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ANTIMICROBIAL POTENTIALS OF TWO FRESHWATER HETEROCYSTOUS CYANOBACTERIA *WESTIELLOPSIS PROLIFICA* AND *NOSTOCHOPSIS LOBATUS* COLLECTED FROM SIMILIPAL BIOSPHERE RESERVE, ODISHA, INDIA

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ABSTRACT

The Similipal Biosphere Reserve, an unique ecosystem in the eastern part of India, occupy the northern boundary of Odisha state, lies between 21°28′ to 22°08′ North latitude and 86°04′ to 86°37′ East longitude, covering a vast area of 5578 sq. km in the district of Mayurbhanj. *Westiellopsis prolifica* and *Nostochopsis lobatus,* are a group of heterocystous, true branched, filamentous cyanobacteria belonging to the order Stigonematales, rarely found in the aquatic bodies of this region in the state of Odisha. In the present investigation, an attempt has been made to evaluate the antimicrobial potentials of *Westiellopsis prolifica* and *Nostochopsis lobatus* collected from flowing streams of Similipal Biosphere Reserve. The organisms were cultured in nitrogen free BG-11 medium and raised to axenic state. Crude metabolites were extracted from cultured test organisms in late log phase using organic solvents of different polarity viz. methanol and chloroform. The metabolites were tested against some clinically significant microorganisms including bacteria and fungi using agar cup diffusion method and the results of antimicrobial activity of cyanobacteria are summarized. The findings revealed that pattern of inhibition varied with respect to the cyanobacterial strains used, nature of solvent extracts and the pathogenic microorganisms tested. *Westiellopsis prolifica* was found to be more potent that inhibit all the pathogenic bacteria and fungi both in methanol and chloroform extract. This study illustrates that heterocystous cyanobacteria from freshwater bodies could be a potent source of antimicrobial agents and further characterization of active metabolites and evaluation of their pharmacological potentials are also needed.

Keywords: Cyanobacteria, Antimicrobial Potentials, Similipal Biosphere Reserve.

1. INTRODUCTION

Cyanobacteria are extraordinarily diverse group of Gram-negative, oxygenic photosynthetic prokaryotes that originated 3.5 billion years ago and distributed in all possible biotopes of the world. Due to their occurrence in diverse habitats, these organisms are excellent materials for investigation by Ecologists, Physiologists, Biochemists, Microbiologists and Biotechnologists. The production of bioactive secondary metabolites by cyanobacteria has an interesting scientific and commercial potential [1]. From a biotechnological point of view, cyanobacteria constitute important group of prokaryotes that needs through investigation for bioactive metabolites particularly those that are less studied and unexplored. Cyanobacteria possess a number of unique biological characteristic and they are considered to be one of the potential organisms which can be useful to mankind in various ways, worldwide attention is drawn towards cyanobacteria for their possible use in production of various bioactive secondary metabolites, vitamins, toxins and other therapeutic substances. The bioactive molecules isolated show a broad spectrum of biological activities including toxins, antibiotics, fungicides and algaecides [2]. The importance of cyanobacteria as a potential drug resource is evident by the launching of the 'Cyanomyces' project in Europe, anticipated to generate novel therapeutic substances by combining genes from actinomyces and cyanobacteria [3]. Several cyanobacterial species like *Anabaena, Nostoc, Lyngbya* and *Phormidium* having activities such as anti-HIV, anticancer and antimicrobial have been reported and *Spirulina* is the important cyanobacteria rich in proteins [4]. With the availability of powerful biotechnological tools and techniques

cyanobacterial biotechnology reached newer heights in terms of process and products developments. In the recent years cyanobacteria have been recognized worldwide as a repository of novel bioactive metabolites with potent cytotoxic, antibacterial, antifungal, anti-HIV and anti-cancer properties [5, 6]. In India many workers have been worked on systematic survey and quantitative enumeration of freshwater cyanobacteria, but very few workers have been done their investigations for screening of antimicrobial potentialities of freshwater cyanobacteria [3, 7-11]. Most of the important antimicrobial bioactive compounds have been isolated from marine cyanobacteria [12].

