



A STUDY OF IN VITRO ANTIMICROBIAL ACTIVITY OF CRUDE EXTRACTS ISOLATED FROM CYANOBACTERIA AGAINST SELECTED MICROORGANISMS

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ABSTRACT

In vitro Antimicrobial activity of two non-heterocystous Cyanobacterial strains i.e. *Gloeocapsa punctata* and *Lyngbya majuscula* was studied. Both Cyanobacterial strains were isolated from various sites of paddy fields of Warangal district, Telangana state, India, during, August, 2012. The crude extracts of both strains in three solvents i.e. Acetone, Methanol and Petroleum ether were screened against four pathogenic bacteria and four fungi. Crude extracts of each strain show differential antimicrobial response to test organisms. The petroleum ether extract of *G. punctata* showed the maximum antibacterial activity (16.00±0.57 mm) against *B. cereus* where as the extract in petroleum ether of *L. majuscula* showed highest antibacterial activity (16.33±0.66 mm) against *P. vulgaris*. The methanol extract of *G. punctata* showed the maximum antifungal activity of (15.66±0.33 mm) against *A.fumigatus* where as the acetone extract of *L. majuscula* showed antifungal activity (15.00±0.57 mm) against *A.niger*. The results clearly indicated that *G. punctata* and *L. majuscula* contain promising antimicrobial compounds. Therefore, further studies are necessary to elucidate the components responsible for antimicrobial activities against microorganisms.

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INTRODUCTION

Cyanobacteria are a primitive, diverse and ubiquitous group of photosynthetic prokaryotes, exhibiting resemblance with Gram-negative bacteria in cellular organization and green plants in oxygenic photosynthesis (Stanier and Cohen, 1977). With long evolutionary history, they are among the oldest life forms existing on earth. They colonize, grow and survive in almost all kinds of terrestrial and aquatic (freshwater and marine) ecosystems which can be attributed to their tremendous adaptability to varying environmental conditions as well as effective protective and tolerance mechanisms against various abiotic stresses (Tandeau de Marsac and Houmard, 1993; Potts, 1999; Ehling and Scherer, 1999). The presence of a wide array of photosynthetic pigments, storage products and primary and secondary metabolites indicates their biochemical diversity. Algae are rapidly proving to be an extremely important source of biologically active secondary metabolites which could be used for the biological control of pathogens. Cyanobacteria (blue-green algae) are one of the richest sources of biomedical relevant compounds with extensive therapeutic pharmaceutical applications (Tan, 2007; Gademann and Portmann, 2008; Martins et al., 2008).

Several strains of cyanobacteria are known to produce intracellular and extracellular metabolites with diverse biological activities such as antibacterial (Mundt et al., 2003; Rao et al., 2007; Kaushik and Chauhan, 2008), antifungal (MacMillan et al., 2002), cytotoxic (Luesch et al., 2000). There are many reports on the inhibition of human pathogens by algal extracts, only few studies reported effects against fish pathogens (Liao et al., 2003; Bansemir et al., 2006; Abdel and Ibraheem, 2008; Vijayakumar et al., 2011; Sulekha Yadav et al., 2016; Mervat et al., 2015; Ramesh Babu et al., 2017).

The aim of the present study was to study the *in vitro* antimicrobial activity of crude extracts of various Cyanobacteria against selected pathogenic microorganisms.

MATERIALS AND METHODS

Sample collection and culture conditions

The Cyanobacterial containing samples collected from various sites of paddy fields of Warangal district, Telangana state, India, during, August, 2012. The collected Cyanobacterial samples were stored in plastic bags and transported to the laboratory under water conditions. The samples were initially washed thoroughly with water to remove adhering substance. The collected sample was cultured initially in BG-11 medium (without nitrate), (Rippka et al., 1979) under white light illumination for a few weeks, and the filaments were isolated after several streaking on BG-11 (without nitrate) agar plates.

