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Research Article

Studies on Biologically Active Compounds Produced by Certain Members of Non Heterocystous Cyanobacteria

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ABSTRACT

Cyanobacteria, also called blue-green algae, are O2-evolving photosynthetic prokaryotes, many (though not all) of which can fix N2. N2-fixing (diazotrophic) cyanobacteria can be broadly grouped as heterocystous and non-heterocystous forms. All heterocystous forms are capable of aerobic N2 fixation and, in the absence of combined nitrogen, 5–10% of their vegetative cells differentiate into specialized cells called heterocysts that provide an environment suitable for the functioning of nitrogenase (the enzyme that catalyses N2 fixation). Unlike vegetative cells, heterocysts are photo synthetically inactive. They do not fix CO2, nor do they produce O2. Cyanobacteria generally remain potential source for investigations as prospective and excellent sources of biologically active constituents produced during primary and especially secondary metabolism. The soil samples containing NHC were collected from different paddy fields in and around Warangal district, Telangana State. About 10 samples were collected from different locations and utilized for enrichment studies in BG–11 medium without nitrogen supplementation. Strains were transferred in liquid nutrient BG 11 medium all the collected Non Heterocystous cyano bacteria strains were maintained at 7.2 pH, 28+20C and with a 14/10-hour light/dark cycle photoperiod. The morphology of Oscillatoria curviceps, Oscillatoria princeps and Oscillatoria acuta was influenced similarly by physicochemical properties of soil. Although Oscillatoria corntiana and Oscillatoria quadrapanculata belong to the same order and family, the latter was influenced differently due to the physicochemical properties of the soil. All extracts exhibited different degrees of antimicrobial activity unrelatedly to the tested pathogen.

1. Introduction

Cyanobacteria, also called blue-green algae, are O2-evolving photosynthetic prokaryotes, many (though not all) of which can fix N2. N2-fixing (diazotrophic) cyanobacteria can be broadly grouped as heterocystous and non-heterocystous forms. All heterocystous forms are capable of aerobic N2 fixation and, in the absence of combined nitrogen, 5–10% of their vegetative cells differentiate into specialized cells called heterocysts (heterocytes) that provide an environment suitable for the functioning of nitrogenase (the enzyme that catalyses N2 fixation). Unlike vegetative cells, heterocysts are photosynthetically inactive. They do not fix CO2, nor do they produce O2. The internal environment of heterocysts is therefore virtually anoxic, which is ideal for nitrogenase, a notoriously O2-sensitive enzyme. In contrast, non-heterocystous cyanobacteria do not show cellular differentiation and until 1960 it was believed that such cyanobacteria were incapable of N2 fixation.Tropical soils are considered to be relatively rich in cyanobacteria (Mague 1977).

This is due to the ability of these strains to resist large climatic variations: such as relative humidity in the daily range of 30-100% and light intensity. Although the procedure of isolation and the relative biomass may make the heterocystous strains more obvious, the presence of non-heterocystous strains is also of real importance, particularly in biotopes newly colonized. Primary metabolites are allow molecular weight compounds that are necessary for growth, these are produced by microorganisms during active growth, which include amino acids. Nucleotides, coenzymes, organic acids and vitamins where as secondary metabolites are not necessary for microbial growth. They have been shown to produce a variety of antibacterial, antifungal, antilarval. antiprotozoan, antialgal, antihelmintic and cytotoxic secondary metabolite. Currently

available drugs are effective against only one-third of the diseases as a result of increased antibiotic resistance in pathogens. Thus, identification of new biologically active compounds is urgently required for development of new drugs. To fulfill the demand for new therapeutic drugs and to decrease the average costs involved in development, scientist should consider screening organisms from over looked microbial sources, such as proteobacteria, bacterioidetes and cyanobacteria. New antibiotics with high activity and without side effects for human and for environment are therefore urgently needed. Some Non Heterocystous Cyanobacteria species could be a prolific resource for substances with antibacterial activity.

The objective of the study is to isolate Non Heterocystous Cyanobacteria species from soil samples collected from various paddy fields in Warangal, Telangana, determine the physicochemical properties of the soil samples, purify the flavonoids from the Non Heterocystous Cyanobacteria crude extracts, and assess their antimicrobial activity

2. Materials and Methods

2.1 **Collection of Soil Samples**

The soil samples containing Non Heterocystous Cyanobacteria were collected from different paddy fields in and around Warangal district, Telangana State. About 10 samples were collected from different locations and utilized for enrichment studies in BG–11 medium without nitrogen supplementation.