Literature reveals that cyanobacteria and other microalgal diversity from Similipal Biosphere Reserve were studied by several workers [13-17]. Antimicrobial evaluation of some freshwater cyanobacteria from rice fields of Mayurbhanj, Orissa; antibacterial and antioxidant potentials of a corticolous cyanobacterium *Hassalia byssoidea* from Similipal Biosphere Reserve were studied [8, 18]. But no work has been done on cyanobacteria to screening the antimicrobial bioactivity potentials from freshwater habitats of Similipal Biosphere Reserve in the district of Mayurbhanj, Odisha.

As no detailed information is available on bioactivity of secondary metabolites from freshwater heterocystous cyanobacteria of Similipal Biosphere Reserve in the district of Mayurbhanj, Odisha, a preliminary investigation has been made to study the bioactivity of cyanobacteria with special reference to antimicrobial screening of cell extracts of two true branched filamentous heterocystous cyanobactria *Westiellopsis prolifica* and *Nostochopsis lobatus* isolated from freshwater habitats of Similipal Biosphere Reserve, Odisha. Considering the present health problem of drug resistant microbes and emerging of new diseases, research priority should be directed to screen microorganisms from diverse habitats for production of novel bioactive molecule to solve health problem. In such cases cyanobacteria occurring in freshwater bodies are no exception.

2. MATERIAL AND METHODS

2.1. Sample Collection, Isolation and Identification

Cyanobacterial samples were collected from free floating freshwater bodies of Similipal Biosphere Reserve in the district of Mayurbhanj, Odisha (Fig.1). Samplings were done with the help of fine forceps, planktonic net, sampling bottles and clean polythene bags. Temporary slides were prepared for identification and observed under trinocular research microscope with attachment of digital camera and computer with software (Olympus). The samples were identified based on their morphological features like colour of thallus, cell shape and cell size (Fig. 2a & 2b) following the standard monographs and literatures [19, 20].

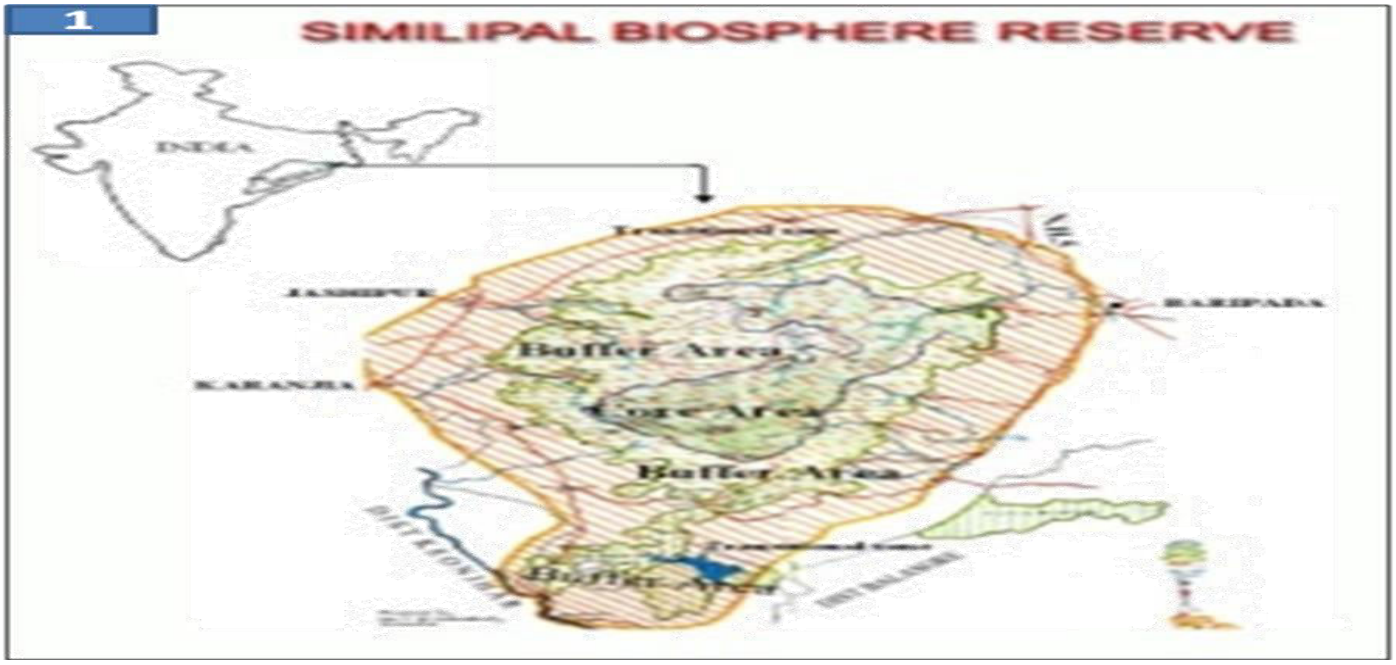

Fig. 1: Map of Study Area (Similipal Biosphere Reserve)

