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Investigation of the toxicity, antioxidant and antimicrobial activities of some cyanobacterial strains isolated from different habitats

Ugotavljanje toksičnosti ter antioksidativne in protimikrobne aktivnosti izbranih sevov cianobakterij, izoliranih iz različnih okolij

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Abstract: Cyanobacteria are known as a source of fine chemicals, renewable fuels, and toxic compounds. The present study aimed at evaluating the toxicity and antioxidant and antimicrobial activities of four cyanobacterial strains isolated from different habitats. Due to the lack of information regarding the *relationship between* toxicity and biological activity of the cyanobacteria in terrestrial and aquatic ecosystems of Iran, we decided to conduct a preliminary study on the cyanobacterial strains in order to identify the potentially toxic cyanobacteria strains. In this respect, biosynthesis genes related to cyanobacterial toxins, anatoxins (*anaC* gene), nodularins (*ndaF* gene) and microcystins (*mcyG* gene) were amplified. In addition, antioxidant, antimicrobial and biochemical properties of cyanobacterial strains have also been evaluated. The results of the molecular analysis demonstrated that only *Fischerella* sp. contained the microcystins (*mcyG)* gene. In fact, this strain encounters numerous predators in its habitat, therefore antibacterial and antioxidant metabolites found in this strain have thought to play an important role in defense mechanisms. This case is the documentation of toxicity and promotion of biological activities of a soil cyanobacterium regarding survival in competitive ecological niches.

Keywords: antioxidant, antimicrobial, toxicity, cyanobacteria

Izvleček: Cianobakterije so znan vir različnih, tudi toksičnih spojin, in obnovljivih goriv. Raziskava se osredotoča na toksičnost ter antioksidativno in protimikrobno aktivnost 4 vrst cianobakterij, izoliranih iz različnih habitatov. Zaradi pomanjkanja podatkov o povezavi med toksičnostjo in biološko aktivnostjo cianobakterij v kopenskih in vodnih ekosistemih v Iranu, smo izvedli prelimirano raziskavo. Pomnožili smo gene, vključene v biosintezo anatoksinov (gen *anaC*), nodularinov (gen *ndaF*) in mikrocistinov (gen *mcyG*). Določili smo antioksidativne, protimikrobne in biokemijske lastnosti cianobakterij. Molekularna analiza je pokazala, da je le *Fischerella* sp. vsebovala gen *mycG*. Ker je vrsta izpostavljena številnim predatorjem v ekosistemu, njeni antioksidativni in protibakterijski metaboliti igrajo pomembno obrambno vlogo. Primer dokazuje pomen toksičnosti in bioloških aktivnosti za preživetje v kompetitivnih ekoloških nišah pri talni cianobakteriji.

Ključne besede: antioksidant, protimikrobno delovanje, toksičnost, cianobakterije

Introduction

Cyanobacterial strains are interesting natural source of new compounds with biological activity that can be used in deterring predatory organisms in the environment (Nowruzi et al. 2019). They are a potential source of antimicrobial and antioxidant substances due to their diversity of secondary metabolites, which are helpful in survival in varied and highly competitive ecological niches (Kalaitzis et al. 2009). Moreover, these compounds are often involved in protection against biotic or abiotic stresses, like bacteria, fungi, nematodes, and insects or grazers.

On the other hand, cyanobacteria produce an unparalleled variety of toxins that can cause severe health problems or even death in humans and wild or domestic animals. They are widely known for their potential to produce a range of neurotoxic, hepatotoxic, and tumor-promoting secondary metabolites (Sivonen and Börner 2008).

Both toxic and nontoxic strains of cyanobacteria synthesize antibacterial and antioxidant compounds that are distinct from cyanotoxins (Corbel et al. 2014). Moreover, cyanotoxins, as antimicrobial and antioxidant compounds of cyanobacteria, affect numerous aquatic and terrestrial organisms, which can be concluded that toxin production predominantly occurs to defense against grazing and/or to reduce resource competition (Holland and Kinnear, 2013). The role of secondary metabolites in defense mechanisms may be due to their toxicity or acting as precursors to physical defense systems (Nowruzi et al. 2018a, Liu et al. 2014).

