ORIGINAL ARTICLE

# Antimicrobial Activity of *Nostoc calcicola* (Cyanobacteria) Isolated from Central India Against Human Pathogens

**Sulekha Yadav[1,2], Monica Agrawal[1,2], Neelima Raipuria[2], Manish Kumar Agrawal[1]**

*[1]Excellent Bio Research Solutions Pvt. Ltd., Napier Town, Jabalpur, Madhya Pradesh, India, [2]Department of Botany and Microbiology, Government M.H. College of Home Science and Science for Women, Napier Town, Jabalpur, Madhya Pradesh, India*

## Abstract

**Introduction:** We have earlier shown that cyanobacteria isolated from Central India possess wide spectrum antibacterial activity. The aim of the study was to add more authenticity by preparing axenic culture of one of the cyanobacteria and to identify the cyanobacterium; *Nostoc calcicola* using 16s rDNA with detailed antibacterial and antifungal activities in a dose-dependent manner. **Materials and Methods:** A local isolate of *Nostoc* was grown in the laboratory and identified as *N. calcicola* using molecular techniques. The cells were extracted with absolute ethanol. Antimicrobial tests were performed by standard methods against five human pathogenic bacteria and four fungi. The data are presented as a mean ± standard deviation for inhibition zones. For statistical analysis, unpaired *t*-test was used using Sigma Graph Pad Prism® version 6.0. **Results and Discussion:** The results concluded that ethanolic extract of *N. calcicola* inhibited all the tested bacteria and fungi. The antimicrobial potency on dry weight basis (5 µg equivalent dry weight in most cases) was better than the 100 µg pure ampicillin and 30 µg miconazole. The activity was pronounced against *Staphylococcus aureus* ATCC 25923 and *Candida albicans* ATCC 10231. The active compound was stable for 6 months at 2-8°C. **Conclusion:** Cyanobacterial antimicrobial compounds being structurally different may have different mechanisms of action and hence can be used as a source of antibiotics and antifungal compounds for various diseases.

**Key words:** Antibacterial activity, antifungal activity, bioactive compounds, cyanobacteria, 16S rDNA

## INTRODUCTION

Cyanobacteria, the oldest photoautotrophic vegetation in the world, have gained much attention as a rich source of bioactive secondary metabolites.[1] The bioactive molecules isolated from diverse taxa of cyanobacteria show a broad spectrum of biological activities including enzyme inhibitors,[2] toxins,[3] and antimicrobials, i.e., antibiotics and antifungal.[4] Cyanobacteria have a capability to grow in diverse habitats, and adaptation of these ecological niches has triggered the production of some of the novel compounds through certain mechanisms. As a result, screening programs of cyanobacteria from different ecological niches have come out with the discovery of novel secondary metabolites with good bioactivity.[5]

*Nostoc* genera are widely distributed worldwide including India. Several species of *Nostoc* are reported as important components of subpolar soil or water biotopes and nitrogen fixers. Few species grow endophytically in fungi (*Geosiphon*), mosses, liverworts, ferns, and vascular plants.[6] *Nostoc* sp. has been reported for the presence of various secondary metabolites including antimicrobial compounds, i.e., tenuecyclamide a-d from *Nostoc spongiaeforme*,[7] noscomin, and coniston a-e from *Nostoc commune*.[8] The diverse polysaccharides in *N. commune* have been shown to possess antibacterial activity along with antitumor,

**Address for correrspondence:**
Manish Kumar Agrawal, Excellent Bio Research Solutions Pvt. Ltd., 1370, Napier Town, Home Science College Road, Jabalpur - 482 001, Madhya Pradesh, India. Phone: +91-7612410074.
E-mail: excellentbioresearch@gmail.com

**Received:** 27-08-2016
**Revised:** 12-09-2016
**Accepted:** 21-09-2016

antivirus, and anti-inflammatory effects.[9] Nostocyclyne A is another antimicrobial compound isolated from *Nostoc* sp.[10]

Earlier studies have highlighted the Central Indian region having good cyanobacterial diversity and having biologically active metabolites.[11] During our hunt for potential cyanobacteria with the wide spectrum antimicrobial activity of pharmaceutical interest, we earlier reported antibacterial activity of some of the local isolates.[12] This study is a step ahead and showcases the antibacterial, and in addition, antifungal activity of an axenic culture extract of locally isolated aquatic (benthic) *Nostoc calcicola* against human pathogenic bacteria and fungi. The molecular identification data for the said culture adds the authenticity of the producing organism.