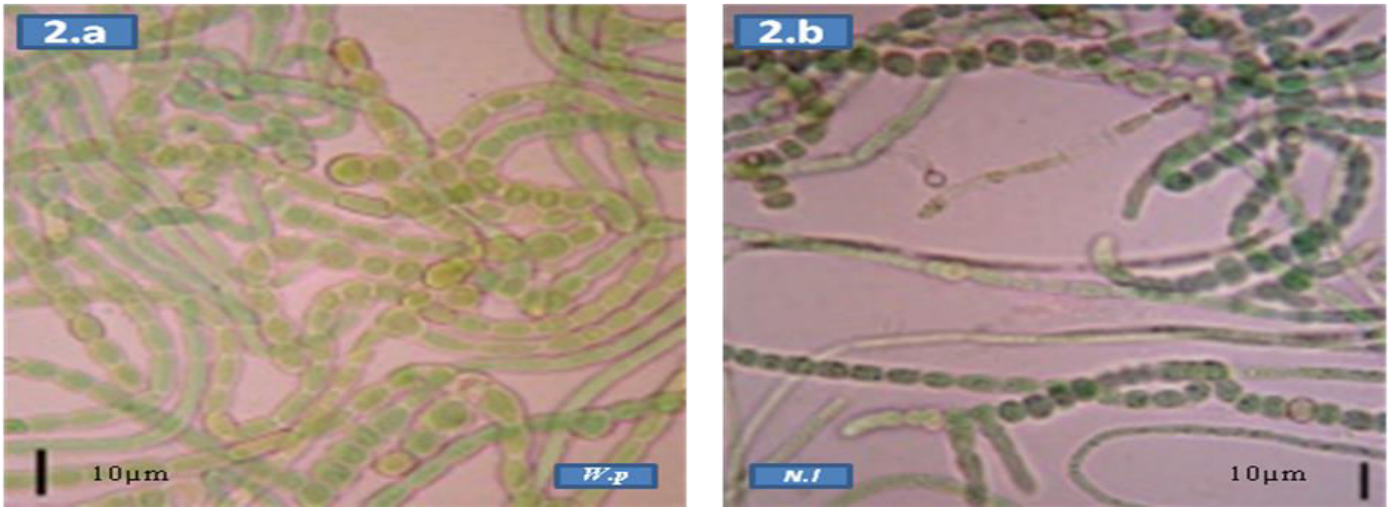

Fig.2a & 2b: Microphotograph of *Westiellopsis prolifica* (*W.p*) & *Nostochopsis lobatus* (*N.l*)

2.2. Purification and Maintenance of Samples

Pure culture was obtained by serial dilution and agar plate methods [21]. The samples were maintained by culturing in freshly prepared BG-11±N medium both in solid and liquid culture [22].

2.3. Mass Cultivation and Extraction of Metabolites

Pure culture of cyanobacteria was cultivated in freshly prepared BG-11±N liquid culture medium [22] and incubated at temperature 28±2°C with illumination of 30-35 µ mol photons m^{-2} s^{-1} white continuous light (12 h:12 h Light and dark) for the period of 120-150 days in large culture vessel for mass cultivation and the biomass were harvested at end in the stationary growth phase. Cyanobacterial mats (*Westiellopsis prolifica* and *Nostochopsis lobatus*) were washed thoroughly with sterile distilled water in order to remove the culture nutrients and suspended particles. Extractions were done by following the standard soxhlet extractor methods [23]. Dry powdered biomass of each sample was dissolved in 100 ml of organic solvents viz. methanol and chloroform for 72 hours. The solvents were finally evaporated to yield the crude bioactive metabolites and dissolved in DMSO (Dimethyl sulphoxide) and put into air-tight sampling bottles and store at low temperature for antimicrobial bioassay.

