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Antibacterial Potential and UV-HPLC Analysis of Laboratory-Grown Culture of *Anabaena variabilis*

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Abstract : The study was undertaken to investigate the antibacterial activity of different organic extracts prepared from biomass and spent medium of laboratory-grown *Anabaena variabilis* against *Staphylococcus aureus* MTCC-740, *Escherichia coli* MTCC-739, *Pseudomonas aeruginosa* MTCC-741, *Salmonella typhi* MTCC-733 and *Klebsiella pneumoniae* MTCC-139. Antibacterial activity was evaluated by disc diffusion assay and Minimum Inhibitory Concentration of all the active crude extracts was determined by broth micro dilution assay. The methanol extract showed more potent activity than hexane and ethyl acetate extracts. However, dichloromethane extract and spent medium were found to be inactive against all the test organisms. No inhibitory effect was found against *Klebsiella pneumoniae*. Minimum Inhibitory Concentration of all the active crude extracts was determined by broth microdilution assay. The MIC of methanol extract against *Staphylococcus aureus* and *Escherichia coli* are 256 and 512 µg/ml, respectively. While it was more than 512 µg/ml against *Pseudomonas aeruginosa* and *Salmonella typhi*. Methanol extracts was further analysed by UV-Vis spectroscopy and HPLC. The antibacterial activity was compared with standard antibiotic Ciprofloxacin.

Key words: Antibacterial activity, Cyanobacteria, HPLC, Minimum inhibitory concentration, *Anabaena variabilis*.

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Introduction

In recent times, the rapid development of multiresistant bacterial strains of clinically important pathogens fetches the interest of scientist to develop newer broad spectrum antimicrobial agents. The less availability and high cost of new generation antibiotics necessitates looking for the substances from alternative medicines with claimed antimicrobial activity. Cyanobacteria (blue-green algae) are a group of extraordinary diverse Gram-negative prokaryotes that originated 3.5 billion years ago. The medicinal and nutrient qualities of cyanobacteria were first appreciated as early as 1500 BC, when *Nostoc* species were used to treat gout, fistula and several forms of cancer (Liu and Chen, 2003). Their diversity ranges from unicellular to multicellular, coccoid to branched filaments. They exist in almost all conceivable habitats. Most species of cyanobacteria are free-living, freshwater, marine or terrestrial: planktonic, or benthic; and comprise major components of microbial mats. Some cyanobacterial species are thermophilic and growing in hot springs. A few cyanobacteria are symbionts of liverworts, water ferns and cycads; while a number of them are found as the phototrophic component of lichens.

Various strains of cyanobacteria are known to produce intracellular and extracellular metabolites with diverse biological activities such as antibacterial (Falch *et al*., 1995; Mundt *et al*., 2001; Rao *et al*., 2007; Kaushik and Chauhan, 2008), antifungal (MacMillan *et al*., 2002), cytotoxic (Luesch *et al*., 2000), algaecide (Papke *et al*., 1997), immunosuppressive (Koehn et al., 1992) and antiviral activities (Hayashi & Hayashi, 1996). The aim of study reported here was to investigate the antibacterial activity of various crude extracts of *Anabaena variabilis*. The cyanobacterium belongs to family Nostocaceae. The study was further extended to find the minimum inhibitory concentrations of active crude extracts. These extracts were further subjected for UV (ultra-violet spectroscopy) and HPLC (high performance liquid chromatography) analysis.

Materials and Methods

Organism and Growth Conditions. Starter culture of *Anabaena variabilis* 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, 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); $MgSO_4$7H2O (0.75 gL^{-1}), $NaNO_3$ (1.5 gL^{-1}), $CaCl_2.2H_2O$ (0.036 gL^{-1}), $K_2HPO_4.3H_2O$ (0.04 g L^{-1}), Na_2.EDTA (.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}), $ZnSO_4.7H_2O$ (0.22 gL^{-1}), $MnCl_2.4H_2O$ (1.81 gL^{-1}), $CuSO_4.5H_2O$ (0.08 gL^{-1}), $Na_2MoO_4.2H_2O$ (0.39 gL^{-1}) and $CO(NO_3)_2.6H_2O$ (0.049 gL^{-1}.