## MATERIALS AND METHODS

### Collection and isolation of *N. calcicola*

During this study, a benthic mat was collected from Devtaal pond, Jabalpur, India. The collected sample was cultured initially in BG11 medium (without nitrate) under white light illumination for a few weeks, and the filaments were isolated after several streaking on BG11 (without nitrate) agar plates. The isolated unialgal culture was maintained in liquid BG11 medium at 25°C with 1500 Lux light intensity and a light-dark cycle of 16/8 h. The culture was primarily identified as *Nostoc* species using morphological identification keys and the strain was numbered as EBRS001. To remove the adherent bacteria and fungi (symbiotic or parasitic), the cultures were grown for several generations with streptomycin (20 mg/L) and cycloheximide (10 mg/L). The sterility of the culture was confirmed by plating the culture on nutrient broth.

The biomass from 15 to 20 days old actively growing laboratory culture was collected by centrifugation. For molecular identification, 16S rDNA sequence analysis was performed. For this, a ~1.5 kb fragment was amplified from isolated genomic DNA. The polymerase chain reaction product was sequenced, and the data were aligned using a combination of NCBI GenBank and RDP database. A phylogenetic tree was constructed using the neighbor-joining method.

### Extraction of secondary metabolites from *Nostoc calcicola*

The antimicrobial compound(s) extraction, 100 mg freeze-dried cells were extracted twice with 100 ml absolute ethanol with stirring, centrifuged (20,000 ×*g*, 30 min), and the supernatant was collected and evaporated to dryness. The residue was redissolved in 1 ml of absolute ethanol.[12] From this stock solution (100 mg/ml), a serial dilution was prepared to get varied concentration of 10, 1, 0.1, 0.01, and 0.001 mg dry weight/ml using ethanol.

### Screening of antimicrobial activity of *Nostoc calcicola* extract

Since there is no idea about the concentration of antimicrobial substance(s) in the culture, the criteria to determine the antimicrobial sensitivity was based on minimum inhibitory concentration (MIC) of extracted dried mass. The test bacteria selected for the study were five clinically important pathogenic bacterial strains, i.e. *Staphylococcus aureus* ATCC 25923, *Bacillus cereus* ATCC 10876, *Escherichia coli* ATCC 35218, *Pseudomonas aeruginosa* ATCC 27853, and *Salmonella typhimurium* ATCC 13311 and four clinically important pathogenic fungal strains, i.e., *Aspergillus fumigatus* MTCC 3785, *Fusarium solani* MTCC 4117, *Penicillium chrysogenum* MTCC 6891, and *Candida albicans* ATCC 10231. The cultures used in the study were not more than four passages old from the master culture. The test bacteria were maintained in nutrient broth while the test fungi were maintained on potato dextrose broth. The test organisms were subcultured for 2-8 h before the test.

The antibacterial tests were performed by agar well-diffusion method[13] using Mueller-Hinton agar plates. The solidified media plates were swabbed with 0.1 ml of each test organism for which the turbidity the broth was set equivalent to 0.5 McFarland just before swabbing. A well punch machine was used to create wells of 6 mm at equal distances. In these wells, 50 µl of *N. calcicola* extract from each dilution was placed (each plate received 5000-0.05 µg dry weight equivalent). In one well, 50 µl of absolute ethanol served as negative control. Ampicillin (100 µg discs) was used as a positive control on separate plates for each tested bacteria.