2.4. Antimicrobial Screening

The bioactive metabolites obtained from cyanobacterial species were screened for their antimicrobial bioactivity against some clinically significant microorganisms using agar cup diffusion methods [24]. The test organisms include three Gram-positive bacteria namely *Bacillus subtilis* (BS), *Staphylococcus aureus* (SA) & *Staphylococcus epidermidis* (SE) and four Gram-negative bacteria *Escherichia coli* (EC), *Shigella dysenteriae* (SD), *Pseudomonas aeruginosa* (PA) and *Salmonella typhi* (ST) and three pathogenic fungi namely *Candida albicans* (CA), *Candida tropicalis* (CT) and *Candida parapilopsis* (CP). For antimicrobial bioassay, the bacterial strains were culture in nutrient broth and fungal pathogens were cultured in potato dextrose broth. Nutrient agar plates were then inoculated with the overnight culture suspension of each test bacterial strains and potato dextrose agar plates were then inoculated with the overnight culture suspension of each test fungal pathogens. The plates with inoculated organisms were then evenly spread out with sterile glass spreader or cotton swabs. Agar cups were prepared by scooping out the media with a sterile cork borer (6mm in diameter) and filled with 50µl of the cyanobacterial extract that already dissolved in DMSO. The Plates were incubated 24 hours at 36±1°C for bacterial strains and 72 hours at 25±2°C for fungal strains, and then the zone of inhibition were recorded and compared with the control (i.e. a cup filled with DMSO solution at the centre of agar plate).

2.5. Determination of Minimum Inhibitory Concentration (MIC)

Minimum inhibitory concentration was determined for those extract which showed considerable zone of inhibition during the antimicrobial bioassay. Broth micro-dilution method using 96 wells micro-titer plates and tetrazolium salt, 2, 3, 5, Triphenyl tetrazolium chloride (TTC) as indicator to microbial growth was

used for determination of minimum inhibitory concentration [25]. 100 µl overnight culture of the test organisms were seeded into the wells serially and at the same time two fold dilution of the crude extracts to be tested were loaded into each wells. Wells containing DMSO (Dimethyl sulphoxide) and antibiotic streptomycin sulphate (100 µg ml^{-1}) were used as negative and positive control respectively. Then 5 µl of 0.5% Triphenyl tetrazolium chloride (TTC) was loaded into each well. The microplate was sealed and incubated at 36±1°C for overnight. Each assay was repeated thrice. The results were read by observing the growth of microorganisms in the wells. MIC was determined at the least concentration of extracts that inhibited the growth of the test pathogens.

2.6. Statistical Analysis

The results obtained were subjected to statistical analysis as mean and standard deviation [26]. The mean values and standard deviations were calculated from the data obtained from three different experiments. Statistical difference at $p < 0.05$ was considered to be significant.

3. RESULTS AND DISCUSSION

3.1. Identification of Organisms

The collected cyanobacterial samples were identified based on their morphological features like colour of thallus, cell shape and cell size following the standard monographs and literatures [19, 20]. The identified organisms are a group of heterocystous, true branched, filamentous cyanobacteria i.e. *Westiellopsis prolifica* and *Nostochopsis lobatus* (Fig. 2a & Fig. 2b) belonging to the order Stigonematales.

3.2. Cyanobacterial Crude Metabolites

Cyanobacterial crude metabolites were extracted from both *Westiellopsis prolifica* and *Nostochopsis lobatus* i.e. 1.030 gm and 1.222 gm of dry biomass respectively from 150 days old 500ml BG-11±N broth culture using two solvents i.e. methanol and chloroform. The crude extracts of each sample were dissolved in 2 ml of DMSO (Dimethyl sulphoxide) for antimicrobial bioassay against some clinically significant microorganisms.

