Antimicrobial effect of a cyclic peptide
Nostophycin isolated from wastewater cyanobacteria, Nostoc calcicola

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ABSTRACT

Different types of peptides are produced by cyanobacteria of the genus Nostoc, which are unique in structure and have a wide spectrum of biological activities. The objective of the study is to explore different habitats of organism and study antimicrobial activities to improve their pharmaceutical application and drug like properties by structure modification. A cyclic peptide nostophycin was isolated from Nostoc calcicola (MK506349) through freeze dried lyophilization method. Its structure has been elucidated using FT-IR, 1H NMR, 13CNMR and LC-MS. Glycine, d-glutamine, l-phenylalanine, d-isoleucine, l-proline and a novel amino acid Ahoa are constituents of nostophycin. 1H NMR, 13CNMR spectroscopy confirmed the number of protons and carbons, and characteristics peak determined the structure and fragmentation pattern through LC-MS. Nostophycin possess Ahoa instead of Adha which makes it different from microcystin. Nostophycin exhibits antimicrobial activity against E. coli, S. aureus, C. albicans and A. niger. Significant antifungal activity (9-52 μg/mL) and moderate antibacterial activity (concentration 18-52 μg/mL) were observed for nostophycin. In case of already known peptides, these molecules may be further exploited to improve pharmaceutical application and future drug development.

KEYWORDS: Ahoa, Cyclic Peptide, 1H NMR, 13CNMR, LC-MS and Microcystine.

INTRODUCTION

Cyanobacterial features conspicuous researchers due to their capability of synthesis of various bioactive compounds, diverse range of habitats, wide diversity and morphological variability. Cyanobacteria are gram negative, photosynthetic and ubiquitous bacteria, which known as a primary producer (Gademann & Portmann, 2008). Availability in the extreme environment and unique feature of cyanobacteria, considered it to be future pioneer for research (Kulasooriya, 2011; Potts, 1999; Scherer et al., 1988; Scherer & Potts, 1989). According to Kalaitzis et al. (2009) cyanobacteria can produce immense range of bioactive compounds which help in survival in endurance and competitive ecological niche. Bioactive metabolites synthesized by Nostoc sp has been applied as a biofertilizer (Ghazal et al., 2018; Win et al., 2018), anticeancer (Moore, 1996), antifungal (El-Sheikh et al., 2014), antibacterial (Ploutno & Carmeli, 2000), antiviral (Botos & Wlodawer, 2003) and enzyme-inhibiting (Mazur-Marzec et al., 2018). These bioactive compounds are explored and identified as peptides, alkaloids, terpenoids, fatty acid and lipopolysaccharides (Chorus, 2012; Dembitsky & Rezanka, 2005; Dittmann et al., 2001; Nowrazi et al., 2012; Parmar et al., 2011). Allelochemicals influence their own growth potential, other microbes in their vicinity, associated microorganisms, higher plants and animals. Cyanobacteria synthesize nitrogen storage material, new proteins, change pigmentation, excrete and store some other compounds, in response to environmental stress, temperature, pH, nutrient availability and light intensity (Mendes & Vermelho, 2013; Priya et al., 2015; Singh, 2014).

High number of metabolites, lipid and lipid like compounds, peptides, oligopeptides and amino acid derivatives produced by different genera of the Nostocaceae family (Rezanka & Dembitsky, 2006). Nostocyclamide a macrocyclic peptide was first reported in Nostoc sp. (Jüttner et al., 2001) and Nostophycin has been isolated from Nostoc calcicola, cyclic peptide with different amino acid including unusual amino acid acid (Fewer et al., 2011). Extracts from various cyanobacteria have already been proved beneficial for Triticum aestivum (Jäger et al., 2005) Orzya sativa (Saadatnia & Riahi, 2009), Zea mays (Saadatnia & Riahi, 2009), Cucumis sativus, Cucurbita maxima and Solanum lycopersicum (Shariatmadari et al., 2011). Cyanobacteria are known to produce different allelochemicals that have the potential for multiple usages in various fields. Drug-like properties of peptide, combine effect of compounds, pharmaceutical applications of peptides can be improved and

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explored widely. The aim of the present work was to elucidate the chemical structure of extracted compound of *Nostoc calcicola* and examine its antibacterial and antifungal potential.