For antifungal tests, Mueller-Hinton agar supplemented with 2% glucose and 0.5 µg/ml methylene blue was used as a medium. 50 µL of absolute ethanol served as negative control. Miconazole (30 µg disc) was used as a positive control on separate plates for each tested fungi. The inoculated plates were incubated at 37°C for 24-48 h. After incubation, the diameter of the inhibition zone was measured. All the tests were performed in three replicates, and the mean zone of inhibition was calculated.

In different data sets, the MIC was identified as the least dilution that showed no visible growth over a period of 48 h on the plates when the tests bacteria/fungi were inoculated in the medium containing extracted biomass.

### Data analysis

The data are presented as mean ± standard deviation for inhibition zones. For statistical analysis, unpaired *t*-test was used using Sigma Graph Pad Prism® version 6.0.

## RESULTS AND DISCUSSION

In this study, a filamentous cyanobacterium was isolated from a local pond. The morphological features revealed it to be *Nostoc* as it showed following morphological characters: Filaments loosely agglomerated, mucilaginous peripheral sheath present in young filaments, cells almost spherical, apical cell not different from other cells, heterocysts present terminally, sometime intercalary [Figure 1].

Based on nucleotide homology and phylogenetic analysis, the isolated cyanobacteria was detected to be *N. calcicola* (GenBank accession no: GQ167549.1) [Figure 2].

### Antibacterial activity of *N. calcicola*

The ethanolic extract of *N. calcicola* was found to inhibit all the test bacteria, though the degree of inhibition varied. Against Gram-negative bacteria, the highest zone of inhibition was observed with *S. typhimurium* ATCC 13311 (mean zone size = 19 ± 0.47, $n$ = 3) and the least was with *P. aeruginosa* ATCC 27853. Among Gram-positive bacteria, *S. aureus* ATCC 25923 was inhibited the most (mean zone size = 23.6 ± 0.47, $n$ = 3). Overall, the highest inhibitory activity was observed with *S. aureus* ATCC 25923 followed by against *S. typhimurium* ATCC 13311 [Table 1, Figure 3]. The MIC was found to be 5 µg equivalent dry weight against these bacteria. The other test bacteria showed increased MIC as 50 µg equivalent dry weight. *P. aeruginosa* ATCC 27853 was less sensitive against the ethanolic extract of *N. calcicola* among all tested bacteria. Further, the crude extract of *N. calcicola* was found to be active against tested bacteria up to 6 months when stored refrigerated (data not shown).

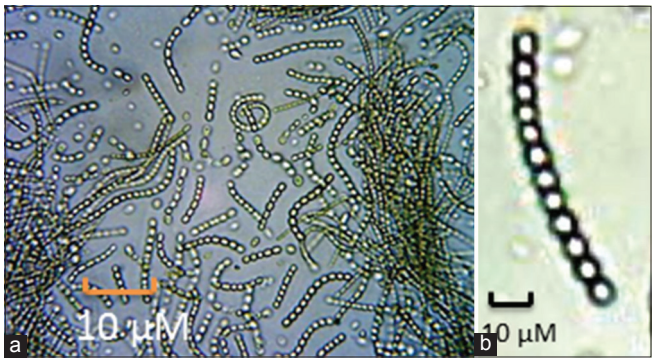


**Figure 1:** Culture characteristics of *Nostoc calcicola* isolated from the Central Indian region of Jabalpur. (a) Filaments of *N. calcicola* in exponential stage, (b) single filament showing peripheral sheath along with the young trichome and spherical cells

Since it is hard to speculate the concentration of active antimicrobial ingredient(s) in *N. calcicola*, we compared the inhibitory zone sizes with those produced by 100 µg ampicillin, to show the effectiveness and usefulness of the study. The activity of the cyanobacterial extract (5 mg dry weight equivalent) was significantly better against *E. coli* ATCC 35218 and *S. aureus* ATCC 25923 and was significantly lower against *S. typhimurium* ATCC 13311 and *B. cereus* ATCC 10876 when compared to the zone sizes produced by 100 µg ampicillin (*t*-test, $P < 0.05$).