Despite the reported antioxidant and antimicrobial properties and the toxic potential of cyanobacteria as sources of these compounds, few studies have focused on the relationship between toxicity and biological activity of the cyanobacteria in terrestrial and aquatic ecosystems of Iran. Therefore, the main objective of this study was to determine the genetic, antioxidant, antimicrobial properties as well as biochemical quantification of some cyanobacterial strains isolated from different habitats.

Materials and Methods

Sampling, culturing and phenotypic analysis

Soil samples with different textures (according to the pedological map of the Kermanshah and Golestan provinces, Iran) were selected and collected from agricultural areas and paddy fields, respectively. The samples were collected from the surface up to 5 cm deep using a sterilized spatula after removing surface debris.

Salt and freshwater samples were collected from Golestan Province at a depth of 30 cm and 1 m away from the shore using cone-shaped bottles. Nitrate-free BG-11 solid media was made and its pH was adjusted to 7.1 after sterilization. Then samples were transferred to sterilized petri dishes (Shokraei et al. 2019, Nowruzi and Ahmadimoghadam 2006,) and incubated in a culture chamber at 28°C, supplied with constant artificial lighting (100-120 µmol photon m^{-2} s^{-1}) for two weeks (Nowruzi et al. 2017 a, b, c).

After 14 days, one or two colonies were isolated for purification, washed thrice with deionized water and transferred to fresh solid media. In order to keep bacteria-free cultures, the colonies were isolated and tested for bacterial contamination in dextrose-peptone broth and caseinate-glucose agar media. Thereafter, bacteria-free colonies were selected and maintained on different agar slants. No further analysis of the cultures was done until pure clonal cultures were established and examined microscopically (Nowruzi et al. 2012 c).

Detailed morphological analyses of these strains were carried out at the time of isolation in order to avoid difficulties in identification. The type of filament orientation, sheath (overall distribution and visibility across the trichome), dimensions and shape of the vegetative cells and heterocysts were determined. Two most frequent heterocytous cyanobacteria strains were selected to investigate the differences for morphological and genotypic studies through a polyphasic approach. Fresh cultures and exsiccates were deposited in the Cyanobacteria Culture Collection (CCC) and herbarium ALBORZ of the Islamic Azad University, Science and Research Branch, Tehran.

DNA extraction and molecular identification and detection of potential toxins from cyanobacteria strains

DNA was extracted and quantified as previously described (Nowruzi et al. 2018b). Presence of 16S rRNA gene and biosynthesis genes for cyanobacterial toxins [anatoxins (*anaC* gene), nodularins (*ndaF* gene) and microcystins (*mcyG* gene)] were evaluated in each species.

DNA fragments within the selected genes were amplified using the oligonucleotide primers listed in Table 1.

Polymerase chain reaction (PCR) reactions were performed using a thermal cycler and the procedure illustrated in Table 2. According to Table 3, the PCR programs 1 and 2 were used. Amplified products were detected in a 1.0 % agarose gel, which was prepared with 0.5% TAE-buffer containing 1 µL of ethidium bromide. The samples (3 µL) and Loading Dye (3 µL) were pipetted to a gel stock. Five µL of the marker (Lambda DNA-Hind III/PhiX-174 RF DNA-HaeIII Digest, Fermentas, USA) was used. Sequencing of the amplified 16S rRNA and *mcyG* genes were performed by cycle sequencing using the ABI Prism 310 Genetic Analyzer (Applied Biosystems). A total volume of 10 pL (10 milllion picoliter –sized) of the PCR master mix included 1 pL of of the primer, 1 x sequencing buffer, 1 pL of BigDye® Terminator v3.1 and 100 ng of DNA for each 1 kb were sequenced. PCR was performed separately for the forward and reverse primers. The PCR program included 25 cycles of 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min, followed by storaging the product overnight at 4°C. The product was then precipitated by adding 40 pL of 0/125 M NaCl and 2/5 x volume of cold 100% ethanol, followed by vortexing and centrifugation for 10 min at 13,000 x G at 4°C. Once the supernatant was removed, a 5 x volume of 70% ethanol was added, and the sample was then centrifuged for 5 min at 13,000 x G at 4°C. The supernatant was again removed, and the pellet was dried at 37°C. Next, 12 pL of HiDi-formamide was added, and the mixture was spun down and denatured for 2 min at 94°C and subsequently analysed with an ABI PRISM® 310 Genetic Analyser (Applied Biosystems, Life Technologies). The runtime for each reaction was 45 min with a running voltage of 15 kV at the temperature of 50°C using POP-6™polymer (Applied Biosystems, Life Technologies). Moreover, the BLAST searching (http://www.ncbi.nlm.-nih.gov/BLAST) of the partial 16S rRNA gene of the studied strains were used to identify similar sequences deposited in the GenBank™ at the NCBI.