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Purified Cyanobacteria cultures were transferred to 100 ml inorganic BG-11 medium (without nitrate). The inoculated conical flasks were incubated for 28 days at 26 ± 2 °C with 4000 lux light intensity and a light dark cycle of 16/8 h. The identification of Cyanobacterial species were made based on the morphological observations using standard monographs and protocols (Desikachary, 1959; Santra, 1993). The photomicrographs were taken using with a fluorescent microscope. The obtained species were identified as *Gloeocapsa punctata* and *Lyngbya majuscula* shown in (Figure-1). At the late exponential growth phase, Cyanobacterial species were harvested by centrifugation at 5000 rpm for 10 min and the pellets were subjected to extraction by different organic solvents of increasing polarity.

Preparation of Extracts

Cyanobacterial biomass was harvested in the stationary growth phase by centrifugation at 5000 rpm for 15 minutes to obtain the biomass. The obtained biomass was dried in the hot-air oven at 60 °C for 24 hrs, resulted biomass powdered was used for the extraction by different organic solvents. The one gram (1.0 grm) of dried powdered of each strain were extracted in 15 ml of organic solvents, i.e., Acetone, Methanol and Petroleum ether left overnight it gets to saturation and filtered the extract. The filtrate crude of each strain was again evaporated to dryness at 40 °C and resultant crude extract 1 mg was weighed and dissolved in 1 ml of same organic solvent as stock solution and it was preserved at 4°C (Perez *et al.*, 1990) until it uses for bioassay. All the crude extracts were used for the antimicrobial screening assays.

Microorganisms: Microorganisms were used for the present study, i.e., bacterial cultures of *Bacillus cereus* (MTCC-430), *Staphylococcus epidermidis* (MTCC-9041), *Proteus vulgaris* (MTCC-1771) and *Escherichia coli* (MTCC-1302) and fungal strains of *Aspergillus fumigatus* (MTCC-4163), *Aspergillusniger* (MTCC-4325), *Trichophyton rubrum* (MTCC-3272) and *Macrophomina sp.* (MTCC-10576) were obtained from Department of Microbiology, Kakatiya University, Warangal, Telangana State, India. The bacterial strains were inoculated onto nutrient broth and incubated at 37 °C for 24 hours. The fungal strains were inoculated onto glucose peptone broth and incubated at 28 °C for 5 days.

Antimicrobial Assay

A disc diffusion assay was used to determine qualitatively the ability of the compounds to inhibit growth of bacteria and fungi. Antimicrobial activity of Cyanobacterial extracts were tested by agar disc diffusion method (Perez *et al.*, 1990). The sterilized 20 ml of Muller Hinton Agar (MHA) and Sabouraud Dextrose Agar (SDA) medium were poured into sterile Petri dishes were allowed to cool and solidify. After solidified MHA & SDA plates, 100 µl of bacterial and fungal suspension were spread with 'L' shaped spreader on MHA and SDA plates with a lawn of cultures. Sterile filter paper discs (6 mm) impregnated with 50 µl of the crude extracts were dried and placed on Muller Hinton Agar (Bacteria) and Sabouraud Dextrose Agar (Fungi) plates. similarly disc (6mm) impregnated with 50 µl of respective organic solvent (Acetone, Methanol and Petroleum ether) were dried and placed on the surface of the agar containing media were used as negative control and Gentamycin 30 µg/disc for bacteria, Nystatin 50 µg/disc for fungi were used as positive standard control. The

plates were incubated for 24 hours at 37°C for bacteria and 48-72 hrs at 28 °C for fungi. At the end of the incubation, the diameter of the inhibition zone formed around the disc was measured. Discs containing organic solvents were used as negative control and all tests were done in triplicate and their mean and standard errors were calculated.

Statistical analysis: The results of the data obtained were statistically analyzed and the values are mean ± standard error (SE) of the three measurements (N=3) by using Graph Pad Prism version 5.03 (Graph Pad Software, Inc.).

RESULTS

The antimicrobial activities of Cyanobacterial strains in three organic solvents were assayed against four bacterial and four fungal strains by evaluating the inhibition zones.

Antibacterial study: The antibacterial activity of *G. punctata* against four strains of pathogenic bacteria (*B. cereus*, *S.s epidermidis*, *P. vulgaris* and *E.coli*) with zone of inhibitions was shown in (Table-1 & Plate-1: A).

