

Screening of *Lyngbya majuscula* for Potential Antibacterial Activity and HPTLC Analysis of Active Methanolic Extract

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The aim of the present study was to investigate the antimicrobial activity of different organic extracts prepared from biomass and culture supernatant of *Lyngbya majuscula* against *Escherichia coli*, *Bacillus cereus*, *Bacillus subtilis*, *Staphylococcus epidermidis*, *Enterococcus faecalis* and a Yeast *Candida albicans*. Antimicrobial activity and minimum inhibitory concentration(s) of various extracts were evaluated by disc diffusion assay and broth micro dilution assay, respectively. Results indicated that this cyanobacterium excreted metabolites having broad spectrum antimicrobial activity. Methanolic extract was found to have significant activity against *Bacillus subtilis* with MIC value 512 µg/ml. HPTLC (high performance thin-layer chromatography) analysis at λ 254 nm was performed for the separation of all the chemical constituents from the methanol extract. The antimicrobial activity was compared with standard antibiotic tetracycline and nystatin.

Key words: Antimicrobial activity, Cyanobacteria, *Lyngbya majuscula*, HPTLC, Minimum inhibitory concentration.

Cyanobacteria, the blue-green algae, are an ancient and diverse group of photosynthetic microorganisms that have evolved to inhabit many different and extreme environments *i.e.* in both freshwater and marine environment (Hagmann and Juttner, 1996). Recently compounds from cyanobacteria have been isolated which display inhibitory effects on bacterial growth (Mundt *et al.*, 2001; Falch *et al.*, 1995; Kaushik and Chauhan, 2008; Kaushik *et al.*, 2008); on *Mycobacterium* spp. (Asthana *et al.*, 2006; Rao *et al.*, 2007); on fungal growth (Kellam *et al.*, 1988; MacMillian *et al.*, 2002); on cancer cells

(Kashiwagi *et al.*, 1980; Carmeli *et al.*, 1990); against viruses (Gustafson *et al.*, 1996; Hayashi and Hayashi, 1996) and enzymes inhibiting (Cannell, 1987). Most of the isolated substance belongs to groups of polyketides, amides, alkaloids and peptides (Borowitzka, 1995; Falch, 1996). In view of emergence of resistance among microorganisms to common antibiotics, the search for new antimicrobial substance has become increasingly important (Trias and Gordon, 1997). In our study, Organic extracts and culture supernatant of *Lyngbya majuscula* were evaluated for potential antimicrobial activity. The study was further extended to determine the minimum inhibitory concentration of active crude substance against the susceptible bacterial species. An attempt was made to determine the number of fraction(s) present in the active extract of the cyanobacterium through HPTLC.

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MATERIAL AND METHODS

Cyanobacterium and Growth Conditions

Starter culture of *Lyngbya majuscula* (Order: Oscillatoriales Family: Oscillatoriaceae) was collected from National Center for Conservation and Utilization of Blue-green Algae, Indian Agricultural Research Institute, New Delhi. The cyanobacterium was grown and maintained in BG-11 (Stanier *et al.*, 1971) growth medium (at pH=7) under rotatory conditions. The culture was illuminated continuously at a light intensity of 3500 LUX.

Chemical composition of BG-11 growth medium

Medium contains the following nutrients (values in parenthesis showed the concentration); $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.75 gL^{-1}), NaNO_3 (1.5 gL^{-1}), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (0.036 gL^{-1}), $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$ (0.04 gL^{-1}), Na_2EDTA (0.001 gL^{-1}), Na_2CO_3 (0.02 gL^{-1}), Ferric ammonium citrate (0.006 gL^{-1}), citric acid (0.006 gL^{-1}) as macronutrients along with 1 ml of micronutrients H_3BO_3 (2.86 gL^{-1}), $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (0.22 gL^{-1}), $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ (1.81 gL^{-1}), $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (0.08 gL^{-1}), $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ (0.39 gL^{-1}) and $\text{CO}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ (0.049 gL^{-1}).

Harvesting of extracts

The cyanobacterial culture was harvested after 30 days of incubation. Biomass was separated to the culture supernatant by centrifugation at 5000 rpm for 15 minutes (Ghasemi *et al.*, 2003). Biomass was further air-dried and then subjected to extraction by mixing well in the appropriated amount of organic solvent. The mixture was left for 24 h at room temperature and then sonicated for 10 min. The mixture was further centrifuged at 5000 rpm for 15 min. After centrifugation, supernatant was collected in a preweighed test tube where it was concentrated using nitrogen gas until completely dried. Test tube was again weighed and dried sample was resuspended in the appropriate solvent to make the solution of known concentration for the antibacterial assay. Standard antibiotic i.e. tetracycline and nystatin were used to compare the antimicrobial activity.

Microbial strains

The microorganisms used in antibacterial assay were collected from Institute of Microbial Technology (IMTECH), Chandigarh,

India. Four Gram-positive bacteria namely *Bacillus subtilis* MTCC-736, *Bacillus cereus* MTCC-430, *Staphylococcus epidermidis* MTCC-435, *Streptococcus faecalis* MTCC 459 and one Gram-negative strains *Escherichia coli* MTCC 739 and one yeast *Candida albicans* MTCC 227 were screened for present evaluation.