Preparation of Extracts. At the stationary phase of growth (25 days), the culture was harvested. Spent media and biomass were separated by filtration. Biomass was dried in a hot-air oven at 60℃ for 1 h. Air-dried biomass was further subjected to extraction by mixing well in the organic solvent. The mixture was left for few hours at room temperature and then sonicated for 10 min. The mixture was centrifuged at 4000 rpm for 10 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 then dried sample was resuspended in the appropriate solvent to make the solution of known concentration for the antibacterial assay.

Bacterial Strains. The microorganisms used in antibacterial assay were collected from Institute of Microbial Technology (IMTECH), Chandigarh, India. Four Gram-positive bacteria namely *Staphylococcus aureus* MTCC-740, four Gram-negative bacteria namely *Escherichia coli* MTCC-739, *Pseudomonas aeruginosa* MTCC-741, *Salmonella typhi* MTCC-733 and *Klebsiella pneumoniae* MTCC-139 were screened for present evaluation.

Inoculum Preparation. The bacterial strains were inoculated on Tryptone Soya Agar (TSA) and incubated for 24 h at 30°C then suspended in saline solution 0.85% NaCl and adjusted to yield approximately $1.0x10^7$-$1.0x10^8$ 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.

Determination of Antibacterial Activity. Antibacterial activity of different crude extracts of was evaluated using the agar well diffusion assay (Perez et al., 1990). Adjusted culture (100 µl) was mixed with 100 ml of Muller Hinton Agar (MHA). Media (25 ml) was poured into sterile Petri dish (90 mm diameter). Media was allowed to solidify and then individual plates were marked for the test organisms inoculated. After solidification, plates were punched to make the well of 6 mm diameter with the help of sterile borer. Respective cyanobacterial extracts (100 µl) were pipetted into the well in assay plates (Kaushik and Goyal, 2008). Plates were incubated at 37°C for overnight. The plates were observed for the zone of inhibition and diameter of these zones was measured. All the tests were performed under sterile conditions and repeated three times. The solvent control revealed no activity.

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 Muller-Hinton Broth (CAMHB from Hi-Media) ranging from 1 to 512 µg/ml. Ciprofloxacin was used as standard antibiotic 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 µl. After 24 h of incubation at 37℃, the calculated amount of nitrogen dried cyanobacterial material present in the most diluted extract that produced a visible inhibition was defined as MIC.

UV-Vis spectroscopic analysis. The absorption spectrum of methanol extract of *Anabaena variabilis* was recorded in the UV-Vis spectrophotometer (Shimadzu; Model No. UV-1700 Pharmaspec) capable of producing monochromatic light in the range of 200-800 nm for measuring the absorbance.

HPLC analysis. High performance liquid chromatography of methanol extract of *Anabaena variabilis* was performed on Shimadzu HPLC (Model No; 10 AVP) equipped with constant temperature column compartment, a sample injector capable of injecting 20 µl aliquots and a programmable variable photodiode array detector and an integrator. The C-18, 4.6 x 250 mm stainless steel column (waters) was used at room temperature. Extracts was eluted with mobile phase water: methanol (80:20) at the wavelength of 260 nm.

Results

Antibacterial activity of different extracts of laboratory-grown cyanobacterium, *Anabaena variabilis* against various test bacteria has been represented in Table 1. Methanol extract of this cyanobacterium was found active against all the tested microorganisms except *Klebsiella pneumoniae*, a Gram-negative bacterium. The highest effective zone of inhibition was observed against *Staphylococcus aureus* (8.56 mm) followed by 7.59 mm against *Escherichia coli*, 4.36 mm against *Pseudomonas aeruginosa* and only 3.38 mm against *Salmonella typhi*. Hexane extract showed little activity against *Staphylococcus aureus* with only 3.32 mm size of effective zone of inhibition. Ethyl acetate extract was found active against *Staphylococcus aureus* and *Escherichia coli* with the effective zone of inhibition 5.37 mm and 4.42 mm, respectively. Dichloromethane extract and spent medium were found inactive against all the test bacteria (Fig. 1).