Table 1: Target genes and oligonucleotide primers used in this study.
Tabela 1: Uporabljeni geni in začetni oligonukleotidni.

Target gene/ sequence	Oligonucleotide sequence 5´-3´	Reference
16S rRNA	pA: (5’-AGAGTTTGATCCTGGCTCAG-3’) B23S: (5’-CTTCGCCTCTGTGTGCCTAGGT-3’)	Taton et al. 2003
mcy G	mcyGF: GAAATTGGTGCGGGAACTGGAG mcyGR: TTTGAGCAACAATGATACTTTGCTG	Rantala et al. 2006
nda F	*ndaF* 8452: GTGATTGAATTTCTTGGTCG *ndaF* 8640: GGAAATTTCTATGTCTGACTCAG	Koskenniemi et al. 2007
ana C	*ana C*: CTCTATTCTCACAAGTTTGGTCT *ana B*: GTTAGTTCAATATCAAGTGGTGGA	Rantala-Ylinen et al. 2011

Table 2. Master-mixes for different PCR-reactions used for detecting the genes of toxins and bioactive compounds synthesis.
Tabela 2: Uporabljene mešanice za PCR pri določanju genov, vključenih v sintezo toksinov in bioaktivnih spojin.

PCR master mix	1 X Buffer (µl)	Primer forward 10 µM (µl)	Primer reverse 10 µM (µl)	DyNazyme 2 U/µl (µl)	dNTP- mix 10 mM (µl)	Sample size (µl)	Amplicom size (bp)
16S rRNA	2	0.5 (pA)	0.5 (B23S)	0.25	0.5	20	1500
nda F	2	0.7 (*ndaF* 8452)	0.7 (*ndaF* 8640)	0.5	0.13	20	188
ana C	2	1.0 (*ana C*)	1.0 (*ana B*)	0.25	0.4	20	263
mcy G	2	1.0 (*mcyGF*)	1.0 (*mcyGR*)	0.2	0.4	20	1000

Table 3: PCR-programs for 16S rRNA and toxic genes.
Tabela 3: Programi za PCR za 16S rRNA in gene za toksičnost.

PCR program	Preheating	Heat cycle	Anneal cycle	Polymerase cycle	Final polymerase	Final T	Number of cycles
PCR 1 (16S rRNA)	94⁰C, 3 min	94⁰C, 30 s	55⁰C, 30 s	72⁰C, 30 s	72⁰C, 5 min	4 ⁰C	30
PCR 2 (*ndaF*, *ana*C and *mcyG*)	95⁰C, 3 min	95⁰C, 30 s	60⁰C, 30 s	72⁰C, 30 s	72⁰C, 5 min	4 ⁰C	30

Antibacterial activity

The toxicity of the three strains was analyzed using the disc diffusion method, with several human pathogenic bacteria: *Staphylococcus aureus* (ATCC-25923), *Escherichia coli* (ATCC-25922), *Streptococcus pneumonia* (ATCC-6305), *Salmonella typhimurium* (ATCC -14028) growing on Müller-Hinton agar and Mueller Hinton agar with Blood. Some colonies from the plates were applied to a saline solution so that the turbidity fitted to 0.5 McFarland standards. At the stationary phase of growth (15th day), the collected biomass was freeze-dried and resuspended in methanol (1 ml) and collected in 2 ml plastic tubes containing approximately 200 ml of 500 mm glass beads (Scientific Industries, New York) using Fast Prep homogenizer (FP120, Bio 101, Savant) with the speed of 6.5 mm/s. After centrifugation at 20 000 rpm for 5 min, the supernatant was collected at a concentration of 500 mg ml^{-1}. Next, 100 µL of the methanolic cyanobacterial extract was pipetted into the 6 mm diameter filter paper discs in assay plates. The plates were incubated overnight at 37°C for a period of 18-24 h. To determine the stability of the methanolic cyanobacterial extract, filter discs were stored in petri dishes at room temperature for 10 days and the antibiogram bioassays were applied again (Nowruzi et al. 2012b). The diameters of the zones with complete inhibition of growth were measured to the nearest millimeters using a ruler. All experiments were performed under sterile conditions in triplicate.

