 2005). Both compounds displayed moderate cytotoxicity against various cell lines. Pahayokolide A isolated from fresh water *Lyngbya* was moderately cytotoxic, inhibiting various human cancer cell lines (Berry et al., 2004).

Although the cytotoxicity of some cyanobacterial compounds is well documented, the mechanisms of such an effect have not been properly described yet. The involvement of several unknown mechanisms, such as apoptosis and nuclear enzymes may be responsible for this. The scytophycins are a newly identified class of natural cytotoxins that have been isolated from cyanobacteria of the family Scytonemataceae.

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