Inoculum preparation

The bacterial strains were inoculated on Tryptone Soya Agar (TSA) and *Candida albicans* was streaked on Chloroamphenicol Yeast Glucose Agar (CYGA) and incubated for 24 h at 30°C and 48 h at 25°C respectively. All the strains were then suspended in saline solution 0.85% NaCl and adjusted to yield approximately 1.0×10^7 - 1.0×10^8 CFU/ml by using spectrophotometer (25% transmittance at 530 nm) as per the guidelines given in Indian Pharmacopoeia (2007). Media component were purchased from Hi Media, Mumbai, India. All the chemicals used were of analytical grade.

Disk diffusion assay

Antimicrobial activity of all extracts was determined as per the guidelines of Kirby and Bauer (1966). Filter paper disks (6mm) were saturated with 60 μl of test solution, dried and placed on the Muller-Hinton agar plate for the bacterial strains and Sabouraud Dextrose agar plate for fungus. All the plates were incubated at an appropriate temperature for fungus (25°C) and bacteria (37°C) for a period of (18-24 h). All the plates were observed for the zone of inhibition following the incubation, diameter of these zones was measured in millimeters. All tests were performed in sterile condition and repeated for three times.

Determination of minimum inhibitory concentration

Minimum inhibitory concentration of active crude extract(s) was determined by broth microdilution method as recommended by National Committee for Clinical Laboratories Standards (NCCLS, 1997). The test was performed in 96 wells microtiter plates. Two fold serial dilutions of all active extracts were made in Cation-Adjusted Mueller-Hinton Broth (CAMHB from Hi-Media) for bacterial strains and Malt Extract Broth for Fungal strains ranging from 4 to 2048 $\mu\text{g/ml}$. Tetracycline and Nystatin were used as standard antibiotics for the assay.

Each inoculum was prepared in the same medium at a density adjusted to a 25% transmittance turbidity standard (10^8 cfu/ml) and diluted to 1:100 for the assay. The final volume in the wells was 200 μ l. After 24 h of incubation at 37°C, the calculated amount of nitrogen dried cyanobacterial material present in the most diluted extract that produced a visible inhibition was defined as MIC.

Statistical analysis

The data of all the parameters were statistically analyzed and expressed as Mean \pm Standard Deviation.

HPTLC analysis

HPTLC analysis was performed on silica gel F-₂₅₄ (E-Merck grade) pre-coated aluminum plate. Before use, plate was pre-washed with methanol and dried in an oven at 105°C for 1 h. A band of 7 mm was applied at a distance of 10 mm from the bottom of the plate. The methanol extract (10 μ l) was applied on a chromatoplate (CAMAG Linomat-5) and run in the solvent system (chloroform and methanol, 7:3). The plate was developed up to 8 cm in a twin trough chamber previously equilibrated with mobile phase for 20 min. Densitometric evaluation of the plate was performed at λ 254 nm.

RESULTS AND DISCUSSION

The antimicrobial activity of *Lyngbya majuscula* is shown in Table 1. Methanolic extract was found more active as compared to hexane and ethyl acetate. The highest activity in terms of effective zone of inhibition (18 mm) was observed against *B. subtilis* followed by *C. albicans*, *B. cereus*, *S. epidermidis*, *E. coli*, *S. faecalis* in decreasing order. Hexane extracts, ethyl acetate and culture supernatant did not exhibit antimicrobial activity against any of the microbial species examined in the present study.

Furthermore, the active extracts obtained from the above process *i.e.* methanolic and ethyl acetate extracts were then subjected to the determination of minimum inhibitory concentrations by 96-well micro broth dilution method. Active crude extracts were diluted in CAMHB in the range from 2048 μ g/ml to 4 μ g/ml using tetracycline and nystatin as a standard antibiotic. Methanol extract was found significant

inhibitory against *Bacillus subtilis* with MIC 512 μ g ml⁻¹. The same extract was found active against *Bacillus cereus*, *Staphylococcus epidermidis* and *Candida albicans* at 1024 μ g ml⁻¹, 2048 μ g ml⁻¹ and 1024 μ g ml⁻¹, respectively. MIC of this extract against *Escherichia coli* and *S. faecalis* was not observed (*i.e.* >2048 μ g ml⁻¹). Hexane and ethyl acetate extract didn't show inhibition in the entire range of extract's dilutions.

Results of HPTLC analysis were summarized in Table 3. The methanol extract contained a mixture of different component, which were eluted at $R_F = 0.18$, $R_F = 0.20$, $R_F = 0.32$, $R_F = 0.36$, $R_F = 0.55$, $R_F = 0.59$, $R_F = 0.66$, $R_F = 0.70$, $R_F = 0.80$ as shown in Fig. 1.