2.2 Isolation and maintenance of cultures

The isolated Non Heterocystous Cyanobacteria strains were maintained under specific culture conditions. Strains were transferred in liquid nutrient BG 11 medium (Rippka et al., 1979). All the collected Non Heterocystous cyanobacteria strains were maintained at 7.2 pH, 28±20C and with a 14/10-hour light/dark cycle photoperiod.

2.3 Determination of physicochemical properties of soil samples

The physicochemical properties of soil such as pH, temperature, conductivity, total dissolved solids, salinity, and dissolved oxygen were measured.

2.4 Temperature and pH

The temperature of the soil of the paddy fields was measured with the help of a deluxe laboratory thermometer and pH of the soil was measured with the help of a Water and Soil Analysis Kit Model 161(EI Products, Panchkula, Haryana, India). The pH rod supplied with the kit worked on the principle of determination of the negative logarithm of [Hþ] ion concentration. Before measuring [H+] ion concentration of the soil, the electrode contained KCl and was properly calibrated by using pH buffer of 4.0 and 9.2. The soil samples were prepared by dissolving 2.5 g of soil sample in 10 mL double distilled water.

2.5 **Determination of total dissolved solids and salinity**

Bicarbonates, carbonates, magnesium salts, phosphates, chlorides, potassium salts, iron salts, and manganese salts are some of the common salts found in dissolved states in natural water. Total dissolved solids were measured in mg/L, whereas salinity range was measured in g/L.

2.6 Determination of dissolved oxygen

Before measuring dissolved oxygen in the soil, the temperature of the samples was evaluated. According to the temperature, oxygen solubility was determined in ppm after proper calibration of the dissolved oxygen.

2.7 Identification of Non Heterocystous Cyanobacteria species (Morphological analysis)

The morphology of the colonies was studied after two to three weeks of growth at 300C on BG-11 medium. Presence of contaminants in the Non Heterocystous Cyanobacteria cultures was checked by microscopic examination and by plating one drop of the culture on a plate containing BG-11 medium with 0.5% glucose and 0.05% casamino acids.

2.8 Structure and distribution pattern of Non Heterocystous Cyanobacteria strains:

The Non Heterocystous Cyanobacteria species were identified and characterized based on microscopic observations. Different Non Heterocystous Cyanobacteria strains from different sites in Warangal showed various sizes of vegetative cells. On consideration of Non Heterocystous Cyanobacteria strains, 7 species were identified on the basis of average filament length, shape and size of the vegetative cell. All the Non Heterocystous Cyanobacteria strains were found to vary in their cell width, cell length and average length of filament (Table-1). Different Non Heterocystous Cyanobacteria strains from different sites in paddy fields of Warangal showed various sizes of vegetative cells. Minimum filament was observed in *Arthrospira sp* (34.7±3.11 um), which was collected from Narsampet paddy field, whereas the largest filament length was reported in *Oscillatoria quadrapanculata* (57.9±3.67 um).

2.9 Antimicrobial Activity

2.9.1 **Collection of Soil Samples**

The soil samples containing Non Heterocystous Cyanobacteria were collected aseptically from different paddy fields (Parkal, Wardhannapet, Sangem, Khanapur and Narsampet) in and around Warangal district, Telangana State . The collected soil samples were stored in sterile plastic bags at 4°C. Before the isolation of Non Heterocystous Cyanobacteria from the soil samples, the soil samples were placed in sterile Petri plates then dried at room temperature for 7 days.

2.9.2 Isolation of Non Heterocystous Cyanobacteria

Non Heterocystous Cyanobacteria Strains were isolated on BG-11 medium (Allen & Stanier 1968) by the serial dilution method (Reynaud & Laloë 1985); and were maintained in the mineral medium BG-11 by incorporating with cycloheximide (50 µg ml−1) and nystatin (30 µg ml−1) as an antifungal. In addition, to inhibit Gram negative bacteria, nalidixic acid (30 µg ml–1) was implemented in the cultivation medium. The cultures, which were grown at 30°C without shaking, were illuminated with white light at an intensity of 1000 lux. Isolation of Non Heterocystous Cyanobacteria was achieved by performing a standard dilution suspension technique. Briefly, 10 g of soil was added to 90 ml of saline water (NaCl 9 g l− 1).