MIC values of *Anabaena variabilis* have been shown in Table 2. The results indicated that the MICs of methanol extract were 256 µg/ml against *Staphylococcus aureus* and 512 µg/ml against *Escherichia coli* whereas it was more than 512 µg/ml against *Pseudomonas aeruginosa* and *Salmonella typhi*. MIC of ethyl acetate extract was 512 µg/ml against *Staphylococcus aureus and more than* against *Escherichia coli*. MIC of hexane extract was found >512 µg/ml against *Staphylococcus aureus* (Fig. 2).

Discussion

Various workers had studied the antimicrobial properties of Cyanobacteria. In different studies, various crude extracts from *Fischerella sp*. (Asthana *et al*., 2006), *Oscillatoria anguistisima* and *Calothrix parietina* (Issa, 1999), *Anabaena, Oscillatoria, Pseudoanabaena, Synechocystis* (Kreitlow *et al*., 1999), *Nostoc* (Bloor & England, 1989), *Phormedium* (Fish & Codd, 1994) ,*Fischerella ambigua* (Ghasemi *et al*., 2004), *Spirulina platensis* (Kaushik and Chauhan, 2008) were evaluated for their antimicrobial effects on pathogenic microorganisms. They have also reported that the extracts prepared in different solvents were effective against both Gram-positive and Gram-negative organisms. This is in agreement with our findings, since the *Anabaena variabilis* extracted in different solvents had similar effects on both types of organisms used in this study. In this work, methanol extract was found to be most active when compared to the other extracts. This extract was further selected for its UV-Vis spectra and HPLC chromatogram as shown in figure 3. Several peaks were observed indicating the presence of active compounds in respective extract. The antibacterial activity of these extracts might be due to the presence of one or more compounds. These extracts can further be subjected to various advanced techniques such as Nuclear Magnetic Resonance (NMR) and Mass Spectroscopy to determine the exact chemical molecule responsible for their bioactivity. This would definitely be turning point for pharmaceutical sciences in determining a novel antibacterial compound.

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Table 1. Antibacterial activity of various extracts of *Anabaena variabilis*

Test Microorganisms	Effective Zone of Inhibition* (in mm diameter)					
	Organic Extracts				Extracellular Substances	Standard Antibiotic[+]
	Hexane	Ethyl Acetate	Dichloro-methane	Methanol	Spent Media	Ciprofloxacin
Staphylococcus aureus	3.32±1.5	5.37±.78	NZ	8.56±1.1	NZ	25.33±.15
Escherichia coli	NZ	4.42±13	NZ	7.59±.63	NZ	23.46±.40
Pseudomonas aeruginosa	NZ	NZ	NZ	4.36±1.4	NZ	21.35±.22
Klebsiella pneumoniae	NZ	NZ	NZ	NZ	NZ	17.35±.22
Salmonella typhi	NZ	NZ	NZ	3.38±1.7	NZ	28.44±.28

Results are the means of diameter values ± standard deviation.
NZ: No Zone of Inhibition,
*Effective zone of inhibition = total zone of inhibition-diameter of well
[+]Ciprofloxacin (5 µg /ml)

Table 2. **Minimum inhibitory concentration(s) of active crude extracts of *Anabaena Variabilis***

	Minimum inhibitory concentration (in µg/ml)			
Test	**Organic Extracts**			**Standard Antibiotic**
Microorganisms	**Hexane**	**Ethyl Acetate**	**Methanol**	**Ciprofloxacin**
Staphylococcus aureus	>512	512	256	<1
Escherichia coli	ND	>512	512	<1
Pseudomonas aeruginosa	ND	ND	>512	<1
Salmonella typhi	ND	ND	>512	<1