The above findings indicate the discovery of novel chemicals that can lead to the

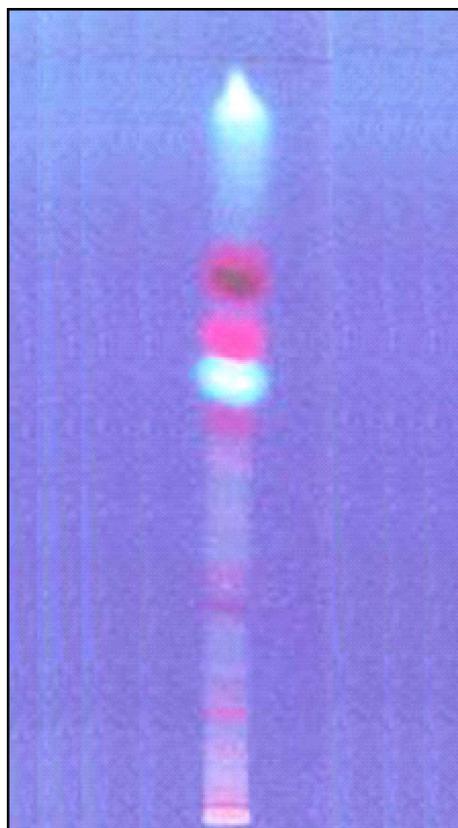


Fig. 1. HPTLC profile of methanol extract of *Lyngbya majuscula*. (HPTLC separation at UV-254 nm)

Table 1. Antimicrobial activity of (organic extracts) and (culture supernatant) of *Lyngbya majuscula*

Type of Extract	Zone of Inhibition* (in mm diameter)						
	<i>Escherichia coli</i>	<i>Bacillus cereus</i>	<i>Bacillus subtilis</i>	<i>Staphylococcus epidermidis</i>	<i>Streptococcus faecalis</i>	<i>Candida albicans</i>	
Organic Extract	Methanol	15±1.8	18±1.0	14±0.80	12±0.90	16±2.0	
	Ethyl Acetate	09±2.1	12±2.1	NZ	NZ	NZ	
	Hexane	NZ	NZ	NZ	NZ	NZ	
Culture supernatant	100 %	NZ	NZ	NZ	NZ	NZ	
Controls	Positive	30.10±0.50	26.20±1.61	28.43±1.89	31.10±1.20	33.±1.2ND	
	Nystatin	ND	ND	ND	ND	25. ±0.40	
	Negative	Methanol	NZ	NZ	NZ	NZ	
		Ethyl Acetate	NZ	NZ	NZ	NZ	
		Hexane	NZ	NZ	NZ	NZ	
		BG-11 Medium	NZ	NZ	NZ	NZ	

*Zone of inhibition (in mm diameter) including the diameter of disc (6 mm). Assay was performed in triplicate and results are the mean of three values ± Standard Deviation.

‘NZ’ -No zone of inhibition, ND- Not detected. Tetracycline and Nystatin (5 µg/ml)

Table 2. Minimum inhibitory concentration of active crude extracts of *Lyngbya majuscula*

Extracts	Type of Active Crude	Test Microorganisms	Concentration of Extracts(in µg/ml)								MIC(in µg/ml)		
			1024	512	256	128	64	32	16	8		4	
Methanol	<i>Escherichia coli</i>	2048	G	G	G	G	G	G	G	G	G	G	>2048
Methanol	<i>Bacillus cereus</i>	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	1024
Methanol	<i>Bacillus subtilis</i>	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	512
Methanol	<i>Staphylococcus epidermidis</i>	NG	G	G	G	G	G	G	G	G	G	G	2048
Methanol	<i>Streptococcus faecalis</i>	G	G	G	G	G	G	G	G	G	G	G	>2048
Ethyl acetate	<i>Bacillus subtilis</i>	G	G	G	G	G	G	G	G	G	G	G	>2048
Methanol	<i>Candida albicans</i>	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	1024
Standard	Tetracycline	<i>Escherichia coli</i>	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG<4
Tetracycline	<i>Bacillus cereus</i>	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	<4
Tetracycline	<i>Bacillus subtilis</i>	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	<4
Tetracycline	<i>Staphylococcus epidermidis</i>	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	<4
Tetracycline	<i>Streptococcus faecalis</i>	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	<4
Nystatin	<i>Candida albicans</i>	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	<4

(NG) represents “No Growth Observed”, (G) represents “Growth Observed”.

Table 3. HPTLC analysis of methanol extract of *Lyngbya majuscula* (Observation under U.V 254 nm)

S. No.	R _f Value	Color of the band
1	0.18	Pink
2	0.20	Purple
3	0.32	Purple
4	0.36	Pink
5	0.55	Pink
6	0.59	Pink
7	0.66	Blue
8	0.70	Pink
9	0.80	Pink

development of pharmaceuticals by cyanobacteria. The current scenario of antibiotics is very threatening with significant emergence of resistance among bacterial pathogens against available antibiotics. The present investigation reveals that cyanobacteria would be the major source in finding such metabolites with greater efficacy.

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