Antiangiogenic and antiproliferative assessment of cyanobacteria

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Biologically active compounds with different modes of action, such as antiproliferative, antioxidant, antimicrotubule, have been isolated from algae and cyanobacteria. The present study was designed to evaluate antiangiogenic and antiproliferative potential of dichloromethane and methanol (2:1) extracts of different cyanobacteria. Further fingerprinting of the activity possessing extracts were carried out using ESI-LC-MS/MS. Extracts (25, 50 and 100 µg) were screened in the vascular endothelial growth factor (VEGF) induced angiogenesis in inovo chick chorioallantoic membrane assay (CAM) at various concentrations using thalidomide and normal saline as positive and untreated control groups respectively. The extracts were also evaluated for their antiproliferative activity by MTT assay using HeLa cancer cell line. The results obtained from the various algal extracts did not show any significant antiangiogenic activity as compared to VEGF control. Oscillatoria sp. and Lyngbya officinalis exhibited significant anti-proliferative activity at IC 50 values of 220 and 260 µg/mL respectively. ESI-LC-MS/MS of L. officinalis showed the presence of lyngbyatoxin-A and that of Oscillatoria sp. reveals the presence of malyngamide-J suggesting the possibility of antiproliferative activity.

Keywords: Algae, Angiogenesis, Chorio Allontoic Membrane, VEGF

Angiogenesis, the formation of new blood capillaries from existing vessels, is an important mechanism for supplying nutrients to cells that are distant from existing blood vessels. Angiogenesis is critically important during embryonic development and certain physiological circumstances in the adult, including wound healing.

Anti-angiogenic therapies have become one of the most promising approaches in the anti-cancer drug development. Several anti-angiogenic agents, alone or in combination with other conventional therapies, are in clinical trials based on the promising and successful preclinical research data. Anti-angiogenic drugs are also reported to offer improvement over conventional cancer treatment in reducing side effects, inhibiting the formation of new blood vessels supplying the tumor and in this process the chances of drug resistance are less likely.

Cyanobacteria are among the most successful and oldest life forms still present on earth. These prokaryotic photoautotrophs populate habitats as diverse as soil, marine and freshwater environments, rocks, plants, animals and ice. This vast variety of different ecological niches requires successful adaptation to vastly different conditions. In order to adapt to these surroundings, cyanobacteria evolved to produce a wide variety of different secondary metabolites. In particular cyanobacteria from marine and freshwater sources produce many metabolites which possess powerful biological activities. Cyanobacteria and algae are reported to produce secondary metabolites that inhibit the transcriptional activation of vascular endothelial growth factor (VEGF), a critical angiogenic factor produced by tumor cells to promote new blood vessel formation and facilitate tumor growth. Therefore this study has been conducted to evaluate antiangiogenic and antiproliferative activity of cyanobacterial strains.

Materials and Methods

The Cyanobacterial strains like Cylindrospermum (NCCU-272), Plectonema sp. (NCCU-204), Anabaena sp. (NCCU-9), Aulosira fertilissima (NCCU-444),


Westiellopsis prolifica (NCCU-331), Microchaete sp. (NCCU-342), Oscillatoria sp. (NCCU-369), Phormidium tenui (NCCU-104), Lyngbya Officials (NCCU-102), Gloecapsa gelatinosa (NCCU-430) and Chroococcus (NCCU-207) were procured from National Center for Conservation and Utilization of Blue Green Algae, Indian Agricultural Research Institute, New Delhi, India. Mass grade reagents were purchased from Merck, Germany. All other reagents were of analytical grade and were purchased from their respective commercial resources. Recombinant VEGF was purchased from Sigma, USA. The fertilized White Leghorn chicken eggs were purchased from local hatchery (Keggs farm Pvt Ltd. Gurgaon, India).

Culture medium—Cyanobacterial test strains were raised in BG-11 medium (pH 7.3). The heterocystous test strains were raised in BG-11 medium without nitrogen source while sodium nitrate was added to the medium for non heterocystous strains. The cyanobacterial strains were raised under illumination by fluorescent tubes providing light intensity of 2000±200 lux with 16:8 h light/dark regime at 30±1 °C. The cells (biomass) were harvested by filtration through nylon cloth and then thoroughly washed with distilled water before drying in lyophilizer. The dried mass was transferred into sterilized sample tubes. The tubes were properly closed and placed in refrigerator (4 °C) till further use.

