Identification and toxigenic potential of a *Nostoc* sp.

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Cyanobacteria are well known for their production of a multitude of highly toxic and / or allelopathic compounds. Among the photosynthetic microorganisms, cyanobacteria, belonging to the genus *Nostoc* are regarded as good candidate for producing biologically active secondary metabolites which are highly toxic to humans and other animals. Since so many reports have been published on the poisoning of different animals from drinking water contaminated with cyanobacteria toxins, it might be assumed that bioactive compounds are found only in aquatic species causes toxicity. However, the discovery of several dead dogs, mice, ducks, and fish around paddy fields, prompted us to study the toxic compounds in a strain of *Nostoc* which is most abundant in the paddy fields of Iran, using polymerase chain reaction and liquid chromatography coupled with a diode array detector and mass spectrophotometer. Results of molecular analysis demonstrated that the ASN_M strain contains the *nosF* gene. Also, the result of ion chromatograms and MS² fragmentation patterns showed that while there were three different peptidic compound classes (anabaenopeptin, cryptophycin, and nostocyclopeptides), there were no signs of the presence of anatoxin-a, homoanatoxin-a, hassallidin or microcystins. Moreover, a remarkable antifungal activity was identified in the methanolic extracts. Based on the results, this study suggests that three diverse groups of potentially bioactive compounds might account for the death of these animals. This case is the first documented incident of toxicity from aquatic cyanobacteria related intoxication in dogs, mice, and aquatic organisms in Iran.

Key Words: cyanobacteria; natural bioactive compound; *Nostoc*; toxicity

INTRODUCTION

The genus *Nostoc* is an ecologically, morphologically, and physiologically diverse genus of microorganisms inhabiting soils, and represents large reservoir of potentially valuable natural compounds (Dembitsky and Řezanka 2005). The ecological significance of the *Nostoc* species extends beyond the compounds which they primarily known to produce, as many of these organisms are

capable of modifying their habitats through the synthesis of biologically active products (Ehrenreich et al. 2005). These compounds demonstrate a diverse range of biological activities and chemical structures, including novel cyclic and linear lipopeptides, fatty acids, alkaloids, and other organic chemicals (Dittmann et al. 2001). A large number of novel antimicrobial agents have been identi-

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fied as having cytotoxic (Bui et al. 2007), antifungal (Kajiyama et al. 1998), antibacterial (Jaki et al. 2000), immunosuppressive, enzyme inhibiting, and antiviral (Kanekiyo et al. 2005) activities.

Blooms of cyanobacteria are not only a significant problem in terms of water quality, but also pose a severe risk to human and animal health. They may contain potent hepato- and neurotoxic, as well as cytotoxic agents produced by strains of several cyanobacterial genera. It has been documented that dogs, birds and young cattle have all been killed by hepatotoxins over the years and forty cows suffered from fatal neurotoxin poisoning at Lake Vesijärvi, Finland in 1928 (Sivonen 2009).

Paddy field ecosystems provide an environment favorable for the growth of heterocytous cyanobacteria due to the moderate light, water, high temperature, and nutrient availability. The paddy fields in question are submerged for 60-90 days during the growing season (Roger and Kulasooriya 1980).

Most researchers in the fields of cyanobacteria toxicology are of the opinion that the toxic compounds produced by toxic aquatic cyanobacteria may leak into the submerged paddy fields when the algae lyse.

As there have been no reports of toxicity from drinking water contaminated with aquatic cyanobacteria in the submerged paddy fields, we decided to conduct a preliminary study of toxic chemicals in order to identify the possible causative compounds of this toxicity in one strain of heterocytous cyanobacteria which is frequently found in paddy fields of Iran.

Discovery of the presence of three diverse groups of potential bioactive compounds with strong anti-fungal and toxigenic properties in the dominant *Nostoc* strain is the first report of these compounds in Iran.

MATERIALS AND METHODS

Culture conditions, morphological characterization and preparation of extracts for chemical and bioactivity analyses

In 2010, soil samples were collected from five paddy fields in Golestan province, Iran. Five grams of soil from each sample were weighed and aseptically transferred to sterile petri dishes with adequate quantities of liquid media BG11₀ (Rippka et al. 1979) without NaNO₃. The pH was adjusted to 7.1 after sterilization. The petri dishes were incubated in a culture chamber at 28°C, and were provided with continuous artificial illumination of approximately

1,500-2,000 lux for two weeks (Kaushik 1987). Three small fragments of growing colonies were then placed equidistantly onto an agar surface for purification. Morphological measurements were made by bright-field microscopy and by phase-contrast illumination of 10-day-old cultures using a Leica DM750 P microscope (Leica, Wetzlar, Germany). The following parameters were selected to describe the morphology of heterocytous cyanobacteria: morphology of vegetative cells (including terminal cell), heterocytes, akinetes; presence or absence of terminal heterocytes; and the shape of the filament and its aggregation in colonies (Rajaniemi et al. 2005). The species were identified according to Desikachary (1959).