ND-Not Detected

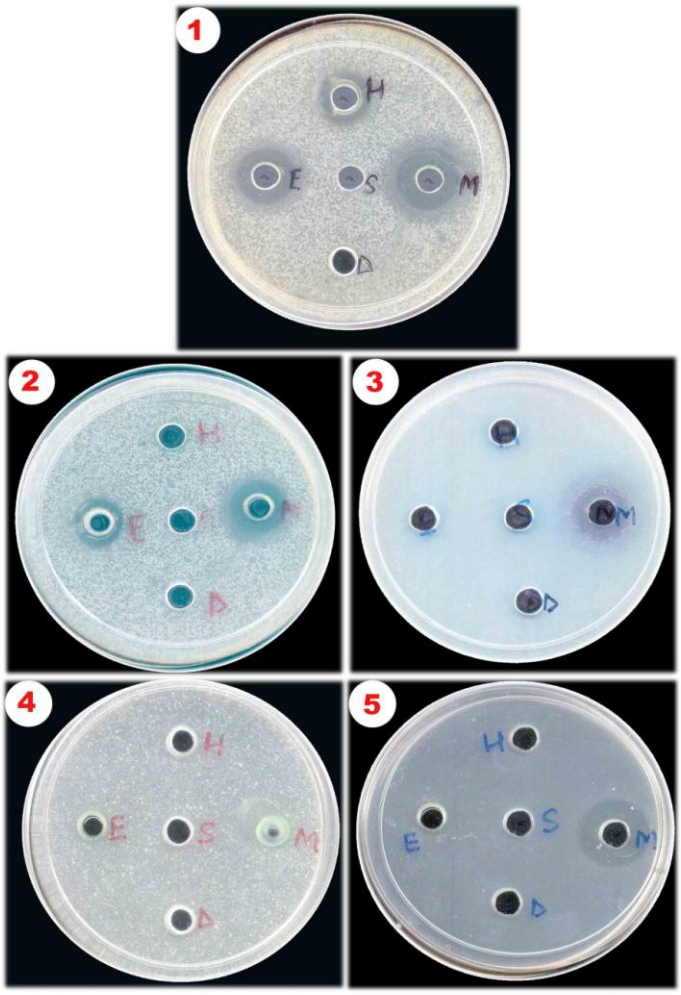


Fig. 1. Zone of inhibition exhibited by different extracts of Anabaena variabilis: 1, S. aureus; 2, E. coli; 3, P. aeruginosa; 4, K. pneumoniae; 5, S. typhi; H, hexane extract; E, ethyl acetate extract; D, dichloromethane extract; M, methanolic extract; S, spent medium.

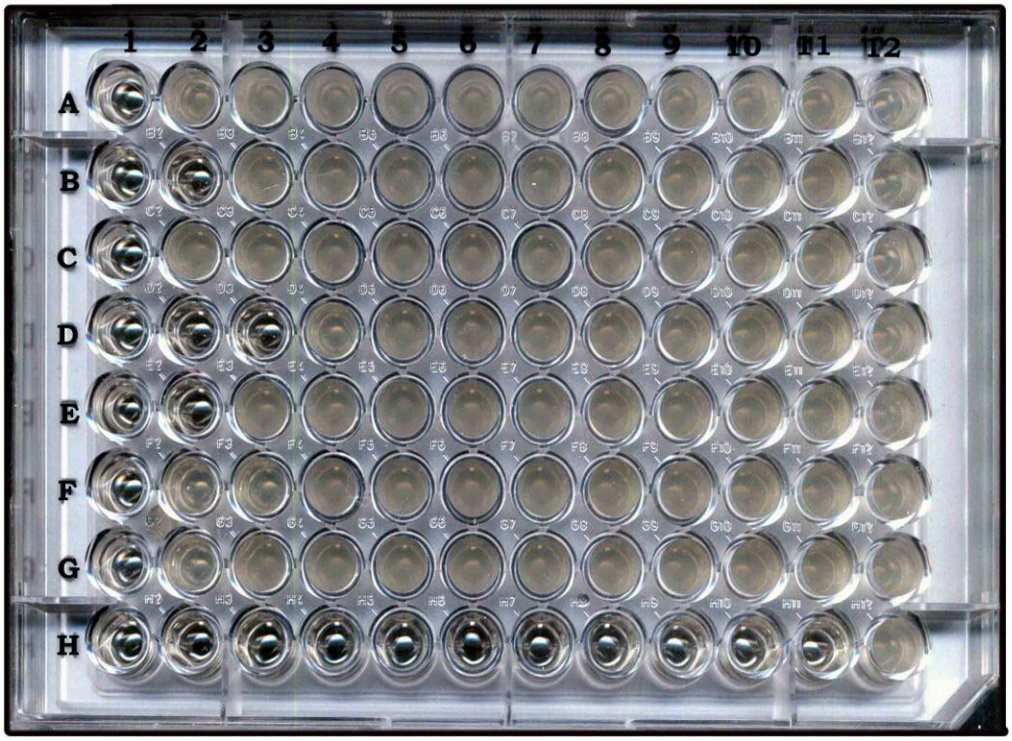
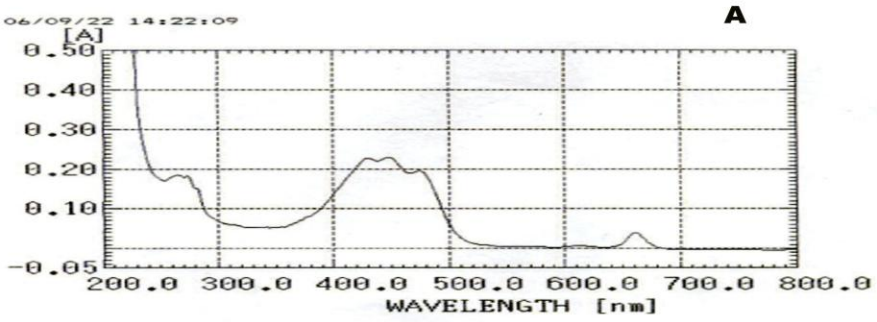