Preparation of algal extracts for performing angiogenic and antiproliferative studies—Lyophilized algal material (100 g) was homogenized in a solvent mixture containing dichloromethane and methanol in the ratio of 2:1 (v/v) using a Polytron high speed homogenizer (Kinematica AG, Switzerland). The homogenate was subsequently subjected for ultrasonication for 3 min using a Sigma bathtype sonicator (Bendelin Sonorex, Berlin). The homogenized material was centrifuged at 5800 × g for 5 min and passed through Whatman (No.1) filter paper. This process was repeated thrice and the extracts were pooled and subjected for vacuum rotary evaporation (Christ, RVC 2-18, Germany) to remove the solvents. The residual moisture (if any) was also removed by overnight lyophilization. This process yielded 1-2 g dark green residue. The extracts were stored at -80 °C till further use.

Vascular endothelial growth factor (VEGF) induced angiogenesis in inovo chick chorioallantoic membrane assay—The chick chorioallantoic membrane (CAM) assay was performed in an identical fashion with slight modification as described by Ribbati et al. The fertilized eggs were incubated at 37 °C and the incubator was humidified by keeping bowl of water inside. On the 3rd day of incubation, 2-3 mL of albumin was removed to detach the developing CAM from the shell. On day 8th, a square window was opened in the egg. VEGF (50 ng) containing ovalbumin pre-coated coverslips (12 mm diameter) were carefully placed over the developing chorionic allantoic membrane and served as untreated control. Similarly, only ovalbumin coated, ovalbumin along with VEGF and thalidomide (10 µg) coated, ovalbumin along with VEGF and algal extracts of various concentrations (25, 50 and 100 µg) coated cover slips were placed individually in every egg and considered as normal, positive control and experimental respectively. The window was sealed with sterile parafilm and placed in incubator.

On 12th day, the cover slip area of the CAM was subjected for light photo microscopy under uniform magnification using digital camera (Scout SCA1390-17 ½° CCD GigE color camera). The digital images in the defined area of the cover slip were analyzed by Aphelion imaging software (Version 2.8 SP1) which was customized and standardized to calculate the blood vessel density. The digital reconstructions were processed through a specific vascular algorithm to get total vascular density in terms of square millimeter area.

Antiproliferative activity of algal extracts by Microculture tetrazolium test assay (MTT)—MTT, 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, is a yellow-coloured compound that is converted by mitochondrial reductases into a blue formazan derivative. Cytotoxicity (MTT) assay was performed following the method described by Mathur et al. and percentage of cell viability was determined by spectrophotometric determination of accumulated formazan derivative in treated cells at 560 nm in comparison with the untreated ones.

Cancer cell line developed from cervical cancer cell (HeLa) was used to study the anti-proliferative activity of different algal extracts. The cells were grown under aseptic conditions in Dulbecco’s modified eagle’s medium (DMEM) enriched with 10% fetal calf serum (FCS), at 7.2 pH. To the medium, antibiotics [ciprofloxacin (10 µg/mL) and amphotericin B (1 µg/mL)] were also added. The medium was prepared aseptically and filtered through 0.22 µm sterile filter membrane. The sterility of the
medium was confirmed by incubating it for 48 h in the incubator at 37 °C with 5% CO₂. Cells grown between passage numbers 11 to 25 were used for all experiments.

Briefly, to perform the MTT assay, the HeLa cells in exponential growth were plated down at a final adjusted density of 7000-8000/100 µL. The cells were plated in 96 well plates at a volume of 100 µL in each well. The cells were allowed to attach and incubated undisturbed for 24 h. The medium was removed after 24 h and the cells were treated with different concentrations of test samples or vehicle in sterile medium. The control wells did not receive any treatment and were maintained in medium.

The cells were subjected to test samples for 24 h and the medium from well plate was removed with simultaneous addition of yellow colored tetrazolium solution. The cells were exposed to the dye for 3 h and completion of the assay was confirmed by microscopic examination of the plate for purple colored formazan crystals. The crystals formed in each well were dissolved with 100 µL of DMSO and protected from light. Absorbance (OD) was immediately read by an ELISA reader at 560 nm. All test samples treatment was conducted in triplicate.