One strain of heterocytous cyanobacteria (ASN_M) which was the most frequently observed species in the paddy fields was selected for evaluation of its toxigenic potential, bioactivity and chemical analysis. ASN_M was grown in liquid media BG11₀ (Rippka et al. 1979). One milliliter of culture was transferred to a sterile 1.5 mL microtube and was stored at -20°C prior to anatoxin analysis. The remaining cell mass was harvested by centrifugation at 7,000 ×g for 7 min, resulting in a cell mass of 336 mg (wet weight).

The harvested biomass was transferred to two separate sterile 1.5 mL microtubes, each containing 50 mg wet mass for DNA extraction, and the remaining 236 mg for chemical and bioactivity analyses.

The extract for the anatoxin analysis was prepared from 1 mL frozen culture. The microtube containing the culture was placed in a water bath at 23°C to thaw. Subsequently, it was supplemented with 300 mg glass beads (disruptor beads, 0.5 mm; Scientific Industries, Bohemia, NY, USA) and 2 µL 50% (v/v) formic acid (Fluka/Sigma-Aldrich, Steinheim, Germany) (final concentration 0.1% [v/v] formic acid). The cells were broken mechanically three times for 15 s at a speed 6.5 m s⁻¹ with a FastPrep instrument (Savant Instruments Inc., Holbrook, NY, USA). The homogenised mixture was centrifuged at 10,000 ×g for 5 min. Twenty microliters of the supernatant was diluted 1:10 with acetonitrile (Chromasolv for LC/MS; Fluka/Sigma-Aldrich), equalling to a final volume 200 µL and the extract was then analysed for chemical analysis by liquid chromatography mass spectrometry (LC-MS) using an Agilent 1100 Series LC/MSD Trap XCT Plus System (Agilent Technologies, Palo Alto, CA, USA).

For the preparation of methanol extracts, 236 mg wet biomass was freeze-dried (Edwards lyophilisator) yielding a dry cell mass of 7.3 mg. The entire dry cell mass was added to a microtube along with 300 mg glass beads (disruptor beads, 0.5 mm; Scientific Industries) and 1 mL

methanol (LC-MS grade; Fischer Scientific, Pittsburg, PA, USA) was homogenised three times for 15 s at a speed of 6.5 m s^{-1} with a FastPrep instrument (Savant Instruments Inc.) The mixture was then centrifuged as described above. The supernatant was stored at 4°C until chemical and bioactivity analyses were performed. For the detection of microcystin, the supernatant was diluted 500 times with methanol (LC-MS grade; Fischer Scientific).

Genomic DNA extraction

Genomic DNA of ASN_M was extracted utilising the E.Z.N.A. SP Plant DNA kit (Omega Bio-tek, Inc., Norcross, GA, USA). The microtube containing 100 mg wet cells was supplemented with 300 mg of two differently sized glass beads (acid-washed, $180 \mu\text{m}$ and $425\text{--}600 \mu\text{m}$; Sigma-Aldrich) as well as lysis buffer and RNase solution, both provided by the kit. In order to ensure proper disruption of the cells, tubes were homogenised three times for 20 s at a speed of 6.5 m s^{-1} with a FastPrep instrument (Savant Instruments). The extraction procedure was continued according to the kit's protocol, as supplied by the manufacturer. DNA was quantified with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA).

16S rRNA gene-based identification

Polymerase chain reaction (PCR) amplification. Two oligonucleotide primers comprised of one forward primer (359F, 5'-GGGGAATYTTCCGCAATGGG-3') and a reverse primer (781Ra, 5'-GACTACTGGGGTATCTAATCCCAT-3') (Nübel et al. 1997) were used for partial amplification of 16S rRNA gene.

One PCR reaction was comprised of 1 time buffer solution (DyNAzyme PCR buffer; Finnzymes, Espoo, Finland), $0.5 \mu\text{m}$ forward primer, $0.5 \mu\text{m}$ reverse primer and 0.5 U Taq polymerase (DyNAzyme II DNA polymerase; Finnzymes) as well as $1 \mu\text{L}$ template DNA and sterile water in a total volume of $20 \mu\text{L}$. The template DNA concentration of the ASN_M strain in the reaction accounted for approximately 140 ng.