Nitric oxide (NO) scavenging activity

The nitric oxide radical scavenging activity was measured using Griess reagent. Accordingly, 50, 100, 150 and 250 µL of the methanolic extract (710 µg mL^{-1}) and 1 mg/mL of the Vitamin C reference standard were taken and diluted up to 1.5 mL with distilled water in test tubes. Then, 1.5 mL of 10 mM Sodium Nitroprusside was added to all tubes and incubated for 150 min at 25°C. After incubation, 1.5 mL of the reaction mixture was transferred to the new tubes and 1.5 mL Griess reagent (1% sulphanilamide, 2% orthophosphoric acid, and 0.1% NEDD) was added to all tubes. Optical density was taken at 545 nm (Kamble

et al. 2013). Vitamin C was used as a positive control and a decrease in absorbance indicated higher scavenging activity. The experiments were performed in triplicate and the percentage of scavenging activity was calculated as follows (Arun et al. 2012):

$$\text{Scavenging (\%)} = \frac{\text{Absorbance (control) - Absorbance (test)}}{\text{Absorbance (control)}} \times 100$$

Qualitative analysis of biochemicals in the methanol extract of cyanobacteria strains

The cyanobacterial cultures were centrifuged at 2500 rpm for 10 min to harvest the biomass. After centrifugation, the supernatant were removed and freshly-harvested cell pastes were air-dried in an oven at 60°C. One gram of the dried samples was extracted with 10 mL of the methanol and was soaked in the solvents for 48 h. The mixture was then centrifuged at 2000 rpm for 10 min at 4°C. The supernatants were filtered through a sterile Whatman filter paper No. 1. The obtained extracts were used for screening their biochemical potential. Detection of proteins and amino acids and carbohydrates was done according to the Millon's test (Arun et al. 2012). Detection of phytosterols was done with modified protocol by Arun et al. (Arun et al. 2012). In this method, the extract (75 mg) was dissolved in 3.5 mL of acetic anhydride. To do this, 3 or 4 drops of the concentrated sulfuric acid was added slowly along the sides of the tube. Moreover, the extract was analyzed for the presence of phenolic compounds, flavonoids, terpenoids, saponins, alkaloids and glycosides (Raaman, 2006). Glycoside analysis was done according to the Keller-Killani test using the protocol developed by El-Karim (2016).

Statistical analysis

The results of each experiment were analyzed by analysis of variance (ANOVA) using SPSS 24 and differences between the groups were detected by Tukey's multiple comparison test. P values of smaller than 0.05 were considered significant. The main values and the standard deviations were calculated using the data obtained from the trials.

Results

Morphological and molecular characterization

The strains were identified using specific keys (Komárek et al. 2013) according to their morphological characteristics. *Morphological* data appeared *congruent* with all *molecular* data (sequenced data). Four strains belonging to different habitats were selected. Based on 16 rRNA BLASTn, the collected strains showed more than 90% similarity with *Fischerella* sp.. *Calothrix* sp., *Nostoc* sp., *Spirulina* sp. and *Fischerella* sp. were collected from agricultural areas of Kermanshah province and *Nostoc* sp., *Spirulina* sp. and *Calothrix* sp. were collected from salt water, freshwater and paddy fields of Golestan province. Morphological and morphometric descriptions of each strain are shown in Figure 1.