3.3. Evaluation of Antimicrobial Activity

The antimicrobial activities of cyanobacterial crude extracts were evaluated against some clinically significant microorganisms that include three Gram-positive bacteria, four Gram-negative bacteria and three pathogenic fungi by agar cup diffusion method. The crude metabolites were extracted using two solvents i.e. methanol and chloroform, and the results of antimicrobial screening of cyanobacteria *Westiellopsis prolifica* and *Nostochopsis lobatus* are summarized in table 1 and 2. *Westiellopsis prolifica* was found to be more potent and showed significant activity against all the pathogenic bacteria and fungi both in methanol and chloroform extract in comparison to *Nostochopsis lobatus*. The methanolic crude extracts of *Westiellopsis prolifica* showed highest zone of inhibition against bacterial pathogens i.e. *Shigella dysenteriae* (24.0±1.0 mm) followed by *Escherichia coli* (23.5±0.75 mm), *Salmonella typhi* (23.0±1.0 mm), *Pseudomonas aeruginosa* (22.0 ±1.0 mm) and *Staphylococcus aureus* (20.0±1.0 mm) respectively and the moderate activity showed against *Bacillus subtilis* and *Staphylococcus epidermidis* each with 13.0±1.0 mm. Similarly, in case of choloform crude extracts highest zone showed against *Escherichia coli* (25.0±1.0 mm) followed by *Salmonella typhi* (24.0±1.0 mm), *Pseudomonas aeruginosa* (23.0±1.0 mm), *Shigella dysenteriae* (21.5±0.75 mm), *Staphylococcus aureus* (19.0±1.0 mm) and the moderate activity showed against *Staphylococcus epidermidis* (15.5±0.75 mm) and *Bacillus subtilis* (14.0±1.0 mm) respectively. Both the methanol and chloroform crude extracts against fungal pathogens i.e. *Candida albicans* showed highest zone of inhibition about 19.0±1.0 mm and 17.5±0.75 mm followed by *Candida tropicalis* (18.0± 1.0 mm & 15.0±1.0 mm) and moderate against *Candida parapilopsis* with 14.5±0.75 mm & 13.5±0.75 mm respectively. *Nostochopsis lobatus* chloroform crude extracts showed highest zone of inhibition against *Bacillus subtilis* (19.0±1.0 mm) followed by *Pseudomonas aeruginosa* (18.0±1.0 mm) and moderate zone of inhibition almost both the solvent extracts against all the rest of bacterial pathogens tested that ranges from 11.0±1.0 mm to 17.0±1.0 mm diameter and less activity against all the fungal pathogens except *Candida albicans* (12.0±1.0 mm) chloroform extract (Table 1 and 2; Fig.3 & Fig.4). In agar cup bioassay the zone of inhibitions were recorded and compared with the control (i.e. a cup filled with DMSO solution at the centre of agar plate) that represented 0.6 mm diameter against all the test pathogens both bacteria and fungi. It was also observed that the crude extracts of all the test organisms showed higher antimicrobial activity against Gram negative bacteria followed by Gram positive bacteria and then pathogenic fungi.

Table 1: Antimicrobial activity of methanol extracts against some clinically significant microorganisms (Zone of inhibition in mm)

Cyanobacteria	BS	SA	SE	EC	SD	PA	ST	CA	CT	CP
Westiellopsis prolifica	13.0± 1.0	20.0± 1.0	13.0± 1.0	23.5± 0.75	24.0± 1.0	22.0± 1.0	23.0± 1.0	19.0± 1.0	18.0± 1.0	14.5± 0.75
Nostochopsis lobatus	14.0± 1.0	10.0± 1.0	08.0± 1.0	11.0± 1.0	17.0± 1.0	16.0± 1.0	10.5± 0.75	09.0± 1.0	08.5± 0.75	09.5± 0.75

All the values are mean ± S.D.; n = 3, BS- B. subtilis, SA- S. aureus, SE- S. epidermidis, EC- E.coli, SD- S. dysenteriae, PA- P. aeruginosa, ST- S. typhi CA- C. albicans, CT- C. tropicalis, CP- C. parapilopsis

Table 2: Antimicrobial activity of chloroform extracts against some clinically significant microorganisms (Zone of inhibition in mm).