Table 1 Antibacterial activity of *Gloeocapsa punctata*

Test Organisms	Zone of Inhibition (ZOI) (diameter in mm)						Standard Control Gentamycin (30 µg/disc)
	Organic extracts (1mg/ml)						
	Acetone		Methanol		Petroleum ether		
<i>Bacillus cereus</i> (MTCC-430)	--	0.00	--	0.00	16.00 ± 0.57	0.00	21.00 ± 0.57
<i>Staphylococcus epidermidis</i> (MTCC-9041)	13.00 ± 0.57	0.00	14.33 ± 0.33	0.00	--	0.00	25.66 ± 0.88
<i>Proteus vulgaris</i> (MTCC-1771)	14.33 ± 1.20	0.00	8.66 ± 0.88	0.00	10.00 ± 0.57	0.00	25.00 ± 0.57
<i>Escherichia coli</i> (MTCC-1302)	14.66 ± 0.33	0.00	13.66 ± 0.88	0.00	15.00 ± 0.57	0.00	29.33 ± 0.33

“ --” No inhibition zone
Diameter of the inhibition zone including disc diameter (6 mm).
Values were with mean ± SE of three separate experiments (n=3).

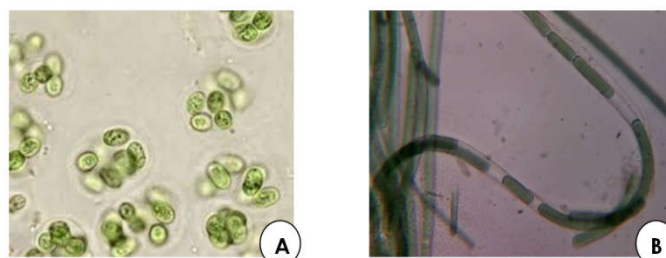


Figure-1 Microphotographs of (A) *Gloeocapsa punctata* (B) *Lyngbya majuscula*

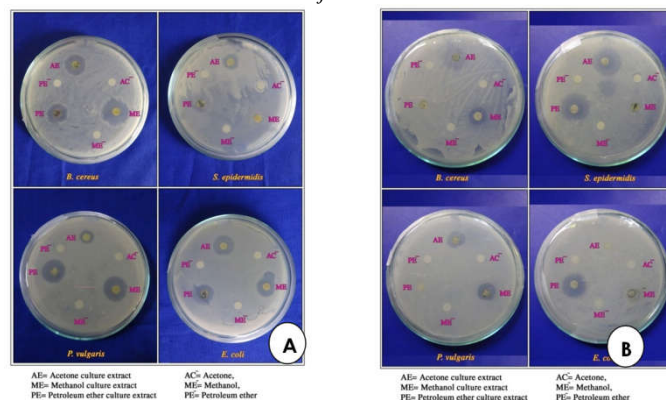


Plate 1 Antibacterial activity of (A) *Gloeocapsa punctata* (B) *Lyngbya majuscula* crude extracts against pathogenic bacteria

The Petroleum ether culture extract exhibited highest zone of inhibition (16.00±0.57 mm) against *B. cereus* followed by 15.00±0.57 mm against *E.coli* and 10.00±0.57 mm against *P. vulgaris*. However, no antibacterial activity was found against *S. epidermidis*. The Acetone culture crude extracts was found to be more active against *E. coli*, *P. vulgaris* and *S. epidermidis* with zone of inhibition 14.66±0.33 mm, 14.33±1.20 mm and 13.00±0.57 mm, respectively. The Methanol culture crude extract was showed a bioassay activity (14.33±0.33 mm, 13.66±0.88 mm and 8.66±0.88 mm) against *S. epidermidis*, *E. coli* and *P. vulgaris*. The culture extracts of Acetone and Methanol solvents did not exhibit any inhibition zone against *B. cereus*. The Methanol culture crude extract exhibited with minimum inhibition zone (8.66±0.88 mm) against *P. vulgaris*. In the present investigation the antibacterial activity of Petroleum ether culture crude extract was found to be higher than the other solvent culture crude extracts under the observation.