Fischerella sp.: Thallus uniseriate, true branching A(a) olive green, vegetative cells in main filaments, spherical to rectangular, 0.7–1.1 × longer than wide, 6.5–13.5 µm long × 6.3–15.5 µm wide A(b); but in branched filaments, spherical or slightly oblong, 3–8 × longer than wide, 11.2–29.5 µm long × 4.5–6.0 µm wide A(c); heterocysts in main filaments, elongate, spherical or even compressed (shorter than broad) intercalary, 10.0–22.5 µm long × 6.5–11.5 µm wide. Akinetes, oblong, mainly in chains, 5.0–6.0 µm broad, 6.5–11.0 µm long (Figure 1A).

Spirulina sp.: Trichomes, unbranched and not forming a regular and definite spiral, in which the cross-walls between adjacent vegetative cells are visible B(a); however, in some filaments, the individual cells may be difficult to distinguish B(b); trichomes, 1–8 µm diameter; multiplication occurs only by fragmentation of a trichome, usually in correspondence of a necridial cell B(c) (Figure 1B).

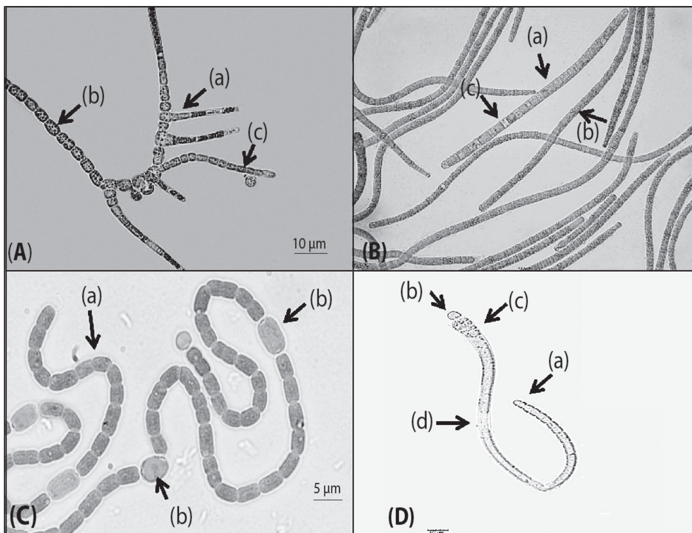

Figure 1: Micrograph of identified cyanobacteria strains. **A**: *Fischerella* sp. (10 µm). **B**: *Spirulina* sp. (10 µm). **C**: *Nostoc* sp. (5 µm). **D**: *Calothrix* sp. (10 µm).

Slika 1: Mikrografije identificiranih cianobakterij. **A**: *Fischerella* sp. (10 µm). **B**: *Spirulina* sp. (10 µm). **C**: *Nostoc* sp. (5 µm). **D**: *Calothrix* sp. (10 µm).

Nostoc sp.: Trichomes, brownish or dark–colored, vegetative cells similar in form, cylindrical, 3.5–4.0 µm broad, 7.0–11.0 µm length, and brownish C(a); heterocysts, slightly spherical or oblong, 5.0–6.5 µm wide, 6.0–12.5 µm long C(b); akinetes, ellipsoidal to oblong, 5.0–5.5 µm broad, 10.0–12.0 µm long (Figure 1C).

Calothrix sp.: filaments, curved, swollen at the base, slightly bent D (a), vegetative cells at the base of trichomes with conical terminal cells D(a); heterocysts, basal, yellowish, single, hemispherical or half-rounded D(b); akinetes, cylindrical, adjacent to heterocysts and usually occurring in series D(c); in some cases, hormogonia is separated from the rest of the trichomes by a necridial cell D(d). (Figure 1D).

Molecular analysis of cyanotoxins-coding genes

Genetic analysis of Microcystin, Anatoxin, and Nodularin was done by amplifying the *mcyG, anaC* and *ndaF* genes and only *mcyG* was found in *Fischerella* sp. collected from agricultural areas. Studied genes were registered at the DNA Data Bank of Japan (DDBJ) with the accession numbers, including KY618863 (*Fischerella* sp.), MK517824 (*Nostoc* sp.), MG356333 (*Calothrix* sp.) for 16S rRNA and MK211496 for *mcyG* gene (*Fischerella* sp.).