**MATERIALS AND METHODS**

**Isolation and Identification of Cyanobacteria**

Samples were collected from waste water of catchment area of Banjara Lake of Sagar, Madhya Pradesh, India, situated between 23.83° North latitude and 78.73° East longitude and unialgal culture was obtained by streak plate method. *Nostoc calcicola* were cultured in BG 11 medium, pH 6.5 at temperature 25±2°C, light intensity 2500-3000 lux for 14 h light/10 h dark (Rippka et al., 1981). Morphological identification of cyanobacterium was done by microscopic analysis (Micron 36620, Carl Zeiss, Germany) using the keys and description of treaties of Desikachary (1959). Total genomic DNA extraction and 16S rRNA gene amplification were done with standard methodology. The sequence for the species was deposited to the prokaryotic rRNA submission portal of GenBank and the accession number was obtained.

**Mass Culture and Crude Extract From Cyanobacterial Samples**

Cells were harvested in mid log phase and centrifuged at 10000×g for 15 min and supernatant was removed and pellet was lyophilized (freeze dried) for 12 h. Furthermore, freeze dried pellet was suspended in methanol and 0.01% TFA (Trifluoroacetic acid), then shaken for 8h by orbital shaker. Later, it was kept overnight at -20 °C and further sonicated in a cold room. Subsequently, centrifuged at 10000×g for 15 minute and supernatant was vacuum dried at 40°C and pellet was re-extracted twice. The mixture was stored in glass vials at -4°C. Yield percentage of extract was calculated by following formula, Yield % = Weight of evaporated extract/ Weight of cyanobacterial powder×100

**SPECTRAL ANALYSIS**

Spectral analysis was carried out by freeze dried extracts namely, *Nostoc calcicola* using standard procedures to identify the components.

**Fourier Transform Infrared (FTIR) Spectroscopy**

Fourier transform infrared spectroscopy (FTIR) performed on Shimadzu 4000S. The spectrum of solid sample was obtained using KBr (Potassium bromide) pellets. About 1 mg of sample and 100 mg of KBr were ground together, dried to remove moisture and mechanically placed in sample holder. The KBr does not absorb infrared radiation in the region 4000 cm⁻¹ and 500 cm⁻¹ and complete spectrum of the solid sample is obtained (Sharma, 1981).

**Nuclear Magnetic Resonance Spectroscopy (NMR)**

¹H NMR and ¹³C NMR spectroscopies were performed by Bruker/Avance II at 500MHz, Advanced Instrumentation Research Facility (AIRF), Jawahar Lal Nehru University, New Delhi using DMSO as a solvent.

**Liquid Chromatography- Mass Spectrometry (LC-MS)**

LC-MS is sophisticated technique used for separation or purification of sample followed by fragmentation pattern. LC-MS analysis was carried out at Indian Institute of Science and Research (IISER) Bhopal, by Agilent technologies 1260 infinity and Agilent technologies 6130 quadrupole. Methanol was used as a solvent for LC-MS. Standard of amino acids were used as control for control in NMR and LCMS.

**Bacterial Strain Preparation**

The bacterial strain Escherichia coli MTCC#1591 (E. coli), Staphylococcus aureus MTCC#3212 (S. aureus) and fungi strains Aspergillus niger MTCC#9652 (A. niger), Candida albicans (MTCC#183) were obtained from The Microbial Type Culture Collection and Gene Bank (MTCC) Chandigarh, India. The test bacteria were maintained in nutrient broth media while the test fungi were maintained on potato dextrose agar plates. The test organisms were subcultured 2-3 h before the test.

**Preparation of Resazurin Solution**

The resazurin solution was prepared at 0.02% (wt/vol) with 0.002 g of resazurin salt powder was dissolved in 10 mL of distilled water and vortexed. The mixture was filtered by Millipore membrane filter (0.2 μm). The resazurin solution can be kept at 4°C for 2 weeks.

**Disk Diffusion Method**

The antibacterial activity of extracted sample against the procured test bacteria was carried out using Kirby-Bauer Disk Diffusion susceptibility Test Method (Hudzicki, 2009). The bacterial strains were spread on Mueller-Hinton agar (Merck, Germany) using sterile cotton swab. Extracted sample was prepared at concentrations of 0.2, 0.4, 0.6, 0.8 and 1 mg/ml. Sterile blank antimicrobial disk was used in the test. The disks were loaded aliquot 5 mL with different concentrations of sample and ampicillin and miconazole was used as positive control for bacteria and fungi respectively, while methanol as a negative control.