As a positive control for the amplification procedure, genomic DNA of the cyanobacterium *Nostoc* sp. 202 (supplied by the University of Helsinki Cyanobacterial Culture Collection, UHCC) was used, whereas sterile water was used as a negative control. The amplification reactions were conducted in a thermocycler (iCycler; Bio-Rad, Foster City, CA, USA) with the following program: initial denaturation at 94°C for 3 min, 30 cycles comprised of

denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and annealing at 72°C for 30 s, as well as a final annealing phase at 72°C for 5 min.

PCR products were checked by electrophoresis on 1% agarose gels (SeaPlaque GTG; Cambrex Corp., East Rutherford, NJ, USA) at 100 V, followed by $0.10 \mu\text{g mL}^{-1}$ ethidium bromide (EtBr; Bio-Rad) staining.

PCR products were visualised in the gel by UV light utilising the Molecular Imager Gel Doc XR system (Bio-Rad). A digital gel image was obtained utilising the image analysis software (software BioRad/Quantity One version 4.6.7).

The size of the products was estimated by comparison to marker DNA (λ /HinfIII + ϕx /HaeIII; Finnzymes). The products were purified using the GeneClean Turbo kit (Qbiogene/MP Biomedicals, Solon, OH, USA) and were quantified with a Nanadrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

Sequencing and analysis. Sequencing of the partial 16S rRNA gene of ASN_M strain was subsequently carried out by the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Life Technologies, Foster City, CA, USA) with $10 \mu\text{L}$ reaction mixtures each containing $2 \mu\text{L}$ sequencing buffer, $1 \mu\text{L}$ big dye, $1 \mu\text{m}$ primer and 42 ng PCR product. Reactions for forward and reverse primers were prepared separately.

BLAST searches (<http://www.ncbi.nlm.nih.gov/BLAST>) of the partial 16S rRNA gene of ASN_M strain were used to identify similar sequences deposited in the GenBank database of NCBI. The 16S rRNA gene sequences obtained in this study, as well as reference sequences retrieved from GeneBank were first aligned with CLUSTAL W with the default settings and were then manually edited in BioEdit version 7.0. The positions with gaps, as well as undetermined and ambiguous sequences were removed for subsequent phylogenetic analyses. Phylogenetic trees using the neighbour-joining method was constructed by the MEGA version 4.1 (Tamura et al. 2007) using the Kimura two-parameter model. The robustness of the tree was estimated by bootstrap percentages using 1,000 replications. The root of the tree was determined using the 16S rRNA of *Aquifex aeolicus* and *Chloroflexus aurantiacus* as outgroups (Fig. 1). To prevent ingroup monophyly, 16S rRNA sequences of *Escherichia coli*, *C. aurantiacus*, and *Agrobacterium tumefaciens* were included in the alignment.

PCR amplification of potential toxin genes

To determine if the ASN_M strain is a potential produc-

Fig. 1. Photomicrograph of *Nostoc* sp. ASN_M. Cells show cell differentiation young hormogonia with the rare proheterocysts (PH) and vegetative cell developmental alternatives with intercalary heterocysts (H). Scale bar represents: 20 μ m.

er of toxins, genomic DNA was amplified by conventional PCR using primers targeting a specific gene of the biosynthetic gene cluster of the toxin to be detected (Tables 1 & 2).

For each reaction (Table 2), 50 ng of genomic DNA was used as a template. As a positive control for each amplification, genomic DNA of the selected cyanobacteria was used (supplied by the University of Helsinki Cyanobacterial Culture Collection, UHCC) (Table 1), whereas sterile water was used as a negative control.