Fig. 2. MIC analysis for *Anabaena variabilis* extracts:
A ninty six well microtiter plate showing the inhibition of growth

Rows:- A-Hexane extracts against *S. aureus.*
B-Ethyl acetate extract against *S. aureus*
C-Ethyl acetate extract against *E. coli*
D-Methanolic extract against *S. aureus*
E-Methanolic extract against *E. coli*
F-Methanolic extract against *P. aeruginosa*
G-Methanolic extract against *S. typhi*
H-Ciprofloxacin against *S. aureus*

Column:- 1-Negative control(MHB)
2-11-Dilution range 512 µg/ml to 1 µg/ml.
2- 512 µg/ml , 3-256 µg/ml, 4-128 µg/ml
5-64 µg/ml, 6-32 µg/ml, 7-16 µg/ml, 8- 8 µg/ml
9-4 µg/ml, 10-2 µg/ml, 11-1 µg/ml.
12-Positive control (MHB+Culture)

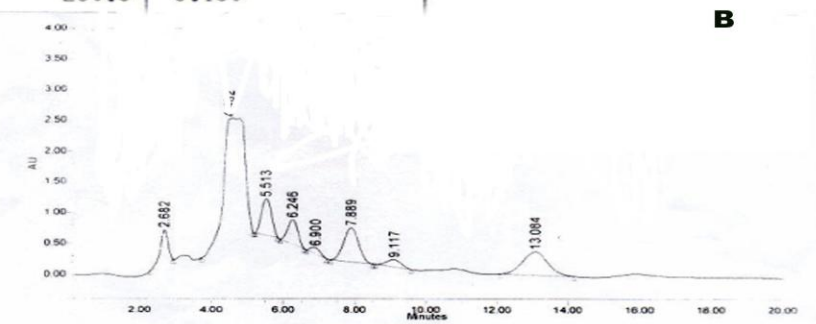
Figure 3. UV-Vis spectrum (A) and HPLC chromatogram (B) of methanolic extract of *Anabaena variabilis*



06/09/22 14:23:20
Peak detection

Abscis.	ABS
661.0	0.040
614.0	0.006
475.0	0.196
448.0	0.231
431.0	0.228
265.0	0.185

Abscis.	ABS



	RT	Area	% Area	Height
1	2.682	8187542	5.52	553820
2	4.482	81128779	54.69	2043562
3	5.513	11419106	7.70	592682
4	6.246	7687594	5.18	393645
5	6.900	1774497	1.20	96309
6	7.889	15967639	10.76	546825
7	9.117	3385382	2.28	116915
8	13.084	18799024	12.67	377578