In order to screen the anti-cancer activity of the cyanobacterial extracts were reconstituted in phosphate buffer saline (final concentration< 0.1%). The extracts were screened at the concentration of 6.25, 12.5, 25, 50, 100, 200, 400, 800 µg/mL for their in vitro antiproliferative activity.

The drug induced cytotoxicity was calculated using the following formula. IC₅₀ was defined as the concentration of drug which inhibited the growth by 50%.

\[
\text{Killing (\%)} = \frac{\text{Average OD of control} - \text{Average OD of treatment}}{\text{Average OD of control}} \times 100
\]

**ESI-LC-MS/MS Fingerprinting of algal extracts**

**Instrument parameters**—Ultra high performance liquid chromatography (UPLC) (Accela, Thermo Surveyor system, Thermo Electron Corp, Waltham, MA, USA) with the in built quaternary pump, online degasser and photodiode array detector (PDA) coupled with triple quadrupole tandem mass spectrometer (4000 Q-Trap, ABS Biosystems, Foster City CA, USA) was used for the identification of compounds. UPLC was operated by using Chromquest software version 4.1 and ESI-MS/MS was operated by Analyst Ver.1.5.2.

**Creation of library of secondary metabolite from cyanobacteria**—All known secondary compounds reported in the literature for the cyanobacterial species were made using MDI interface of Chache (Software, Fujitsu Japan) and pooled to make a library in MDL format for their proposed use for analysis using fragmentation pattern interpretation protocol of the Analyst (Ver. 1.5.2).

**Information Dependent Acquisition (IDA) protocol for analysis of extract**

Sample preparation—Crude organic extract of algal biomass amounting to 1 mg/mL in methanol were serially diluted with 50% methanol:water to reach the final concentration of 100 µg/mL. After centrifugation at 5500 g for 10 min the supernatant was subjected for analysis using ESI-LC-MS/MS. Chromatographic separation was achieved using C₁₈ column (Phenomenex Luna column; dimension of 250 x 4.6 mm, 5.0 µm; Merck, Darmstadt, Germany). The linear gradient mobile phase reaching 90% acetonitrile (with 0.1% formic acid) from 10% over the period of 30 min was used to elute the compounds. The mobile phase was used at the rate of 0.5 mL/min. For mass spectrometry source dependent parameters such as Gas 1 and 2 were kept at 30 and 60 psi and temperature was kept at 450 °C. The IDA protocol consisting Enhanced Mass Spectrometry (EMS), Enhanced resolution (ER) and Enhanced Product Ion Scan (EPI) were performed at the speed of 4000 Da/sec between 100-800 a.m.u. at the positive ESI (+5500 V). Post acquisition of data, UV Photodiode array spectrum guided analysis of secondary metabolite was conducted using EMS scan of the extract at the same retention time. The unprotonated molecular weight matching the known mass values of the secondary metabolite library was subjected for fragment analysis using inbuilt algorithm for fragment interpretation tool. The identified metabolites were also verified and confirmed using product ion spectrum using flow infusion analysis.

**Results**

Effect of VEGF and algal extracts on CAM-On 12th day of incubation, on the developed CAM which was treated with VEGF showed profuse growth of blood vessels around the cover slip (Fig. 1b). At microscopic level, a higher vascular density was recognizable below the VEGF containing ovaalbumin coated cover slips as compared to the normal (Fig. 1a). At the boundary between cover slip and CAM mesenchyma, numerous capillaries were detectable around the cover slip. In contrast, treatment with algal extracts did not decrease the blood vessel density with the increase of the dose of
the extract. Figure 1 (d-n) indicate the representative sample showing the effect of normal, VEGF, VEGF+thalidomide, VEGF with different concentrations of algal extracts. The thalidomide showed reduction in blood vessel density and was found to be statistically significant as compared to untreated VEGF control. However at start Cylindrospermum (Fig. 1d) has shown angiogenic activity but with subsequent studies the angiogenic activity was found to be insignificant as compared to untreated control (Table 1).