The PCR program A included the following phases: initial denaturation at 95°C for 3 min, 30 cycles comprised of denaturation at 95°C for 30 s, annealing at 58°C for 30 s and annealing at 72°C for 30 s, as well as final annealing phase at 72°C for 5 min. The PCR program B was comprised of the same phases as program A, with the exception of the annealing phase being carried out at 60°C for

Table 1. Primers used to amplify genes of the microcystin, nodularin, anatoxin and hassallidin, as well as methylproline-containing compounds biosynthetic gene clusters

Primer pair (forward/reverse)	Gene	Genus	Toxin	Positive control	Amplicon size (bp)	References
mcyEF2/12R	<i>mcyE</i>	<i>Anabaena</i>	Microcystin	<i>Anabaena</i> sp. 202	247	Vaitomaa et al. 2003
mcyEF2/R8	<i>mcyE</i>	<i>Microcystis</i>	Microcystin	<i>Microcystis</i> sp. 205	247	Vaitomaa et al. 2003
mcyEF2/plaR3	<i>mcyE</i>	<i>Planktothrix</i>	Microcystin	<i>Planktothrix</i> sp. 49	249	Vaitomaa et al. 2003, Rantala et al. 2006
ndaF8452/8640	<i>ndaF</i>	<i>Nodularia</i>	Nodularin	<i>Nodularia</i> sp. HEM	188	Koskenniemi et al. 2007
anaC/anab	<i>anaC</i>	<i>Anabaena</i>	Anatoxin	<i>Anabaena</i> sp. 86	263	Rantala-Ylinen et al. 2011
anaC-osc	<i>anaC</i>	<i>Oscillatoria</i>	Anatoxin	<i>Oscillatoria</i> sp. PCC 9029	216	Rantala-Ylinen et al. 2011
hasA-F/R	<i>hasA</i>	nonspecific	Hassallidin	<i>Anabaena</i> sp. 90	920	Vestola et al. unpublished
nosF/R	<i>nosF</i>	nonspecific	Several ^a	<i>Nostoc</i> sp. UK1	1,000	Luesch et al. 2003

^aAmplifies compounds containing methylproline.

Table 2. Polymerase chain reactions for the amplification of genes involved in toxin biosynthesis (Table 1) in a total volume of 20 μ L

Primer pair (forward/reverse)	Forward primer (μ M)	Reverse primer (μ M)	dNTP ^a (μ M)	Taq polymerase ^b (U)	Buffer ^c	Program
mcyEF2/12R	0.5	0.5	250	0.5	1×	A
mcyEF2/R8	0.5	0.5	250	0.5	1×	B
mcyEF2/plaR3	0.5	0.5	250	0.5	1×	A
ndaF8452/8640	0.35	0.35	65	1	1×	B
anaC/anab	0.5	0.5	200	0.5	1×	B
anaC-osc	0.5	0.5	200	0.5	1×	B
hasA-F/R	0.5	0.5	200	0.4	1×	C
nosF/R	0.5	0.5	200	0.4	1×	C

^adNTP mixture (Finnzymes).

^bDyNAzyme II polymerase (Finnzymes).

^cDyNAzyme PCR buffer (Finnzymes).

Fig. 2. Neighbour-joining tree based on the partial 16S rRNA gene sequenced in this study or taken from the GenBank. The studied strain is shown with bold square. Numbers near nodes indicate bootstrap values over 50%. The scale indicates 0.05 mutations per amino acid position.

30 s. PCR program C was 94°C for 3 min, 36 cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 1 min, as well as 72°C for 10 min. Amplification products were checked by electrophoresis on 1.5 % (w/v) agarose gels followed by 0.10 µg mL⁻¹ EtBr (Bio-Rad) staining and visualization on an UV transilluminator.

Evaluation of antifungal activity

Extracted compounds of the ASN_M strain were examined for bioactivity against the yeast *Candida albicans* (Hambi 261) and the fungus *Aspergillus flavus* (Hambi 829) by disk diffusion assays. *C. albicans* culture was streaked onto Sabouraud agar plates and were grown for 24 h at 28°C, and *A. flavus* culture was streaked onto potato dextrose agar plates and also grown for 7 days at 28°C.

Colonies of the *C. albicans* subculture were transferred to 3 mL of saline solution (0.145 M NaCl) until the suspension reached a turbidity of 0.5 McFarland turbidity standard. *A. flavus* biomass was transferred into a saline solution until the inoculum suspension reached a turbidity value between 0.09 and 0.11 at 530 nm. Absorbance values were measured using a microplate spectrophotometer (Infinite M200; Tecan, Mannedorf, Switzerland). Müller-Hinton agar plates supplemented with 2% of dextrose and 0.5 µg of methylene blue were streaked with the suspension, as described by Espinell-Ingroff (2007), and were allowed to dry for 5 to 10 min. Disks (21-blank disks; Abtek Biologicals, Liverpool, UK) containing the extracted compounds were placed firmly against the agar. Plates were subsequently sealed with parafilm and were incubated for 48 h at 28°C (*A. flavus*) and 35°C (*C. albicans*).