Antimicrobial activity of cyanobacterial strains

Antibacterial activity was observed after 18-24 h in all tested Gram positive and Gram negative bacteria. It was found that the potentially toxic cyanobacterium (*Fischerella* sp.) exhibited the highest inhibitory activity (15.10 ± 0.03 mm) against only for *Streptococcus pneumonia* in comparison to other strains against all tested bacteria, probably due to the presence of the toxic compounds. *Nostoc* sp., *Calothrix* sp. and *Spirulina* sp. showed strong inhibition against *Streptococcus pneumoniae* with the following zone of inhibition: 13.93 ± 0.06 mm, 13.96 ± 0.03 mm and 8.03 ± 0.03 mm, respectively. Moreover, *Fischerella* sp. revealed no significant decrease in the inhibitory effect after 10 days, whereas there was a significant decrease in the inhibitory effect of *Nostoc* sp. (Table 4).

Table 4: Antibacterial activity of the cianobacterial extracts presented as growth inhibition (diameter of zone inhibition) of Gram positive and Gram negative bacteria. Data are mean ± standard deviation. Different letters indicate statistically significant differences (P>0.05, ANOVA followed by Tukey›s grouping tests).

Tabela 4: Protibakterijska aktivnost ekstraktov, prikazana kot zavrta rast (premer cone inhibicije) pri Gram pozitivnih in negativnih bakteriah. Prikazane so povprečne vrednosti ± standardni odklon. Različne črke pomenijo statistično značilno razliko (P>0,05 ANOVA in Tukey post-test).

	Diameter of zone inhibition (mm)			
Extract	***Streptococcus pneumonia***	***Salmonella* sp.**	***E. coli***	***Staphylococcus aureus***
			After 18-24 h	
***Nostoc* sp.**	8.0 ± 0.05 (a)	6 ± 0.07 (b)	9.9 ± 0.09 (a)	7.9 ± 0.07 (c)
			After 10 days	
	4.1 ± 0.03	3.2 ± 0.05	5.1 ± 0.06	3.1 ± 0.05
			After 18-24 h	
***Calothrix* sp.**	13.9 ± 0.05 (a)	8.5 ± 0.1 (b)	9.9 ± 0.06 (c)	7.1 ± 0.1 (d)
			After 10 days	
	10.1 ± 0.04	5.1 ± 0.03	4.7 ± 0.06	3.2 ± 0.06
			After 18-24 h	
***Fischerella* sp.**	15.1 ± 0.05 (a)	8.5 ± 0.05 (b)	11.0 ± 0.06 (c)	10 ± 0.07 (d)
			After 10 days	
	14.6 ± 0.02	5.1 ± 0.04	10.3 ± 0.03	9.1 ± 0.05
			After 18-24 h	
***Spirulina* sp.**	13.9 ± 0.07 (a)	6.2 ± 0.07 (b)	9.9 ± 0.04 (c)	7.9 ± 0.05 (c)
			After 10 days	
	9.9 ± 0.07	4.5 ± 0.06	5.1 ± 0.04	3.2 ± 0.03

Antioxidant activity of cyanobacterial strains

Free radical scavenging potential of the studied strains was found to be concentration-dependent. Toxic cyanobacterium (*Fischerella* sp.) had strongest percentage of inhibition of nitric oxide generation, and significantly scavenged 70.33%, 73.75%, 76.33% and 77.5% the nitric oxide radicals in the volumes of 50, 100, 150 and 250 µl, respectively (Table 5).

Table 5: Nitric oxide scavenging activity of cyanobacteria (reagent stock concentration 710 µg mL^{-1}). Data are mean ± standard deviation. Different letters indicate statistically significant differences (P>0.05, ANOVA followed by Tukey›s grouping tests).

Tabela 5: Odstranjevanje dušikovega oksida pri cianobakterijah (založna raztopina reagenta 710 µg mL^{-1}). Prikazane so povprečne vrednosti ± standardni odklon. Različne črke pomenijo statistično značilno razliko (P>0,05 ANOVA in Tukey post-test).