Table-1 Physicochemical properties of collected microbial soil samples

Area	pH	Temperature (^{0}C)	Salinity (g/L)	Total dissolved solid (mg/L)	Dissolved oxygen (ppm)
Parkal (*Oscillatoria princeps* and *Microcoleus major)*	7.86	31.4	68.3±5.31	129±16.4	62.3±2.32
Narsampet *(Oscillatoria corntiana* and *Arthrospira spirulinoides)*	8.0	32	40±3.01	103±5.49	39.9±1.06
Khanapur *(Oscillatoria quadrapanculata)*	6.8	33	38±1.02	99±3.9	51±2.08
Sangem (*Oscillatoria curviceps)*	8.1	31	39±1.05	104.6.1	67±3.0
Wardhannapet *(Oscillatoria acuta)*	8.2	37	59±3	103.5.1	69±4.84

From the diluted samples, approximately 100 µl was spread on the surface of isolation media and incubated at humid atmosphere for 2–4 weeks at 28±2 °C. After the incubation time, suspected Non Heterocystous Cyanobacteria colonies were identified based on morphological characters and purified on BG-11 medium for routine laboratory studies.

2.9.3 Preparation of Non Heterocystous Cyanobacteria crude extracts

The extractions were performed by soaking the dried Non Heterocystous Cyanobacterial material (0.1: 2 w/v) in (85%) methanol, n-hexane and ethyl acetate solvents. The extract mixture was sonicated at 30% amplitude, interval 5 sec, pulse 5 sec for 15min. The extract was then shaken on a rotary shaker at 120 rpm at 28°C for 48 hrs; and filtered using Whatman No 4 flter paper. The solvent was evaporated under reduced pressure up to dryness and the obtained residue (crude extract) was stored at -20°C in air tight bottles until used .

2.9.4 Preparation of Bacterial Cultures

The Non Hetrocystous Cyanobacteria crude extracts of different solvents were evaluated for its antibacterial activity against two Gram positive bacteria- *Staphylococcus aureus* (ATCC 25923) and *B. cereus* (ATCC 10102) and two Gram negative bacteria- *E. coli* (ATCC 25922) and *P. aeruginosa* (ATCC 27853). The fungal strains (*C. albicans and A. flavus*).

Mueller-Hinton agar was employed in all of the cultures, and by choosing three to five well-isolated colonies from agar plate cultures, the turbidity of all of the bacteria was adjusted to 0.5 McFarland standard. An additional dilution (1000-fold) was carried out to produce an inoculum with 1 × 106 CFU. Before being compared to a control, the cultures were incubated at 37°C for 24 hours to measure the amount of turbidity brought on by microbial growth.

2.9.5 Antimicrobial activity assay

The susceptibility of the tested pathogenic bacteria to various cyanobacterial extracts was assessed disk diffusion method on Muller Hinton agar medium (Oxoid). Similarly, the anti-fungal activities were tested using Sabouraud dextrose agar medium (Oxoid). Each extract material was dissolved in DMSO solution (1 mg /ml). Previously sterilized filter paper discs soaked in the extract solutions were placed aseptically in the Petridishes containing agar media and previously seeded with the tested microorganisms (at a concentration of 106 cfu/ml). The Petridishes were incubated at 37°C and the inhibition zones were recorded after 24 h and 48 h of incubation for bacteria and fungi, respectively.

Each treatment was replicated three times. Ampicillin (100 µg/ml) and fluconazole (100 µg/ml) were used as common standard for antibacterial and antifungal activity while DMSO (1%) was used as a negative control using the same procedure as above.

2.9.6 Determination of Minimum Inhibition Concentration (MIC)

The MIC of the Non Heterocystous Cyanobacteria was calculated using the broth micro dilution method (Ericsson and Sherris, 1971). 25 µL of Mueller-Hinton broth (MHB) and 25 µL of the prepared Non Heterocystous Cyanobacteria methanol extract and standards were added to each well of a 96-well microtiter plate, respectively.