Inhibition zones were measured and documented. The disks (21-blank disks; Abtek Biologicals) were prepared in microplates and were supplemented with sample and control extract and allowed to dry.

For each assay the following disks were prepared: a disk containing 730 µg of sample, another disk containing 10 mg of sample as well as a negative control disk containing 100 µL of methanol (LC-MS grade; Fischer Scientific) and a positive control disk containing 10 µg of nystatin.

Chemical analysis

ASN_M strain cell extracts were analysed by LC-MS using an Agilent 1100 Series LC/MSD Trap XCT Plus System (Agilent Technologies). Samples for analysis of anatoxin were separated with a Cogent Diamond Hydride column (100 Å, 150 mm × 2 mm, particle size 4 µm; MicroSolv, Eantontown, NJ, USA), whereas samples for quantitation of microcystin were separated with a Luna C18(2) reverse phase column (100 Å, 150 mm × 2 mm, particle size 5 µm; Phenomenex, Torrance, CA, USA) and samples for the analysis of other bioactive compounds were separated with a Luna C8(2) reverse phase column (100 Å, 150 mm × 2 mm, particle size 5 µm; Phenomenex). The injection volume of each sample was 10 µL. For anatoxin analysis, a commercial anatoxin-a standard (Enzo Life Sciences International, Farmingdale, NJ, USA) was used.

RESULTS

Morphological characterization and phylogenetic analysis of the 16S rRNA gene of ASN_M strain

The isolate was identified as a filamentous cyanobacterial strain. The vegetative cells were spherical or slightly longer than broad (4-5 µm broad and 5.5-7 µm long), olive, heterocysts somewhat globose (4.5-7 µm broad and 4-8.5 µm long), akinetes oblong, many in a chain (5-6 µm broad and 6.5-11 µm long). Thallus gelatinous, adhering by under surface, dull olive in color (Fig. 2).

Based on the description of the morphology provided by Desikachary (1959) this cyanobacterium was identified as a strain of *Nostoc*.

An 837 bp portion of the 16S rRNA gene was successfully amplified from the PCR amplicon obtained from the ASN_M strain. The phylogenetic tree which was constructed using the neighbor-joining method is shown in Fig. 1. Among available 16S rRNA sequences, the closest

Fig. 3. Zone of inhibition exhibited by methanolic extracts of ASN_M strain against of *Aspergillus flavus* (Hambi 829).

relative to ASN_M strain was *Nostoc_callicola_99* (Fig. 1). The partial 16S rRNA gene sequence *Nostoc* sp. ASN_M has been registered in the Data Bank of Japan (DDBJ) under the accession No. JF795282.1.

Identification of potential of bioactive compounds producers by PCR

PCR results confirmed the bioactivity potential of the ASN_M strain. A 1,000 bp portion of the *nosF* gene was successfully amplified from DNA extracted from *Nostoc* sp. ASN_M. No other toxin genes were amplified from this strain by PCR. Positive and negative controls work for *nosF* and all the other toxin genes were tested.

Assaying *Nostoc* strain for antifungal activity

The methanolic extract of ASN_M strain had an antifungal effect on *A. flavus* (Hambi 829) (Fig. 3). The inhibition zone diameter of *Nostoc* strain was 11 mm against the positive extract and 24 mm against *A. flavus* (Hambi 829). No allelopathic effect against *Candida albicans* (Hambi 261) was observed. The solvent control revealed no activity.

Chemical identification of cyanobacterial toxins and bioactive compounds

LC-MS analysis revealed the presence of three different classes of peptidic compounds (anabaenopeptins, cryptophycin, and nostocyclopeptides) from the ASN_M strain (Figs 4-7). No anatoxin-a, homoanatoxin-a, hassalidin or microcystin were detected in the ASN_M strain.

Fig. 4. Ion chromatograms of protonated cryptophycins (655.5 m/z), nostocyclopeptides (Nos; 757.5, 775.5, and 791.5 m/z) and anabaenopeptins (Ana; 809.5, 844.5, 858.5, 828.5, and 842.5 m/z) of the ASN_M strain. LC-MS, liquid chromatography mass spectrometry; EIC, extracted ion chromatogram.

Fig. 5. MS^2 fragmentation patterns of the protonated cryptophycins (655.5 m/z) of the ASN_M strain.

DISCUSSION

The classification of cyanobacteria has routinely relied on morphological characteristics which are not always trustworthy, as they may show variation depending on the culturing and environmental conditions, leading to misidentifications (Komárek and Anagnostidis 1989). In the present study, through a polyphasic approach, morphometric and genetic (16S rRNA) data were used to characterize the strain in a liquid suspension culture.