### Antifungal activity of *N. calcicola*

A local isolate of *N. calcicola* showed good antifungal activity against all test fungi. The maximum diameter of inhibition was noticed with the extracts having 5000 µg/ml dry weight equivalent concentrations of *N. calcicola* against *C. albicans* ATCC 10231 (zone size = 15.6 ± 0.47, $n$ = 3) while *F. solani* MTCC 4117 was the least inhibited fungus [Table 2, Figure 4]. The MIC was found to be 50 µg dry weight equivalent against all test fungi except *C. albicans* ATCC 10231 where the MIC was tenfold lower.

When the antifungal activity of *N. calcicola* extract having 5 mg equivalent dry weight was compared with the activity of 30 µg miconazole, the zone sizes were significantly better against *A. fumigatus* MTCC 3785 and *C. albicans* ATCC10231 (*t*-test, $P < 0.05$) and comparable, though not significantly different or higher with the other tested fungi.

This study shows that the active compound(s) from *N. calcicola* is able to encounter all the tested bacteria and fungi *in vitro*. No other antibiotic, known till date is able to

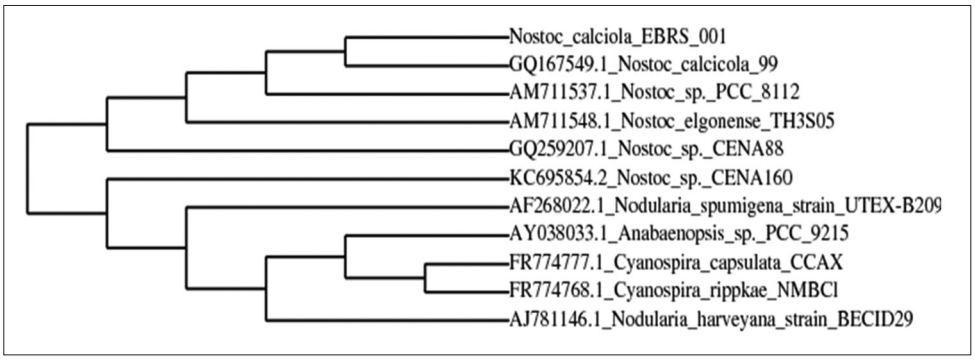


**Figure 2:** Phylogenetic tree of *Nostoc calcicola* made using the neighbor-joining method with nearest homologs with their GenBank accession numbers based on partial 16S rDNA gene sequences

**Table 1:** Antibacterial activity of *Nostoc calcicola* against human pathogenic bacteria

| Microorganism | Zone of inhibition in mm (mean±SD, *n*=3) | | | | | | |
|---|---|---|---|---|---|---|---|
| | ***Nostoc calcicola* extract (µg dry weight equivalent extract per well)** | | | | | | **Control** |
| | **5000** | **500** | **50** | **5** | **0.5** | **0.05** | **Ampicillin** |
| *Escherichia coli* ATCC 35218 | 17±0.81* | 12.9±5.24 | 10±4.70 | - | - | - | 13.0±1.0 |
| *Salmonella typhimurium* ATCC 13311 | 19±0.47 | 14.8±6.22 | 10±5.72 | - | - | - | 20.3±0.57 |
| *Pseudomonas aeruginosa* ATCC 27853 | 14.6±0.47* | 11.8±4.49 | 8.66±4.20 | - | - | - | 17.6±0.57 |
| *Staphylococcus aureus* ATCC 25923 | 23.6±0.47* | 17.6±7.5 | 11.6±6.90 | - | - | - | 20.0±1.0 |
| *Bacillus cereus ATCC* 10876 | 15.6±0.47* | 11.5±5.03 | 7.6±4.55 | - | - | - | 30.3±0.57 |

The inhibitory zone sizes are presented as mean±standard deviation of three replicates. The asterisk indicates that the mean zone size of inhibition at 5 mg dry weight equivalent was significantly different from that produced by 100 µg ampicillin