Reagent volume	***Nostoc* sp.**	***Calothrix* sp.**	***Fischerella* sp.**	***Spirulina* sp.**
50 µl	64.6 ± 0.1 (a)	69.2 ± 0.3 (b)	70.3 ± 0.1 (c)	68.0 ± 0.3 (c)
100 µl	66.0 ± 0.1 (a)	71.7 ± 0.2 (b)	73.7 ± 0.1(b)	68.2 ± 0.3 (c)
150 µl	68.0 ± 0.1 (a)	75.5 ± 0.3 (b)	76.3 ± 0.1 (c)	70.5 ± 0.3 (d)
250 µl	69.9 ±0.1 (a)	76.5 ± 0.1 (b)	77.5 ± 0.3 (c)	77.0± 0.1 (d)

Table 6: Qualitative screening of cyanobacterial species for the presence of certain phytoconstituents.
Tabela 6: Kvalitativno ugotavljanje fitokemijskih spojin v vrstah cianobakterij.

Compound	***Nostoc* sp.**	***Fischerella* sp.**	***Calothrix* sp.**	***Spirulina* sp.**
Carbohydrate	-	-	-	+
Alkaloids	+	+	+	+
Glycoside	+	+	+	+
Saponin	+	+	+	+
Phytosterols	+	-	-	+
Phenolic compound	+	+	+	+
Flovnoids	+	+	+	+
Amino acids and proteins	+	+	+	+

Qualitative biochemical analysis of methanol extract

Phenolic compounds, flavonoids, saponins, alkaloids, glycosides and proteins were detected in all extracts. Carbohydrates was only found in *Spirulina* sp. and phytosterols was only found in *Nostoc* sp. and *Spirulina* sp. Carbohydrates were absent in *Nostoc* sp. *Fischerella* sp., and *Calothrix* sp., whereas phytosterols were absent in *Fischerella* sp. and *Calothrix* sp. Saponin, phytosterols and phenolic compound were found with high color intensity in *Spirulina* sp. (Table 6).

Discussion

In recent years, secondary metabolites of various cyanobacterial strains are often believed to have an important ecological role in competition against other algae and plants (Sukenik et al. 2015).

Cyanobacteria are a rich source of novel bioactive compounds, which are widely used in human and animal medicine. A promising strategy for the replacement of antibacterial and antifungal chemicals is to promote the natural biological control products obtained from cyanobacteria (Kultschar and Llewellyn, 2018, Nowruzi et al. 2012a, Nowruzi et al. 2013b, Nowruzi et al. 2018b). Although several chemical libraries have constructed for screening new drugs, natural products are still an important resource for drug leads (Cragg and Newman 2013).

The presence of microcystin-producing cyanobacterium (*Fischerella* sp.) in agricultural areas showed that the source of contamination was possibly microcystin-contaminated water. Among cyanobacterial toxins, microcystins are the most widespread group (Corbel et al. 2014). Microcystins can persist in agricultural soils for relatively long times, with a half-life ranging between 6 and 17.8 d (Bouaïcha and Corbel 2016). Jones et al. (1995) reported that the scums of *Microcystis aeruginosa* that dry on the shores of lakes may contain high concentrations of multiple chemical sensitivities (MCS) for several months. Thus, the persistence of these toxins within dried cyanobacterial cells for a long period suggests that they will be released back into the soil by leaching, runoff and drainage processes or can be accumulated in soils and, therefore, may cause contamination of vegetation by absorption from soils or by surface pollution of the plants (Bouaïcha and Corbel 2016). In addition, it seems that cyanotoxins sorption in soils is low, which can potentially result in their high bioavailability to soil organisms and plants (Victory 2009). Recently, much attention is given to cyanobacteria, due to the presence of biologically active compounds, such as phenolic compounds, flavonoids, saponins, alkaloids, glycosides, proteins, carbohydrates and phytosterols (Mazard et al. 2016). Carbohydrate storage is a crucial element of metabolism in cyanobacteria cells during diurnal growth. Storage of carbohydrates is also an important factor toward harnessing cyanobacteria for energy production.

All members of the cyanobacteria strains stored different form of carbohydrates. The reason why some cyanobacteria form different carbohydrate storage granules is still unknown. Accordingly, in this study, we found the absence of carbohydrates in 3 out of 4 cyanobacterial strains, which this result is in agreement with bioinformatic analysis of *Cyanothece*, *Cyanobacterium*, and *Synechocystis* species that revealed varying copy numbers of all genes involved in the synthesis and degradation of storage polysaccharides (Welkie et al. 2016).