The wells were then filled with precisely 25 µL of each standardized bacterial inoculum (S. aureus, B. cereus, E. coli, and P. aeruginosa), and they were all cultured for 24 hours at 37°C. The MBC was established after the MIC was established by removing 25 µL from each well and subcultured on freshly made nutrient agar plates. The MIC was established as the lowest concentration that resulted in no discernible bacterial growth.

Table-2 Evaluation antibacterial activity of Non Heterocystous Cyanobacteria extracts

NHC species	Solvent	Diameter inhibition zones (mm)					
		Gram positive bacteria		*Gram negative bacteria*		*Fungal strains*	
		S. aureus	*B. cereus*	*E. coli*	*P. aeruginosa*	*C. albicans*	*A. flavus*
Oscillatoria princeps	Hexane	11±0.4	12±0.2	11±0.2	11±0.1	NA	NA
	Ethyl acetate	10±0.1	11±0.1	10.0.1	NA	NA	NA
	Methanol	NA	18±0.1*	NA	13±0.4*	NA	NA
Oscillatoria corntiana	Hexane	NA	10±0.1	10±0.1	NA	10±0.1	NA
	Ethyl acetate	NA	11±0.1	NA	12±0.3	13±0.4	14±0.2
	Methanol	14±0.2*	15±0.2*	11±0.2	13±0.3*	11±0.2	16±0.3*
Oscillatoria quadrapanculata	Hexane	10±0.4	11±0.1	10±0.3	11±0.3	12±0.1	14±0.2*
	Ethyl acetate	11±0.2	13±0.5	12±0.2	NA	14±0.1*	16±0.4*
	Methanol	NA	16±0.2*	NA	NA	16±0.4*	21±0.2*
Oscillatoria curviceps	Hexane	14±0.3	16±0.3*	NA	12±0.1	11±0.1	19±0.1
	Ethyl acetate	12±0.3	14±0.6	NA	11_0.1	NA	17±0.3
	Methanol	19±0.3*	21±0.2*	13±0.4	16±0.2*	14±0.5	25±0.5*
Oscillatoria acuta	Hexane	20±0.4*	20±0.3*	13±0.4	16±0.2	19±0.2*	26±0.6*
	Ethyl acetate	17±0.1*	18±0.4*	NA	15±0.1	20±0.4*	24±0.3*
	Methanol	24±0.2*	26±0.2*	17±0.6	20±0.5*	24±0.7*	30±0.7*
Microcoleus major	Hexane	14±0.2	18±0.1*	12±0.4	11±0.3	12±0.1	13±0.2
	Ethyl acetate	15±0.1	16±0.3	15±0.1	NA	NA	13±0.1
	Methanol	22±0.3*	23±0.1*	11±0.5	16±0.3*	20±0.4*	26±0.2*
Arthrospira spirulinoides	Hexane	16±0.5	15±0.3	16±0.4	16±0.1	NA	11±0.1
	Ethyl acetate	13±0.9	18±0.2	13±0.9	13±0.3	NA	15±0.5
	Methanol	21±0.2*	20±0.4*	18±0.2*	19±0.1*	NA	21±0.3*
Ampicillin		27±0.2	29±0.6	25±0.8	25±0.2	--	--
Fluconazole		--	--	--	--	27±0.5	31±0.4

2.9.7 Statistical Analyses

The active Non Heterocystous Cyanobacteria extracts were analyzed for the presence of secondary metabolites such as tannins, phenolics, flavonoids, saponins, terpenoids and sterols according to the standard phytochemical methods (Edeoga et al. 2005). Total phenolic content was estimated as Gallic acid (GA) equivalent per gram extract dry weight (Taga et al. 1984).

3. Results and Discussions

3.1 Antibacterial Activity

All extracts exhibited different degrees of antimicrobial activity unrelatedly to the tested pathogen. Generally, the methanolic extracts showed the highest antimicrobial activity followed by ethyl acetate and then hexane extracts. Methanolic extracts of *Oscillatoria princeps* showed broad activity with the highest inhibition zone recorded against *B. cereus* (18 mm) while all *Oscillatoria princeps* extracts showed no activity against the two tested fungal species.

Similarly, methanolic extracts of *Oscillatoria corntiana* recorded the highest inhibition zones against *A. flavus* (16 mm) and *B. cereus* (15 mm). Regarding *Oscillatoria quadrapanculata* methanolic extract, significant inhibition zones were recorded only for *B. cereus*, *C. albicans* and *A. flavus* (16, 16, and 21 mm), respectively, with a noticeable antifungal activity. Hexane and ethyl acetate extracts of *Oscillatoria quadrapanculata* showed comparable activities against the tested bacterial pathogens.