**Table 2:** Antifungal activity of *Nostoc calcicola* against human pathogenic fungi

| Microorganism | Zone of inhibition in mm (mean±SD, *n*=3) | | | | | | |
|---|---|---|---|---|---|---|---|
| | ***Nostoc calcicola* extract (µg equivalent dry weight per well)** | | | | | | **Control** |
| | **5000** | **500** | **50** | **5** | **0.5** | **0.05** | **Miconazole** |
| *Aspergillus fumigatus* MTCC 3785 | 11.6±0.47* | 9.51±3.56 | 7.66±0.47 | - | - | - | 17.7±1.5 |
| *Fusarium solani* MTCC 4117 | 10±0.81 | 8.60±3.05 | 7.33±0.47 | - | - | - | 10.3±0.6 |
| *Penicillium chrysogenum* MTCC 6891 | 13.3±0.94 | 11.0±3.97 | 9.33±0.94 | - | - | - | 13.0±1.0 |
| *Candida albicans* ATCC 10231 | 15.6±0.47* | 12.2±4.84 | 10±0.81 | 8.72±3.04 | - | - | 12.3±0.6 |

The inhibitory zone sizes are presented as mean±SD of three replicates. The asterisk indicates that the mean zone size of inhibition at 5 mg dry weight equivalent was significantly different from that produced by 30 µg miconazole. SD: Standard deviation

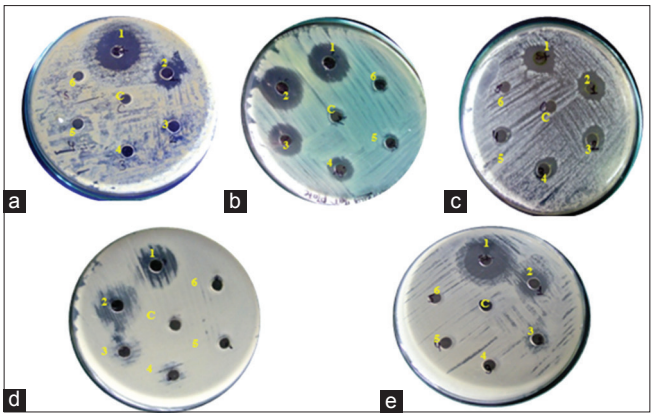


**Figure 3:** Antibacterial activity of ethanolic extract of *Nostoc calcicola* against (a) *Escherichia coli* ATCC 35218, (b) *Salmonella typhimurium* ATCC 13311, (c) *Pseudomonas aeruginosa* ATCC 27853, (d) *Staphylococcus aureus* ATCC 25923, and (e) *Bacillus cereus* ATCC 10876, in various concentrations (µg equivalent dry weight extract per well). 1 = 5000 µg, 2 = 500 µg, 3 = 50 µg, 4 = 5 µg, 5 = 0.5 µg, and 6 = 0.05 µg dry weight. C: Control (50 µl absolute ethanol)

encounter all types of organisms that include Gram-positive and Gram-negative bacteria, filamentous fungi and yeast. Asthana *et al.*[14] also discussed the possibility of the presence of broad spectrum antimicrobial compound from another cyanobacteria *Fischerella* sp. based on the results obtained with *Mycobacterium tuberculosis* and comparing the data with the MIC of three well-known antibiotics, i.e., rifampicin, streptomycin, and chloramphenicol.

The antimicrobial compounds from cyanobacteria include a structurally diverse group of compounds that include cyclic peptides, phenolic group compounds, fatty acids, and linear peptides.[14,15] This study deals with an axenic and identified culture that may avoid erratic and false results arising out due to the different environmental conditions of the cyanobacteria as well as resistance patterns of test organisms. Further, the results indicate that antibacterial activity has been pronounced over antifungal activity. Martins *et al.*[16] showed that out of 1000 strains of cyanobacteria, only 90 strains (9%) inhibited fungal growth. Further, the activity was higher against Gram-positive bacteria.