The Petroleum ether culture crude extract of *L. majuscula* expressed with the widest inhibition zone 16.33±0.66 mm against *P. vulgaris* and then followed by *E. coli*, *B. cereus* and *S. epidermidis* with inhibition zones (13.66±0.88 mm, 12.66±0.66 mm and 9.66±0.88 mm), respectively (Table-2 & Plate-2: B). The Methanol culture crude extract exhibited antibacterial activity against *B. cereus* and *P. vulgaris* with the inhibition zone (15.66±0.88 mm) each and followed by the 12.33±0.88 mm against *E.coli* and 8.33±0.33 mm against *S. epidermidis*, respectively. The Acetone culture crude extract inhibited the growth of *B. cereus*, *E. coli*, *S. epidermidis* and *P. vulgaris* with zone of inhibitions (14.00±0.57 mm, 14.00±0.57 mm, 11.00±0.88 mm and 8.66±0.33 mm), respectively. On the other hand it was observed that Methanol extract has shown with minimum inhibition zone (8.33±0.33 mm) against *S. epidermidis*. The discs saturated with Acetone, Methanol and Petroleum ether solvents were used as a negative control and in all cases, no antibacterial activity (no inhibition zone) was found in chosen Cyanobacterial strains under study. In the comparison with standard Gentamycin (30 µg/disc) (Plate-3: A), all the culture extracts under the observations were exhibited with low zone of inhibition.

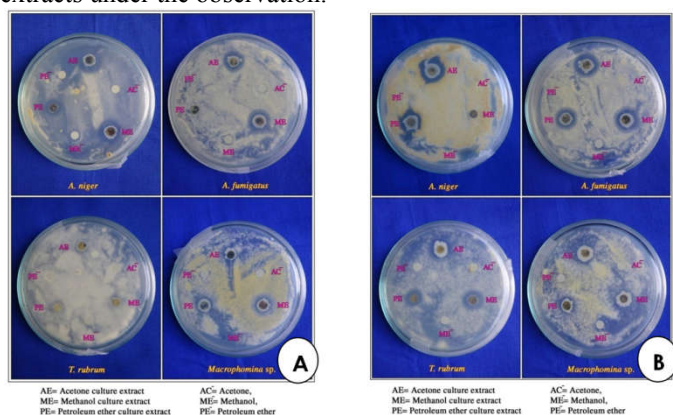


Plate 2 Antifungal activity of (A) *Gloeocapsa punctata* (B) *Lyngbya majuscula* crude extracts against pathogenic fungi

Antifungal study: The present study was carried to investigate the presence of antifungal property in *G. punctata* culture crude extracts against four fungal strains (Table-3 & Plate-2: A). The methanol extracts of *G. punctata* culture extract show with maximum widest inhibition zone (15.66±0.33 mm) against *Aspergillus fumigatus*, followed by the 14.00±0.57 mm and 13.00±0.57 mm against *Macrophomina sp.* and *A.niger*, respectively.

Table 2 Antibacterial activity of *Lyngbya majuscula*

Test Organisms	Zone of Inhibition (ZOI) (diameter in mm)						
	Organic extracts (1mg/ml)						Standard Control Gentamycin (30 µg/disc)
	Acetone		Methanol		Petroleum ether		
	Extract	Control	Extract	Control	Extract	Control	
<i>Bacillus cereus</i> (MTCC-430)	14.00 ± 0.57	0.00	15.66 ± 0.66	0.00	12.66 ± 0.33	0.00	21.00 ± 0.57
<i>Staphylococcus epidermidis</i> (MTCC-9041)	11.00 ± 0.57	0.00	8.33 ± 0.33	0.00	9.66 ± 0.88	0.00	25.66 ± 0.88
<i>Proteus vulgaris</i> (MTCC-1771)	8.66 ± 0.33	0.00	15.66 ± 0.88	0.00	16.33 ± 0.66	0.00	25.00 ± 0.57
<i>Escherichia coli</i> (MTCC-1302)	14.00 ± 0.57	0.00	12.33 ± 0.88	0.00	13.66 ± 0.88	0.00	29.33 ± 0.33

Diameter of the inhibition zone including disc diameter (6 mm). Values were with mean ± SE of three separate experiments (n=3).

