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Cave Cyanobacteria showing antibacterial activity

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Abstract: Cave Cyanobacteria - thriving in an 'extreme' environment with interesting species biodiversity - are supposed to be a potential source of bioactive compounds. Lipid extracts from pure cultures of two recently established Cyanobacteria from Greek caves, *Toxopsis calypsus* and *Phormidium melanochroun*, were used for antibacterial screening against human pathogenic bacteria (reference and clinical isolates). Antimicrobial Susceptibility testing for both taxa was carried out using the disc-diffusion (Kirby Bauer) method, while preliminary data applying the standard broth microdilution method for the determination of the Minimal Inhibitory Concentration (MIC) are given only for *T. calypsus*. Antibacterial activity was demonstrated against the Gram-positive clinical and reference bacteria, mostly pronounced in enterococci; no activity was observed against the Gram-negative bacteria. The above screening is the first record of antibacterial activity from lipid extracts of cave Cyanobacteria enhancing the importance of cave microbiota and the necessity for cave conservation.

Keywords: cave Cyanobacteria; *Toxopsis calypsus*; *Phormidium melanochroun*; antimicrobial susceptibility screening

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INTRODUCTION

Cyanobacteria represent a group of Gram-negative photoautotrophic prokaryotes thriving in almost all aquatic and terrestrial habitats on earth, including extreme environments. This widespread distribution reflects the tolerance of Cyanobacteria towards environmental stress due, *inter alia*, to a broad spectrum of specific properties in physiology (Uzair et al., 2012). Generally, microorganisms forming microbial mats in extreme environments have been recently identified as a good source of bioactive compounds for different biotechnological applications (Harvey, 2000; Dobretsov et al., 2011).

Modern research has focused on a variety of bioactive compounds produced by Cyanobacteria. After analysis of a great number of marine cyanobacterial natural products, lipopeptides seem to prevail followed at much lesser proportions by amino acids, fatty acids, macrolides and amides (Burja et al., 2001; Singh et

al., 2011; Engene et al., 2013). These interesting and biochemically active compounds possess biological activity covering a wide range of antibacterial (Mundt et al., 2003; Kaushik & Chauhan, 2008; Ramadan et al., 2008; Asthana et al., 2009; Kaushik et al., 2009; Khairy & El-Kassas, 2010; Suhail et al., 2011), antifungal (MacMillan et al., 2002), antialgal (Papke et al., 1997), antiviral (Hayashi et al., 1996; Zainuddin et al., 2002), anti-thrombotic (Antonopoulou et al., 2002; 2005a,b) and also anticancer effectiveness (Luesh et al., 2001; Simmons et al., 2005).

Many Cyanobacteria from various biotopes have been tested for antibacterial activity, e.g. marine (Luesh et al., 2001; Simmons et al., 2005; Mathew et al., 2008; Vijaya Baskara Sethubathi & Ashok Prabu, 2012), freshwater (Østensvik et al., 1998; Mian et al., 2003; Madhumathi et al., 2011) and terrestrial (Mian et al., 2003; Abdel-Raouf & Ibraheem, 2008; Ramamurthy et al., 2012). Considering cave ecosystems as an extreme environment (due to the insufficient light

and nutrient limitation), antibiotic effectiveness by cave bacteria has recently been recorded (Montano & Henderson, 2013); however, no studies have yet identified the antibacterial potential of cyanobacterial isolates from caves.

Exploitation of new natural products as antibacterial agents against resistant pathogens is very important for clinical medicine and public health, and a limited number of new antimicrobial classes have been developed by the international pharmaceutical industry in the last 20 years (Infectious Diseases Society of America, 2007). The aim of the present study is to assess the potential antibacterial activity of extracts from two recently established Cyanobacteria from Greek caves, i.e. *Toxopsis calypsus* and *Phormidium melanochroun* (Lamprinou et al., 2012, 2013). It is noted that cave environments are still relatively underexploited, and may prove to be a rich source of novel biodiversity possessing bioactive compounds potentially useful in biotechnology.