The cyanobacterial genera *Nostoc* highlights the potential of cyanobacteria as a resource of secondary metabolites.[1] Biondi *et al.*[17] reported that *Nostoc* strain ATCC 53789 showed a wide range of activities against a variety of plant pathogenic fungi along with its pesticide activity. Becher *et al.*[18] isolated nostocarboline from *Nostoc linckia, Nostoc muscorum,* and *N. ellipsosporum*, which antagonized both bacteria and fungi. Apart from *Nostoc,* antibacterial and antifungal activity has been shown by other cyanobacteria, i.e., *Oscillatoria subrevis, O. amphibian, Anabaena* sp., *Scytonema* sp., *Microcystis* sp., *Phormidium* sp., and *Lyngbya majuscule.*[19] Asthana *et al.*[20] isolated and identified new antibacterial compound from *Nostoc CCC* which were similar to anthraquinone and indane derivatives of a diterpenoid.

This study shows that ethanolic extract of *N. calcicola* was more effective against *S. aureus* and *E. coli*. Asthana

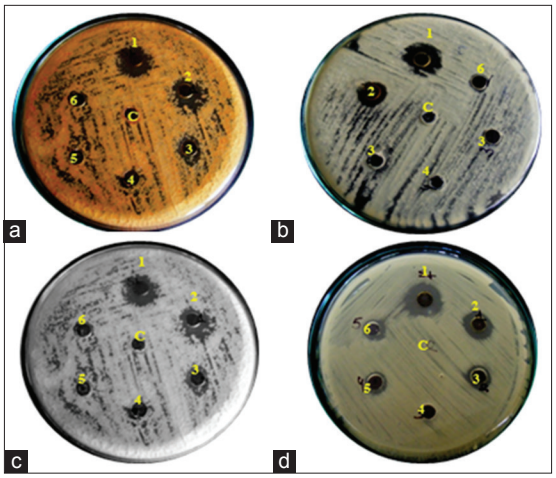


**Figure 4:** Antifungal activity of ethanolic extract of *Nostoc calcicola* against (a) *Aspergillus fumigatus* MTCC 3785, (b) *Fusarium solani* MTCC 4117, (c) *Penicillium chrysogenum* MTCC 6891, and (d) *Candida albicans* ATCC 10231 in various concentrations (µg equivalent dry weight extract per well). 1 = 5000 µg, 2 = 500 µg, 3 = 50 µg, 4 = 5 µg, 5 = 0.5 µg, and 6 = 0.05 µg dry weight. C: Control (50 µl absolute ethanol)

*et al*.[20] reported similar results with *Nostoc* CCC 537 though with higher MIC values. Further it has been shown that the yield of active antimicrobial compound from Indian cyanobacteria ranges between 0.001% and 0.002%.[20] Taking this into account, 5 mg dry weight of *N. calcicola* should not contain more than 10 µg of active principal. The results show that the 5 mg dry weight (~10 µg active principal) amount was better than ampicillin (100 µg) and miconazole (30 µg) during *in vitro* studies in dose-dependent manner.

Keeping in mind the easiness and cost-effectiveness of culturing the cyanobacteria, coupled with rich biodiversity in India, *N. calcicola* produces a good opportunity toward the commercialized production of clinically and agriculturally important antimicrobial compounds. Such compounds may be used as a single compound or may be formulated with other compounds (cyanobacterial and/or synthetic origin) to get synergistic effects. Cyanobacterial compounds have been shown to possess synergistic effects. One example is combined enhanced effect of laxaphycin A 77 and B78, isolated from *Anabaena laxa*, against *C. albicans*, and lymphoblastic cell lines.[21] However, the active principals of *N. calcicola* need to be isolated and identified before depicting further speculations.

## ACKNOWLEDGMENT

The authors would like to thank Merck Specialities Private Limited, Bangalore, India for assistance in 16S rDNA experiments.