For antifungal test, Mueller-Hinton agar supplemented with 2% glucose and 0.5 μg/ml methylene blue was used as a medium. The inoculated plates were incubated at 37 °C for 24-48 h and the diameter of the inhibition zone was measured after incubation. All the tests were performed in three replicates, and mean of inhibition zone was calculated.

**Determination of Minimum Inhibitory Concentration**

Minimum inhibitory concentration (MIC) was determined by using the method described in standard CLSI guidelines (Wayne, 2012). The MIC test was performed in 96 well round
bottom microtiter plate using standard broth microdilution method while MBC was performed on the MHA plates. The bacterial inoculums were adjusted to the concentration of 10^6 CFU/mL. For the MIC test, five dilutions were prepared with 4.5, 6.5, 8.5, 10.5 and 12.5 mg/mL extracted sample in methanol. A total of 50 μL of the microbial suspension, at 0.5 McFarland concentrations, was inoculated into each well and then the microtiter plate was incubated at 37°C for 24h. The lowest concentration at which no turbidity was observed was considered the MIC. A well of the microtiter plate with no microorganisms was considered as the negative control and with no extract was considered as the positive control.

The MBC was defined as the lowest concentration of the antibacterial agents that completely kill the bacteria. MBC test performed by plating the suspension from well of microtiter plates into MHA plates. The plates were incubated at 37 °C for 24 h. The lowest with no visible growths on the MHA plate was taken as MBC value.

**Time-kill Curve**

Time-kill assay was done in MHB medium as described by Zainin *et al.* (2013) and Lau *et al.* (2018). The bacterial inoculums were adjusted to 10^6 CFU/mL. The nostophycin solution was diluted with MHB media containing bacterial inoculums to obtain the final concentration of 0×MIC, 1×MIC, 2×MIC, 4×MIC, and 8×MIC for each type of bacteria in the total final volume of 1 mL. The cultures were then incubated at 37°C with 150 rpm agitation. The culture (100 μL) was spread on MHA plates at time 0, 0.25, 0.5, 1.2 and 4h in triplicates. The number of colonies on the MHA plates was quantified in CFU/mL after incubation at 37°C for 24h. For statistical analysis, SPSS (v.26) statistical package was used to determine the significant (P < 0.05) difference among the tested bacteria.

**DATA ANALYSIS**

The data are presented as mean ± standard deviation for inhibition zones. For statistical analysis, unpaired t-test was used using SPSS version 26.

**RESULT AND DISCUSSION**

With the help of microscopic study and camera lucida sketching, morphological characteristics i.e. subspherical heterocyst, barrel shaped cells, and molecular study confirmed it as *Nostoc calcicola* (MK506349) (Figure 1).

**SPECTRAL ANALYSIS OF NOSTOC CALCICOLA (NPC) EXTRACTED SAMPLE**

**FTIR Study**

FTIR spectrum was recorded using KBr pellets in range 4000-3545, 3250, 2895, 1745, 1550 and 1195 cm⁻¹. The characteristic stretching frequency for carbonyl group attributed to the presence of amide group in sample. Based on the previous literature and standard protocol, the functional classes were characterized as –OH stretching, N-H bond of amine, –C-H stretching carbonyl, C=C (aromatic ring) stretching and C-N stretching respectively (Table 1; Figure 2).

**NMR Study**

After confirming the presence of carbonyl group in the form of amide group, sample was further characterized by using 1H NMR and 13C NMR spectroscopic techniques. According to literature survey it was noted that the sample consists of peptide bonds (Fuji *et al.*, 1999). The expected structure of sample was further characterized by using 1H NMR and 13C NMR spectroscopic techniques. According to literature survey it was noted that the sample consists of peptide bonds (Fuji *et al.*, 1999).