The result of morphological characteristics of the ASN_M strain and genetic criteria showed congruency. Thereby, this cyanobacterium was named as *Nostoc* sp. ASN_M (JF272482) strain.

Cyanobacteria produce a great variety of secondary metabolites. These include cyanobacterial toxins but also compounds interesting for therapeutic use (Fewer et al. 2009).

Sixteen metabolites with bioactivities, such as anticancer, antibiotic, antifungal, and antiviral properties, have

Fig. 6. MS² fragmentation patterns of the protonated anabaenopeptins (845.2, 859.1, 829.0 and 842.9 m/z) of the ASN_M strain.

Fig. 7. MS² fragmentation patterns of the protonated nostocyclopeptides (Nos; 776.0, 757.4, 809.9 and 791.7 m/z) of the ASN_M strain.

already been found in seven *Nostoc* strains (Namikoshi et al. 1990, Fujii et al. 1999, Oksanen et al. 2004). The most prevalent cyanobacterial natural products were produced in mixed pathways like mixed PKS / NRPS systems, as the cryptophycins (Sivonen 2009).

Molecular identification of bioactive compounds showed the amplification of *nosF* gene in ASN_M strain. *NosF* showed strong homology to Δ^1 -pyrroline-5-carboxylic acid (P5C) reductases (Luesch et al. 2003). Several studies have performed similar screening of these genes in chemical structures of the nostopeptolides (Golakoti et al. 2000, Hoffmann et al. 2003, Becker et al. 2004, Hunsucker et al. 2004). It has been emphasized that some of the genes responsible for the biosynthesis of this unusual amino acid appeared to be embedded in the 3' end of the nostopeptolide gene cluster.

Interestingly, the presence of the peptidic compound nostocyclopeptide in the ASN_M strain was confirmed by LC/MS screening (Fig. 7). Nostocyclopeptides, a cyclic hexapeptide, showed weak cytotoxicity against KB (a human nasopharyngeal carcinoma) and LoVo (a human colorectal adenocarcinoma) cell lines and were devoid of antifungal and antibacterial activities (Golakoti et al. 2001, Becker et al. 2004, Jokela et al. 2010).

Anabaenopeptins have been reported to cause a relaxation of noradrenaline-induced contraction of aortic preparations in rats and anabaenopeptolides have been documented to cause inhibition of the serine protease chymotrypsin (Harada 2004). Moreover, Murakami et al. (2000) found that anabaenopeptins, ureido bound-containing cyclic peptides, have potent carboxypeptidase-A inhibitors (Fig. 6).

Besides nostocyclopeptides and anabaenopeptins, the obtained results showed the presence of the cryptophycin from the ASN_M strain (Fig. 5). The ability of the ASN_M strain to produce cryptophycins and the potency of its extract against *A. flavus* in comparison with that of the control, support the hypothesis that the activity is due to a cryptophycin (Fig. 3). This finding is in an agreement with that of Schwartz et al. (1990), who isolated cryptophycin-1 from the cyanobacterium *Nostoc* sp. ATCC 53789 on the basis of its antifungal activity.

Although, the use of nostocyclopeptides and anabaenopeptins in medicine is not as wide spread as it once was, the use of cryptophycins in pharmacy, medicine, and biochemistry is well established.

Several types of semisynthetic analogue of cryptophycins with greater therapeutic efficiency and lower toxicity than cryptophycin are currently in clinical trials or pre-clinical trials or are undergoing further investigation.

Certainly, the exploitation of ASN_M strain as an organism with potential anti-fungal properties in the pharmaceutical industry has the potential to prevent many fungal diseases. Moreover, cryptophycins, by exerting antiproliferative and antimitotic activities by binding to the ends of the microtubules can be effective in treating many diseases, as the studies of Liang et al. (2005) showed excellent activity of cryptophycin against tumors implanted in mice.

Actively growing soil cyanobacterial mats may pose little risk as the majority of cyanotoxins are likely to be intracellular and not released into water. However, when mats die, as could be the case once a paddy field is drained, there could be large-scale release of cyanotoxins (Smith et al. 2011).

These findings indicate that soil cyanobacteria represent a promising but still unexplored natural resource possessing many bioactive compounds useful for the pharmaceutical industry. However, the toxicity of these soil cyanobacteria on soil microorganisms remains to be evaluated. This merits further and more detailed investigations.

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