The highest inhibition zones were recorded for *A. flavus* followed by *C. albicans* hexane and ethyl acetate extracts of *Oscillatoria quadrapanculata*, respectively. The majority of *Oscillatoria curviceps* extracts exhibited considerable antifungal and antibacterial effects. Among which *Oscillatoria curviceps* methanolic extracts recorded the highest inhibition zones against *A. flavus* (25 mm), *B. cereus* (21 mm) and *S. aureus* (19 mm).

The highest inhibition zones were recorded by the methanolic extracts of *Oscillatoria acuta* as 30, 26 and 24 mm against *A. flavus*, *B. cereus*, *S. aureus* and *C. albicans*, respectively. Similarly, ethyl acetate and hexane extracts displayed signifcant antifungal inhibition zones of 24 and 26 mm, respectively against *A. flavus* strain.

For *Microcoleus major*, the methanolic extract presented promising antimicrobial inhibition zone diameters of 26, 23, 22, and 20 mm against *A. flavus, B. cereus, S. aureus, and C. albicans* pathogens, respectively (Table-3). These results indicated that the antimicrobial activity of the extracts depended mainly on the type of NHC species, the used solvent and the tested pathogen.

3.2 Evaluation of MIC of Non Heterocystous Cyanobacteria extracts.

The MIC showed fluctuated values with methanolic extracts of different tested Non Heterocystous Cyanobacteria species. However, the values compared well as potent

Table-3. The Minimum Inhibitory Concentration (MIC) of the NHC methanol extracts against bacterial and fungal cultures

NHC species	MIC (µg/mL)					
	Gram positive bacteria		*Gram negative bacteria*		*Fungal strains*	
	S. aureus	*B. cereus*	*E. coli*	*P. aeruginosa*	*C. albicans*	*A. flavus*
Oscillatoria curviceps	3.5±2.4	17.7±1.5	24.0±1.5	9.27±0.5	6.15±1.3	3.03±0.3
Oscillatoria acuta	3.12±0.3	2.24±0.1	4.58±0.8	3.03±0.3	1.07±0,1	0.68±0.2
Microcoleus major	9.27±0.5	11.5±1.5	11.4±1.5	6.15±1.3	4.59±0.7	1.46±0.2
Ampicillin	0.68±0.2	1.07±0.2	1.46±0.3	2.24±0.1	--	--
Fluconazole	--	--	--	--	1.07±0.2	0.48±0.1

antifungal agents against *C. albicans* (4.59; 1.07 µg/ml) and *A. flavus* (1.46 µg/ml; 0.68 µg/ml) for *Microcoleus major*, and *Oscillatoria acuta* methanolic extracts, respectively.

Lower MIC values were also recorded for *Oscillatoria acuta extract* against *E. coli* (4.58 µg/ml), *P. aeruginosa* (3.03 µg/ml), and *B. cereus* (2.24 µg/ml). These values were non-significant to those recorded for Ampicillin (except for S. aureus) and Fluconazole standard antibiotics implying feasible antibacterial and antifungal activities for this extract (Table-4).

4. Conclusion

The study compared the impact of physicochemical properties of soil on Oscillatoria corntiana and Oscillatoria quadrapanculata, which belong to the same order and family. It was observed that the latter was influenced differently by the soil properties, while Arthrospira sp. showed the optimum impact. The extracts from all species exhibited varying degrees of antimicrobial activity against tested pathogens, with methanolic extracts showing the highest activity, followed by ethyl acetate and then hexane extracts. Oscillatoria princeps' methanolic extract displayed broad activity, with the highest inhibition zone against B. cereus. The minimum inhibition concentration (MIC µg/ml) of methanol extract for three species of Non Heterocystous Cyanobacteria showed the highest inhibition zone, and the MIC values fluctuated with methanolic extracts of different tested species. The study also highlighted the potential of flavonoids as effective antimicrobial agents and suggested the need to purify the phenolic compounds present in Oscillatoria acuta methanolic extract to further test its antimicrobial activity.

Conflicting Interests

The authors have declared that no conflicting interests exist.

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