**Figure 1:** Photomicrograph and camera lucida sketch illustrating the morphological features of isolated *Nostoc calcicola* (MK506349). Bar denotes 20 μm

**Figure 2:** Characteristics IR peaks of NPC

| Serial No. | Frequency (cm⁻¹) | Functional Group | Type of Vibration |
|------------|-----------------|------------------|------------------|
| 1          | 1745            | C=O              | Stretching       |
| 2          | 3250            | N-H              | Stretching       |
| 3          | 2895            | C-H              | Stretching       |
| 4          | 3545            | O-H              | Stretching       |
| 5          | 1550            | C=C (Aromatic ring) | Stretching     |
| 6          | 1195            | C-N              | Stretching       |
confirmed by the presence of different types of hydrogen atoms present in it. As the literature reports that the whole structure of sample is constructed with different types of amino acids. The set of amino acids as follows proline I, isoleucine, phenylalanine, proline II, glycine and glutamine. The presence of all amino acids and formation of particular peptide bond were taken into consideration. The obtained 1H NMR spectrum for sample was compared with 1H NMR spectra of individual amino acids. The change in chemical shift value of –NH₂ in all amino acids were observed. The chemical shift values obtained in range 6-8 δ ppm corresponds to proton present in amino acid in the form of –NH in Nostophycin molecule. The compared values suggest that the signals obtained for amino acids in sample shows larger chemical shift value (δ ppm) as compared to spectrum obtained for pure amino acids, it may happen due to removal of proton and formation of hydrogen atom (Figure 3).

Some remarkable changes can be mention; the original spectrum of isoleucine displays sharp singlet around 7.05 δ ppm but in present sample it shows some shift towards higher chemical shift value 7.37 δ ppm as doublet with J=8.4 Hz. Furthermore, phenylalanine and glycine also exhibit the same behavior and their chemical shift values are around 7.39 and 6.89 δ ppm respectively due to removal of one proton and formation of new bond. Presence of glutamine was confirmed by singlet obtained at 8.7 δ ppm corresponds to -N-H proton whereas singlet at 6.60 δ ppm value corresponds to -NH₂ bond. Additionally one unusual amino acid is determined to be a β-amino acid (3 amino-2,5-dihydroxy-8-phenyloctanoic acid) or (Ahoa), which exhibits two hydroxyl groups in its structure. The 1H NMR spectrum shows following chemical shifts values 5.76 and 7.52 δ ppm corresponds to –OH and –NH proton respectively. The chemical shift value obtained around 3.62 δ ppm (J=8.72) can be assigned to protons of -CH₂ present in proline amino acid.

The 13C NMR spectrum of sample shows the presence of carbonyl group in structure. The carbonyl group displays its characteristic δ ppm value around 168-170 δ ppm. In this context 13C NMR spectrum obtained for sample are in two different ranges. Figure 4 displays characteristics peaks for different types of carbonyl group present in compound. The chemical shift values obtained at 171.1, 170.5 and 168.1 δ ppm assigned for carbonyl group present in proline, isoleucine and glycine molecule. The singlet obtained for each carbonyl group suggests that no direct proton is attached to the carbon atom. The other singlets obtained at 170.1, 174.2 and 175.2 corresponds to carbonyl group present in glutamine, phenylalanine and 2,5-dihydroxy-8-phenyloctanoic acid.

Liquid Chromatography- Mass Spectrometry (LC-MS) Study

As, it is important to confirm the molecular mass of sample, LC-MS technique was applied to confirm the established structure of sample. Therefore, a confirmation of the amino acids spectra and structure of cyanobacterial peptides by LC-MS at 1000 m/z was carried out (Fujii et al., 1999).

In the product ion spectrum (Figure 5) for the [M + H]+ at m/z 889 of sample, several ions were prominently observed, which were assigned as the ions of each constituent amino acid residue lost from the precursor ion [M-Glycine]+ at m/z 833, [M-Phenyalanine]+ at m/z 741, [M-3-amino-2,5-dihydroxy-8-phenyloctanoic acid (Ahoa)] and [M-Glycine+glutamine]+ at m/z 532, [M-Glutamine]+ at m/z 744.

**STRUCTURE ELUCIDATION**

Based on the results of FT-IR, NMR (13C and 1H) and mass spectroscopy the molecule was tentatively predicted as cyclic peptide and the structure is composed of six amino acids and a novel β-Ahoa; named as Nostophycin. Molecular formula of the nostophycin was C₃₀H₃₅N₇O₁₀ and molecular weight was predicted as 887. The structure was shown in Figure 6.