Table 3 Antifungal activity of *Gloeocapsa punctata*

Test Organisms	Zone of Inhibition (ZOI) (diameter in mm)						
	Organic extracts (1mg/ml)						Standard Control Nystatin (50 µg/disc)
	Acetone		Methanol		Petroleum ether		
	Extract	Control	Extract	Control	Extract	Control	
<i>Aspergillus niger</i> (MTCC-4325)	14.66 ± 0.33	0.00	13.00 ± 0.57	0.00	9.66 ± 0.33	0.00	23.00 ± 0.57
<i>Aspergillus fumigatus</i> (MTCC-4163)	14.33 ± 0.33	0.00	15.66 ± 0.33	0.00	--	0.00	23.33 ± 0.88
<i>Trichophyton rubrum</i> (MTCC-3272)	8.66 ± 0.33	0.00	--	0.00	10.00 ± 0.57	0.00	21.66 ± 0.88
<i>Macrophomina sp.</i> (MTCC-10576)	10.33 ± 0.33	0.00	14.00 ± 0.57	0.00	12.33 ± 0.88	0.00	20.66 ± 0.33

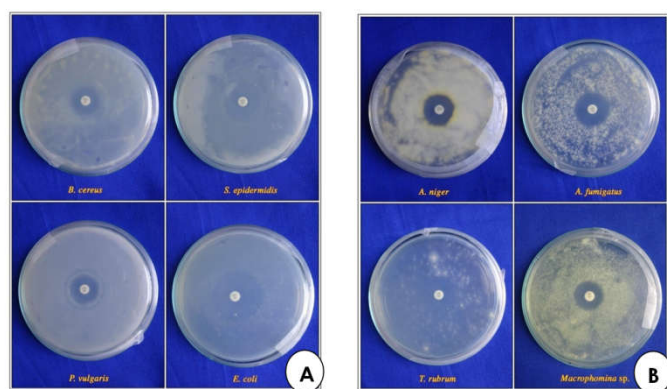


Plate 3 Standard Control (A) Gentamycin (30 µg/disc) (B) Nystatin (50 µg/disc)

The minimum inhibition zone (9.33±0.66 mm) was found in the Methanol extract of *Macrospora* sp. and solvent Methanol culture extract have not shown any bioassay against *A. niger* fungal pathogen. The discs saturated with Acetone, Methanol and Petroleum ether solvents were used as a negative control and in all cases, no antifungal activity (no inhibition zone) was found in chosen cyanobacterial strains under study. In the comparison with the standard Nystatin (50 µg/disc) (Plate-3: B), all the culture extracts under the observations were exhibited with low zone of inhibitions.

DISCUSSION

A few studies have been made to screen cyanobacteria from paddy- fields for the production of antimicrobial substances.

Table 4 Antifungal activity of *Lyngbya majuscula*

Test Organisms	Zone of Inhibition (ZOI) (diameter in mm)						Standard Control Nystatin (50 µg/disc)
	Organic extracts (1mg/ml)						
	Acetone		Methanol		Petroleum ether		
	Extract	Control	Extract	Control	Extract	Control	
<i>Aspergillus niger</i> (MTCC-4325)	15.00 ± 0.57	0.00	--	0.00	13.66 ± 1.45	0.00	23.00 ± 0.57
<i>Aspergillus fumigatus</i> (MTCC-4163)	14.33 ± 0.88	0.00	14.66 ± 0.66	0.00	12.66 ± 0.88	0.00	23.33 ± 0.88
<i>Trichophyton rubrum</i> (MTCC-3272)	12.33 ± 0.88	0.00	13.00 ± 0.57	0.00	10.66 ± 0.33	0.00	21.66 ± 0.88
<i>Macrospora</i> sp. (MTCC-10576)	10.33 ± 0.33	0.00	9.33 ± 0.66	0.00	11.00 ± 0.57	0.00	20.66 ± 0.33

"--" No inhibition zone

Diameter of the inhibition zone including disc diameter (6 mm).

Values were with mean ± SE of three separate experiments (n=3).