## REFERENCES

1. Nunnery JK, Mevers E, Gerwick WH. Biologically active secondary metabolites from marine cyanobacteria. Curr Opin Biotechnol 2010;21:787-93.
2. Skulberg OM. Microalgae as a source of bioactive molecules – Experience from cyanophyte research. J Appl Phycol 2000;12:341-8.
3. Agrawal M, Ghosh S, Bagchi D. Occurrence of microcystin-containing toxic water blooms in Central India. J Microbiol Biotech 2006;16:212-8.
4. Svircev Z, Cetojevic-Simin D, Simeunovic J, Karaman M, Stojanovic D. Antibacterial, antifungal and cytotoxic activity of terrestrial cyanobacterial strains from Serbia. Sci China C Life Sci 2008;51:941-7.
5. Patterson GM, Larsen LK, Moore RE. Bioactive natural products from blue-green algae. J Appl Phycol 1994;6:151-7.
6. Whitton BA, Potts M. The Ecology of Cyanobacteria: Their Diversity in Time and Space. Netherlands: Kluwer Academic Publishers; 2000. p. 37-59.
7. Whitton BA. Cyanobacterial diversity in relation to the environment. In: Evangelista V, Barsanti L, Frassanito AM, Passarelli V, Gualtieri P, editors. Environment. Berlin: Springer Verlag; 2008. p. 17-43.
8. Jaki B, Orjala J, Heilmann J, Linden A, Vogler B, Sticher O. Novel extracellular diterpenoids with biological activity from the cyanobacterium *Nostoc commune*. J Nat Prod 2000;63:339-43.
9. Sheng J, Zeng L, Sun H, Huang A. Biological activities of protein-polysaccharides from *Nostoc commune*. J Guang Acad Sci 2001;17:20-3.
10. Ploutno A, Carmeli S. Nostocyclyne A, a novel antimicrobial cyclophane from the cyanobacterium *Nostoc* sp. J Nat Prod 2000;63:1524-6.
11. Agrawal M, Bagchi D, Bagchi S. Acute inhibition of protease and suppression of growth in zooplankter, *Moina macrocopa*, by *Microcystis* blooms collected in Central India. Hydrobiologia 2001;464:37-44.
12. Yadav S, Agrawal M, Raipuria N, Agrawal MK. Potential of cyanobacteria collected from Central India against human pathogenic bacteria *in vitro*. Int J Pharm Sci Res 2015;6:4047-52.
13. Abed RM, Dobretsov S, Sudesh K. Applications of cyanobacteria in biotechnology. J Appl Microbiol 2009;106:1-12.
14. Asthana RK, Srivastava A, Singh AP, Singh SP, Nath G, Srivastava R, *et al*. Identification of an antimicrobial entity from the cyanobacterium *Fischerella* sp. Isolated from bark of *Azadirachta indica* (Neem) tree. J Appl Phycol 2006;18:33-9.
15. Reehana N, Parveez A, Thajuddin N. *In- vitro* studies on bactericidal effect of selected cyanobacteria against bacterial pathogens. Int J Mediciobiol Res 2012;1:345-7
16. Martins RF, Ramos MF, Herfindal L, Sousa JA, Skaerven K, Vasconcelos VM. Antimicrobial and cytotoxic assessment of marine cyanobacteria - *Synechocystis* and

*Synechococcus*. Mar Drugs 2008;6:1-11.
17. Biondi N, Piccardi R, Margheri MC, Rodolfi L, Smith GD, Tredici MR. Evaluation of *Nostoc* strain ATCC 53789 as a potential source of natural pesticides. Appl Environ Microbiol 2004;70:3313-20.
18. Becher PG, Beuchat J, Gademann K, Jüttner F. Nostocarboline: Isolation and synthesis of a new cholinesterase inhibitor from *Nostoc* 78-12A. J Nat Prod 2005;68:1793-5.
19. Ramamurthy V, Raveendran S. Antimicrobial activity of heterocytic *Cyanobacteria*. Int J Adv Life Sci 2012;1:32-9.
20. Asthana RK, Tripathi MK, Srivastava A, Singh AP, Singh SP, Nath G, *et al*. Isolation and identification of a new antibacterial entity from the antarctic cyanobacterium *Nostoc* CCC 537. J Appl Phycol 2008;21:81-8.
21. Bonnard I, Rolland M, Francisco C, Banaigs B. Total structure and biological properties of laxaphycins A and B, cyclic lipopeptides from the marine cyanobacterium *Lyngbya majuscula*. Lett Pept Sci 1997;4:289-92.

**Source of Support:** Nil. **Conflict of Interest:** None declared.