Effect of algal extract on antiproliferative study by MTT assay—In preliminary experiments, the algal extracts were screened at concentration of 6.25, 12.5, 25, 50, 100, 200, 400, and 800 µg/mL for duration of 24 h. Oscillatoria sp. and Lyngbya officinalis extracts negatively affected the viability of HeLa cells. About 40% increment in killing was seen when the dose of the extract were increased from 6.25 to 400 µg/mL. IC$_{50}$ values of the extracts were found to be 220 and 260 µg/mL and that of positive control taxol was 14.7 µg/mL respectively (Fig. 2).

LC-MS/MS Fingerprinting—LC-positive ESI-MS analysis of organic extract of Oscillatoria sp. and Lyngbya officinalis organic extract showed the
branching and changed direction abruptly with VEGF treated group (Fig. 1). In the treatment group, VEGF with cyanobacterial extracts did not disturb the pattern and the result obtained was insignificant when compared with control group, which has indicated that the tested algae do not possess such anti-angiogenic effect (Fig. 1). In the present study, initially Cylindrospermum at the concentrations of 25, 50 and 100 µg showed a dose dependent increase in angiogenic activity but later with subsequent studies it was found that the results were insignificant as compared to untreated control.

Angiogenesis inhibitors combined with chemotherapeutic drugs have significant efficacy in the treatment of a variety of cancers. Pre-clinical and clinical studies have identified successful chemopreventive agents against lung cancer acting by angiogenesis pathway. As cyanobacteria are largely unexplored, they represent a rich opportunity for discovery. Oral administration of Spirulina fusiformis as a supplement resulted in regression in patients with homogenous leukolakia. The extracts of Spirulina and Dunaliella inhibited the chemically induced carcino genesis in hamster buccal pouches.

Angiogenesis is an important process in the body both during normal and pathological conditions. VEGF is the best characterized angiogenic factor and it is the main driving force behind in tumour angiogenesis and in all blood vessel formation. There are many experimental evidences that anti-angiogenic strategies will contribute to the future therapy of cancer and several compounds with anti-angiogenic properties are now under clinical investigation.

An effective glucosidase inhibitor di (hydroxymethyl) dihydroxy pyrrolidine (DMDP; 2(R), 5(R)-bis-(hydroxymethyl)-3(R),4(R) dihydroxy pyrrolidine) was isolated from the cyanobacterial genus Cylindrospermum that effectively inhibits digestive glucosidases of aquatic insects and crustacean grazers. Cyanobacteria were isolated having positive antimicrobial activity were identified morphologically as Lyngbya-Phormidium-Plectonema Group B, Oscillatoria sp., Leptolyngbya sp., Phormidium sp., Dunaliella sp., Aulosira fertilissima (100 mg/kg body weight). Normalization of enzymes levels like super oxide dismutase, catalase, glutathione peroxidase and glutathione transferase were observed in rats treated with Aulosira fertilissima (100 mg/kg body weight). Evidences were obtained with Anabaena sp. extracts as a potential source of new antibacterial agents.

Table 1—Quantification of anti-angiogenic response of the VEGF vs normal, thalidomide and Cylindrospermum, Oscillatoria sp. and Lyngbya officinalis, (each 100 µg/egg) in the CAM assay on 12th day incubation. Number of experiment is three (n=3)

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Blood vessel density</th>
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<tbody>
<tr>
<td>Normal</td>
<td>41.51 ± 1.57</td>
</tr>
<tr>
<td>VEGF(50 ng/egg)</td>
<td>91.6 ± 1.45</td>
</tr>
<tr>
<td>VEGF(50ng/egg) + Thalidomide (10 µg/egg)</td>
<td>27.86 ± 1.01</td>
</tr>
<tr>
<td>Cylindrospermum extract (100µg/egg) + VEGF(50 ng/egg)</td>
<td>103±2.38</td>
</tr>
<tr>
<td>Oscillatoria sp.extract (100µg/egg) + VEGF(50 ng/egg)</td>
<td>96.08±2.3</td>
</tr>
<tr>
<td>Lyngbya officinalis extract (100µg/egg) + VEGF(50 ng/egg)</td>
<td>98.38±3.24</td>
</tr>
</tbody>
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Fig. 2—Antiproliferative activity of dichloromethane/methanolic extract of Oscillatoria sp. and Lyngbya officinalis on HeLa cells. Cells were incubated with different concentrations of the plant extracts (ranged from 6.25–800 µg/mL) for 48 h. Cell viability was determined by the MTT assay (n=4). Viability curves: Percentage viability = absorbance of test wells/absorbance of control wells) × 100 plotted against the concentration of extract.