Moreover, Cyanobacteria strains have been reported to prevent oxidative damage by scavenging free radicals and active oxygen, resulting in the reduced risk of cancer indirectly in human body (Bouaïcha and Corbel 2016). The presence of alkaloids, in all extracts of the studied strains might exert a remarkable antibacterial activity against Gram positive and Gram negative bacteria. Moreover, cellular presence of phenolic compounds has also been coupled with both antioxidant and antibacterial activities. Phenolic compounds are a class of antioxidant agents, which act as free radical terminators. Phenolic compounds have been extensively studied for their antioxidant properties not only in fruits and vegetables, but also in cyanobacteria (Rajeshwari and Rajashekhar 2011). Cyanobacteria phenolic compounds have reported to be potential antioxidants against free radicals, which are harmful to our body and food systems. Several epidemiological studies have revealed that phenolic compounds present in diet are helpful in treating coronary heart disease. Furthermore, phenols have been reported to exhibit pharmacological properties, such as anticarcinogenic, antiviral, antimicrobial, anti-inflammatory or anti tumoral activities (Nowruzi et al. 2020). Miranda et al. (1998) studied the antioxidant activity of carotenoids, phenolic and tocopherols extracted from *Spirulina maxima* and found that the phenolic compounds are responsible for the antioxidant properties of their extracts. This hypothesis was agreed with our results, as all four species containing phenolics and flavanoids exhibited antioxidant activity. Although high amount of saponin, phytosterols and phenolic compound were found in *Spirulina* sp. in comparison to the other strains, the obtained results confirmed that microcystin-producing cyanobacterium (*Fischerella* sp.) was the most effective free radical scavenger and antimicrobial agent. This potential activity might be related to the toxic compounds detected in cyanobacteria strain terrestrial ecosystems. However, it is also possible that the primary function(s) of cyanotoxins cannot be associated with their toxic properties (Bouaïcha and Corbel 2016). Given that toxin production by cyanobacteria has been retained over long evolutionary periods, and despite the metabolic costs of production, it seems highly likely that these compounds have important biological function(s) (Rajeshwari and Rajashekhar 2011). For example, recent studies examining cyanotoxin production have found a close relationship between these compounds and certain physiological functions that may be considered as a part of the primary metabolism of the cell (Bouaïcha and Corbel 2016). Several other physiological roles have been speculated for cyanotoxins, such as cell signaling, nutrient uptake, iron scavenging, maintenance of homeostasis, and protection against oxidative stress. Moreover, flavonoids are a group of polyphenolic compounds with known properties, including free radical scavenging, inhibition of hydrolytic and oxidative enzymes and anti-inflammatory activity. Bouaïcha and Corbel suggests that the biological actions of these compounds are related to their antioxidant activity (Bouaïcha and Corbel 2016). However, in the present research, we found a weak amount of flavonoids in four studied strains.

Comparatively, few studies have considered the ecological role of secondary metabolites, and their possible applications on protection of microorganism against grazing (Kultschar and Llewellyn 2018, Holland and Kinnear 2013). Therefore, this study explored the genetic evidence for cyanotoxin production in natural environments. These metabolites are often produced by cyanobacteria in response to biotic or abiotic stress in the surrounding environment by providing protection and survival resulting in an advantage over other species. Agricultural paddy field ecosystems provide an environment favorable for the growth of heterocystous cyanobacteria, due to the moderate light, water, high temperature, and nutrients. However, they have close associations and intense competition compared to other species and a higher frequency of encounters with numerous predators, including grazers and phage

(Falaise et al. 2016). Actually, toxins, antibacterial and antioxidant compounds are released when the cells lose its integrity and they seem to be involved in an "activated" defense mechanism to protect them against grazing predators. Therefore, the toxicity and antibacterial and antioxidant activities in microcystin-producing cyanobacterium (*Fischerella* sp.) are thought to play an important role in defense mechanisms to attempt to get the upper hand and thrive within their niche choice. However, further investigations with a broader selection of toxic cyanobacteria are necessary to confirm their biological activities. Moreover, it is necessary to perform successful separation, purification and identification of the responsible metabolites with different biological activities.

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