Cyanobacteria	BS	SA	SE	EC	SD	PA	ST	CA	CT	CP
Westiellopsis prolifica	14.0± 1.0	19.0± 1.0	15.5± 0.75	25.0± 1.0	21.5± 0.75	23.0± 1.0	24.0± 1.0	17.5± 0.75	15.0± 1.0	13.5± 0.75
Nostochopsis lobatus	19.0± 1.0	09.5± 0.75	08.0± 1.0	12.0± 1.0	13.5± 0.75	18.0± 1.0	11.0± 1.0	12.0± 1.0	08.0± 1.0	10.0± 1.0

All the values are mean ± S.D.; n = 3, BS- B. subtilis, SA- S. aureus, SE- S. epidermidis, EC- E.coli, SD- S. dysenteriae, PA- P. aeruginosa, ST- S. typhi, CA- C. albicans, CT- C. tropicalis, CP- C. parapilopsis

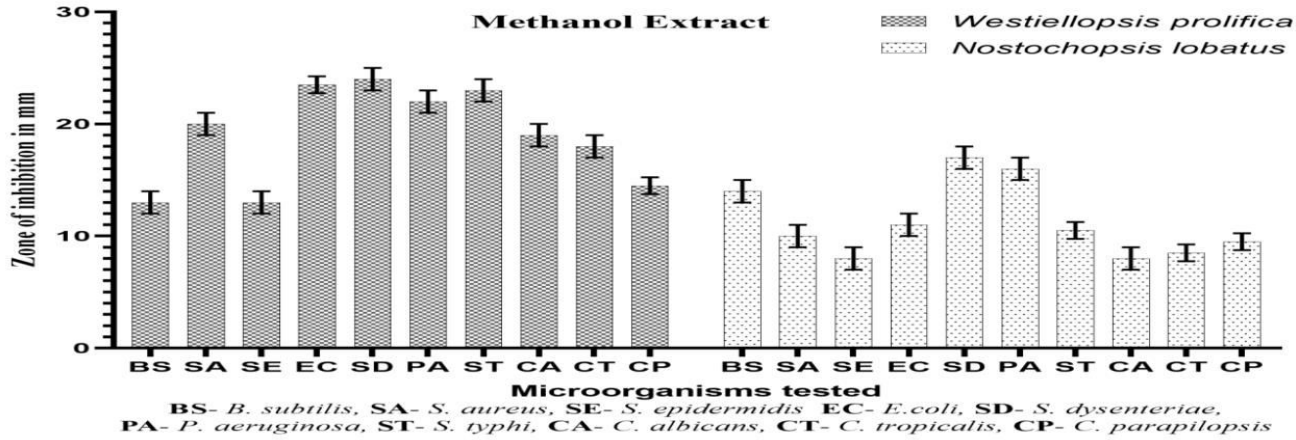

Fig. 3: Antimicrobial activity of methanol extracts against some clinically significant microorganisms (Zone of inhibition in mm).

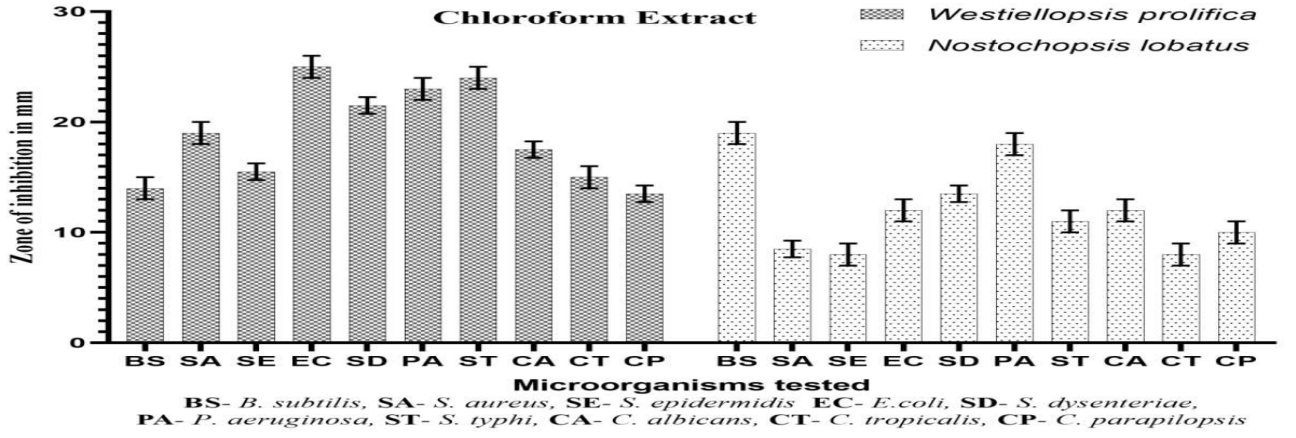

Fig. 4: Antimicrobial activity of chloroform extracts against some clinically significant microorganisms (Zone of inhibition in mm)