**Antimicrobial activity of Nostophycin**

The antibacterial activity of nostophycin was determined against two bacterial (E. coli and S. aureus) and two fungal species (A. niger and C. albicans). The results for disk diffusion test, MIC and MBC of the nostophycin are summarized in Table 2. For the disk diffusion test, the presence of clear zone around the nostophycin disk suggesting that the nostophycin possessed antimicrobial activity which is able to inhibit the growth of bacteria and fungus. As previous study reported that nostophycin extracted from Nostoc CCC537 produced a maximum inhibition zone (29 mm) for S. aureus followed by P. aeruginosa (11 mm), S. typhi (10 mm), E.coli (10 mm) and Enterobacter aerogens (7 mm), it has also been reported that doubling in the concentration increased the inhibition zone for
the same microorganism (Asthana et al., 2009). Agrawal (2016) also reported antibacterial and antifungal activity of extract of N. calcicola against different human pathogens through inhibition zone ranging from 7.5 to 20 mm.

Disk diffusion test was described as the preliminary study in screening the antibacterial activity of an antimicrobial agent; therefore, a further evaluation in determining the antibacterial activity of nostophycin using MIC value was needed. MIC was defined as the lowest concentration of the antibacterial agent to inhibit the growth of bacteria by serial dilution. As showed in Table 2, the MIC values of nostophycin against the bacteria and fungi were ranged from 4.5 to 8.5 mg/mL. It has been reported that lower quantity of active principle 2.5 μg/mL of Nostoc CCC 537 was effective against M. tuberculosis H37Rv (Asthana et al., 2009). MBC is the lowest concentration of antibacterial and antifungal agent to kill the bacteria and fungus (showed no growth on the agar plate). MBC for Candida albicans was killed after 1 h of incubation at 2 × MIC (9 μg/mL), 4 × MIC (18 μg/mL), 8 × MIC (36 μg/mL).

**Table 2: The diameter of inhibition zone (mm), MIC value (μg/mL), and MBC value (μg/mL)**

| Organisms                | Inhibition Zone Diameter (mm) | MIC (μg/mL) | MBC (μg/mL) |
|--------------------------|-------------------------------|-------------|-------------|
| Escherichia coli         | 22                            | 4.5         | 6.5         |
| Staphylococcus aureus    | 38                            | 6.5         | 8.5         |
| Aspergillus niger        | 20                            | 6.5         | 6.5         |
| Candida albicans         | 24                            | 4.5         | 4.5         |

S. aureus (8.5). It has been reported that lower quantity of active principle of Nostoc CCC 537 is more effective on high quantity of streptomycin and rifampicin against Enterobacter aerogens (Asthana et al., 2009). Pesticidal effects of methanolic extract of Nostoc strain ATCC 53789 from 0.25g/L to 100g/L has been used to treat various pathogenic fungi (Biondi et al., 2004). Resazurin dye was used in the study to determine cell growth, especially in cytotoxicity assays (McNicholl et al., 2007). Oxidoreductase within viable cells reduced the resazurin salt to resorufin and changed the color from blue non-fluorescent to pink and fluorescent. According to McNicholl et al. (2007), resazurin dye has been applied for decades to check for the bacterial and yeast contamination milk.