Discussion

Blood vessels in the CAM exhibited an organized branching pattern. High order vessels branch into several lower order vessels, which then again branch into smaller vessels. Implantation of the cover slip coated with vehicle control did not disturb this pattern. In contrast, blood vessels seemed to exhibit presence of secondary metabolites which were further confirmed by their respective fragmentation patterns. Organic extract of Oscillatoria sp. showed a distinct peak with retention time of 5.39 min. confirming the presence of malyngamide-J with [M+H]⁺ value of 622.3(C₃₈H₅₅NO₁₈) (Fig. 3a).

Organic extract of Lyngbya officinalis showed the presence of a secondary metabolites with retention time of 20.45 confirming the presence of lyngbyatoxin-A with [M+H]⁺ values of 438.6(C₂₇H₄₀N₃O₂) (Fig. 3b).

Discussion

Blood vessels in the CAM exhibited an organized branching pattern. High order vessels branch into several lower order vessels, which then again branch into smaller vessels. Implantation of the cover slip coated with vehicle control did not disturb this pattern. In contrast, blood vessels seemed to exhibit
In the present study of MTT study indicated that the extracts of the algae Oscillatoria sp. and Lyngbya officinalis were shown to induce significant and dose-dependent inhibitory activities against human cervical cancer cell lines. The American National Cancer Institute assigns a significant cytotoxic effect of promising anticancer product for future bioguided studies if it exerts an IC\textsubscript{50} value \leq 30 \mu g/mL\textsuperscript{28}. In this preliminary study, the focus of interest was on crude extracts, the anti-proliferative activity could be due to the presence in the dichloro methane/methonolic extracts of active products that could probably have highly anti-growth effects. Veraguamide-A obtained from Oscillatoria margaritifera has potent cytotoxicity activity in the H-460 human lung cancer cell line (LD\textsubscript{50}=141 nM)\textsuperscript{29}. Similarly, Pahayokolide A, a bioactive metabolite from a freshwater species of Lyngbya isolated from the Florida Everglades was shown to inhibit a number of cancer cell lines over a range of concentrations (IC\textsubscript{50} varied from 2.13 to 44.57 \mu M)\textsuperscript{30}.

Structures of all known secondary compounds reported in the literature for the cyanobacterial species were made using MDI interface of Chache (Software, Fujitsu Japan). They were further pooled together to make a library in MDL format and were used by fragmentation pattern interpretation protocol of the Analyst (Ver. 1.5.2). The positive ESI–LC-MS/MS studies conducted in the present study showed the presence of lyngbyatoxin-A in Lyngbya officinalis and Oscillatoria sp. showed the presence of malyngamide-J.

The structural diversity of natural products generated by microorganisms appears to be predominantly a result of modifications and combinations of reactions from primary metabolic pathways\textsuperscript{8}. If the production of the bioactive compound is advantageous with a new biosynthetic capability it is expected to be adopted by the organism for its defense. This process has been noted several times within L. majuscula, most notably within the structurally novel metabolite ypaoamide\textsuperscript{31}. Structural similarities between this compound and the malyngamides\textsuperscript{32}, pukeleimides\textsuperscript{33}, majusculamides-D\textsuperscript{34} and microcolins A and C\textsuperscript{35} suggests that a common biosynthetic pathway may be employed by different chemotype of
L. majuscula. This may be the reason that Oscillatoria sp. and Lyngbya officinalis may be evolutionary adopted for the production of such compounds and the antiproliferative activity may be due to presence of such secondary metabolites. To conclude, the present study showed the possible antiproliferative activity of the organic extract of fresh water algae Oscillatoria sp. and Lyngbya officinalis. This study further confirmed the presence of lyngbyatoxin-A in Lyngbya officinalis and malyngamide-J in Oscillatoria sp. These compounds may be responsible for the proposed antiproliferative activity. However, further studies are required to evaluate their individual activity and mechanism of action which are in progress using other complementary techniques.

References
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