3.4. Evaluation of Minimum Inhibitory Concentration (MIC)

Minimum inhibitory concentration (MIC) was carried out both the cyanobacterial species i.e. *Westiellopsis prolifica* and *Nostochopsis lobatus* against the test pathogens i.e. Gram positive, Gram negative bacteria and pathogenic fungi during agar cup diffusion bioassay. The MIC ranges from 1000 µg ml^{-1} to 125 µg ml^{-1} in both cyanobacterial crude extracts (both methanol and chloroform) (Table 3).

Table 3: Minimum inhibition concentration (MIC) of cyanobacterial crude extracts against pathogenic microorganisms

Microorganisms Tested	MIC Value (µg/ml)			
	Cyanobacterial methanol extracts		Cyanobacterial chloroform extracts	
	Westiellopsis prolifica	*Nostochopsis lobatus*	*Westiellopsis prolifica*	*Nostochopsis lobatus*
B. subtilis	500	500	500	125
S. aureus	125	1000	125	1000
S. epidermidis	500	ND	500	ND
E. coli	125	1000	125	1000
S. dysenteriae	125	250	125	500
P. aeruginosa	125	500	125	250
S. typhi	125	1000	125	1000
C. albicans	250	1000	250	1000
C. tropicalis	250	ND	500	ND
C. parapilopsis	500	1000	500	1000

ND - Not determined

Cyanobacteria are recognized as a rich but not yet extensively examined source of pharmacological as well as structurally interesting secondary metabolites [5]. Most of the bioactive metabolites identified so far have been derived from marine cyanobacteria. However, freshwater cyanobacteria remain less explored for bioactive metabolites. Secondary metabolites excreted by cyanobacteria were active against Gram positive and Gram negative bacteria [27]. Similar results were also obtained in our present study where crude extracts of both the cyanobacterial strains not only showed activity against both Gram positive and Gram negative bacteria but also against pathogenic fungi. Such finding suggests that cyanobacteria produce diverse secondary metabolites with antimicrobial activity. Antimicrobial activity of acetone and hexane extracts of freshwater cyanobacteria *Westiellopsis* sp. isolated from the River Diyala in Nhrawan Baghdad were examined [28]. This is very much similar with our present finding. In the present study two organic solvents (methanol and chloroform) were used for extraction of metabolites. In general all the crude solvent extracts showed antimicrobial activity. In most cases organic solvent like methanol has been commonly used for extraction of metabolites and it has proved to be efficient in inhibiting both bacterial and fungal pathogens [29]. Similar results were obtained in our present finding where both methanolic as well as chloroform extracts displayed both antibacterial and antifungal activity, but very few workers have used solvent like chloroform for extraction of cyanobacterial metabolites. Further, production of secondary metabolites might vary among different cyanobacterial strains based on different habitats and ecological niches. Such variations have been noticed from freshwater cyanobacteria collected from different parts of the world. Similarly, in our study both the cyaanobacterial strains *Westiellopsis prolifica* and *Nostochopsis lobatus* were collected from same ecological habitats but their bioactivity is different as our results showed. In nature all organisms need to compete in order to survive in their habitat. This task is achieved by developing secondary metabolites, enzymes and toxins that inhibit other organisms. Our finding revealed that pattern of inhibition varied with respect to the cyanobacterial strains used, nature of solvent extracts and the pathogenic microorganisms tested. In such cases the present findings illustrates that heterocystous filamentous cyanobacteria from freshwater bodies could be a potent source of antimicrobial agents and further characterization of active metabolites and evaluation of their pharmacological potentials are also needed.

4. CONCLUSION

Considering the present health problem of drug resistant microbes and emerging of new diseases, research priority should be directed to screen microorganisms from diverse habitats for production of novel bioactive molecule to solve the growing health problems. Since cyanobacteria have been identified as repository of bioactive metabolites, research investigations on less explored heterocystous filamentous cyanobacteria occurring in freshwater bodies could be very promising in acquiring novel metabolites.

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Conflict of interests

The authors declare that they have no any conflict of interests.

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