The Acetone culture extracts have inhibited the four fungal pathogens *A. niger*, *A. fumigatus*, *Macrospora* sp. and *T. rubrum* with inhibition zones (14.66±0.33 mm, 14.33±0.33 mm, 10.33±0.33 mm and 8.66±0.33 mm), respectively. The Petroleum ether culture extracts were expressed with varying inhibition zones like 12.33±0.88 mm, 10.00±0.57 mm and 9.66±0.33 mm against *Macrospora* sp., *T. rubrum* and *A. niger*. Regarding the effect of different inhibition zones of Acetone culture extract expressed minimum inhibition zone (8.66±0.33 mm) against *T. rubrum*. However, there was no inhibition activity in the Methanol and Petroleum ether culture crude extracts against *T. rubrum* and *A. fumigatus* in the present investigation.

The culture extracts of *L. majuscula* species have shown significant inhibitory bioassay on the fungal pathogens (Table-4 & Plate-2: B). The fungal species, *A.niger* was inhibited by the Acetone culture crude extract with highest zone of inhibition of 15.00±0.57 mm, followed by 14.33±0.88 mm, 12.33±0.88 mm and 10.33±0.33 mm against *A. fumigatus*, *T. rubrum* and *Macrospora* sp. respectively. The methanol culture extract showed considerable inhibition effect with three fungal pathogens under study, in which it showed 14.66±0.66 mm, 13.00±0.57 mm and 9.33±0.66 mm against *A. fumigatus*, *T. rubrum* and *Macrospora* sp. In the Petroleum ether culture extract, it was exhibited with antifungal activity against four fungal species 13.66±1.45 mm against *A. niger*, 12.66±0.88 mm against *A. fumigatus*, 11.00±0.57 mm against *Macrospora* sp. and 10.66±0.33 mm against *T. rubrum*, respectively.

Possibly the synthesis of highly active toxin is a defense option of cyanobacteria in these environments against other organisms like bacteria, fungi and viruses (Mundt *et al.*, 2001). In one study reported that the culture media of cyanobacteria belonging to Nostocaceae, Microchaetaceae and Scytonemataceae isolated from paddy-fields of Argentinean were found to active against *S. aureus* and *C. albicans* (De Caire *et al.*, 1993). In another study, cyanobacteria from the paddy-fields of Northern Thailand induced bioactive substances with antibiotic activity against *B. subtilis* reported by (Chetsumon *et al.*, 1993) but no antifungal activity. Certain cyanobacteria have drawn much attention as prospective and rich sources of biologically active constituents and have been identified as one of the most promising groups of organisms capable of producing bioactive compounds (Fish and Codd, 1994 and Schlegel *et al.*, 1999).

These results are in agreement with those earlier reports, The extracts from *O. princeps* were active against *B. subtilis*, *S. aureus*, *E. coli* and *B. bronchiseptica* (Gupta and Shrivastava, 1965). Partially purified compounds from marine cyanobacterium *O. willei* enhanced immunoreactive cells of Swiss albino male mice (Thirunalasundari *et al.*, 2003). The methanol extracts of cyanobacteria was recorded maximum antimicrobial activity against *P. vulgaris*, *B. cereus*, *E. coli*, *P. aeruginosa*, *A. niger* and *A. flavus* (Prashantkumar *et al.*, 2006). The ethanol extracts of cyanobacteria mat gave maximum inhibition zone against *S. aureus*, *S. pyrogenes* and *P. aeruginosa* (Abed *et al.*, 2011).

CONCLUSION

In our study the cyanobacteria from paddy fields of Warangal District, Telangana State made for assessment of antimicrobial activity by isolates two genera of cyanobacteria showed significant *in vitro* antimicrobial activity in different organic solvents used. Among the two species studied for antibacterial and antifungal activity, it proved that *L. majuscula* found to produce affective bioactive compounds against *P.vulgaris* in the solvent of Petroleum ether and *G. punctata* has been reported as highly effective against *A.fumigatus* in the solvent of methanol. Therefore, the results of this work indicates that this group of organisms displays a potential that warrants further investigation with regards to their isolation and purification of the biologically active compounds.

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