**TIME-KILL CURVE**

The time kill activity of bacteria and fungi is shown in Figure 7. The reduction in the number of CFU/mL effective against bacteria and fungi were ≥3 Log units (99%) for bactericidal and fungicidal activity of nostophycin. The bactericidal endpoint of nostophycin for E. coli was reached after 2 h of incubation at 4 × MIC (18 μg/mL) and 8 × MIC (36 μg/mL), while for Staphylococcus aureus, the bacteria was killed after 2 h of incubation at 4 × MIC (26 μg/mL) and 8 × MIC (52 μg/mL). The fungicidal endpoint of nostophycin for Aspergillus niger was reached after 2 h of incubation at 4 × MIC (26 μg/mL) and 8 × MIC (52 μg/mL). Candida albicans was killed after 1 h of incubation at 2 × MIC (9 μg/mL), 4 × MIC (18 μg/mL), 8 × MIC (36 μg/mL).
Antibacterial activity of Nostoc sp. is already reported by Asthana et al. (2009), and antifungal activity is reported by Agrawal (2016). Structurally diverse group of Nostophycin exhibits antibacterial activity against gram negative bacteria E. coli and gram positive bacteria S. aureus, similarly antifungal activity against A. niger and C. albicans. No significant differences were found between gram positive and negative bacteria, and fungus, which indicates that nostophycin is broad spectrum antimicrobial and antifungal agent. In this study, nostophycin compound extracted from Nostoc calcicola include a structurally diverse group i.e. cyclic and linear peptides, phenolics and fatty acid. Different bioactive compounds like nostocine A (Hirata et al., 1996) and tenuecyclamide A- D (Banker & Carmeli, 1998) from N. spongiaeformae, noscomin (Jaki et al., 1999) and commostin A-E (Jaki et al., 2000) from N. commune, borophycin (Hemsechidt et al., 1994), muscoride (Nagatsu et al., 1995), cryptophycin (Biondi et al., 2004) and nostocarboline (Becher et al., 2005) from N. lineka, N. muscorum, N. ellipsoseporum, Nostoc ATCC 55789 and Nostoc 18-12A respectively have been reported. Methanol as a control was not able to inhibit microbial activity but methanol extracted nostophycin exhibits antimicrobial activity against bacteria, different workers also adopted methanol extracts for evaluating antibacterial activity (Asthana et al., 2009; Jaki et al., 2000; Mundt et al., 2001). The inherent capacity to produce specific bioactive compounds from methanolic extract of Nostoc strain ATCC 55789 seems to play an antifungal role against variety of pathogens of different agricultural importance fungi such as Armillaria sp., Colletotrichum coffeum, C. trifolii, Fusarium solani, F. oxysporum f. sp. melonis, Penicillium expansum, Phytophthora cambivora, P. cinnamomi, Rhizoctonia solani, Rosellinia sp. Sclerotinia sclerotiorum and Verticillium albo-astrum (Biondi et al., 2004). Various Nostoc species behave as an antifungal agent such as extract of N. linkia inhibit the growth of wilt disease causing Fusarium oxysporum f. sp. lycopersici, which help in biological control and better yield for tomato plants (Alwatha & Perveen, 2012). Extract of Nostoc commune FA-105, Nostoc endophytum and Nostoc muscorum suppress the effect of Fusarium oxysporum f. sp. lycopersici and soyabean root rot causing Rhizoctonia solani respectively (Ismail & Ismail, 2011; Kim & Kim, 2008).

CONCLUSION

Cyanobacterial cyclic peptide was isolated and identified from Nostoc calcicola through 1H NMR, 13C NMR and LC-MS and named as nostophycin, it contains Gln, Gly, Pro I, Pro II, Phe, Ile and Ahoa. Nostophycin exhibits resemblance with microcystin with all variables except amino acid ADHA ((2S,3S,5R)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid), which is replaced by Ahoa ((2S,3R,5R)-3-amino-2,5-dihydroxy-8-phenyloctanoic acid). The β-amino acid Ahoa from nostophycin is structurally quite similar to (Ahda) in scytomenin. Similar β-amino acids are also reported in several peptides isolated from cyanobacteria. But unusual β-amino acid Ahoa is only found in nostophycin (Fewer et al., 2011). Cyclic peptides from natural resource exhibits a variety of significant biological profiles. The anti-microbial activity of cyanobacterial secondary metabolites has been reported by many researchers. However, the MIC values from previous study showed a large variation. Therefore, the comparison of the result is difficult as there is no standard method for determination of antimicrobial activity of nostophycin. In this study, nostophycin exhibits a good antimicrobial activity against gram positive and gram negative bacteria and fungi. Lower concentration of nostophycin is able to kill bacteria and fungi in a short time, the reproduction time of bacteria is main cause of infection and treating with nostophycin could be a viable way to prevent infections. Unique structure of nostophycin and a wide spectrum of biological activity shows a remarkable biotechnological potential of the Nostoc. Newly discovered or already known bioactive peptides can be used to improve drug-like properties through structure alteration or conjugation with antibodies or small molecules. It is necessary to explore chemical synthesis, exact mechanism of secondary metabolites against bacteria and fungi and search for new chemicals from cyanobacteria.

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