MATERIAL AND METHODS

Sampling

Fresh material, as scrapped mats and pieces of rocks of ≤ 5 g, was collected from 'Francithi' Cave ($37^{\circ}25'21.01''N$, $22^{\circ}17'51.18''N$; altitude 12.5 m a.s.l.), an exposed, non typical cave, with partly collapsed roof, located in Argolida (Peloponnese, Greece). Sampling was conducted seasonally at seven selected sites from the entrance inwards. Temperature (average $18.26^{\circ}C$, min $11.53^{\circ}C$, max $25.94^{\circ}C$), Relative Humidity (average 66.20%, min 50.73%, max 93.51%) and photosynthetically active radiation (average $3.09 \mu\text{mol}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$, min $0.08 \mu\text{mol}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$, max $26.70 \mu\text{mol}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$) were measured at each sampling site and sampling date by a LI-1400 data logger (LI-COR Biosciences, USA). Four subsamples were collected from each sampling site. Two of them were incubated *in situ* into sterile transparent vials and, the other two were partly fixed with formaldehyde solution at 2.5%. Enrichment cultures were obtained in flasks and petri dishes with culture media (BG11_o and BG11, Stanier et al., 1971) and under proper conditions (Gallenkamp, Sanyo incubator; $23^{\circ}C$, 80% RH, $7 \mu\text{mol}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$). The two Cyanobacteria selected for antibacterial screening (Tables 1 a,b) were: (i) *Toxopsis calypsus* (type strain: ATHU-CY 3314, GenBank acc. Nr. JN695681-JN695685) found at the nearest to the entrance site, and (ii) *Phormidium melanochroun* (type strain: ATHU-CY 3315, GenBank acc. Nr. JQ692233) found in almost all sampling sites. The required time for attaining sufficient biomass for lipid extraction was 150-200 days.

Lipids Extraction and Thin Layer Chromatography (TLC)

Total lipids were extracted from cell suspensions of cultures using the Bligh Dyer method (Bligh & Dyer, 1959). Total lipids (TLs) were then separated into polar (PLs) and neutral lipids (NLs) by countercurrent distributions in a binary system formed by mixing three volumes of pre-equilibrated petroleum ether and

one volume of pre-equilibrated 87% ethanol (Galanos & Kapoulas, 1962). The PLs were further fractioned by Thin Layer Chromatography (TLC) on ten (10) TLC plates using chloroform/acetone/methanol/acetic acid/water at a ratio of 100:40:34:10:10 (v/v/v/v/v) as developing system. Appropriate standards of phospho- and glycol-lipids were also used. After exposure of the TLC plate to I_2 vapor, the fractions of PLs were scrapped off separately, centrifuged, and the organic solvents were phased by adding appropriate volumes of chloroform, methanol and water at a ratio of 1:2:0.8 (v/v/v). All reagents and chemicals were of analytical grade and supplied by Merck (Darmstadt, Germany). The chromatographic material used for TLC was silica gel H-60 (Merck, Darmstadt, Germany).

Antibacterial activity determination

The potential antibacterial activity was tested in both Cyanobacteria strains by the disk diffusion method with Mueller-Hinton II agar (OXOID, UK) according to CLSI guidelines. Dried extracts were dissolved in methanol. The plates were inoculated with a suspension of each strain adjusted to a turbidity of 0.5 McFarland. Sterilized blank (3 mm Chr Whatman) paper disks (6 mm diameter) were applied to the surface of the inoculated agar and were loaded with a total amount of 10 μl and 20 μl of each extract solution. The antibiotic disks (BIORAD, UK) gentamicin 10 μg (GEN), ampicillin 10 μg (AMP), cefoxitin 30 μg (FOX), tetracycline 30 μg (TET), ciprofloxacin 5 μg (CIP) and co-trimoxazole 1.25 / 23.75 μg (SXT) were used as positive controls depending of the bacterial species. Methanol alone was used as a negative control since a volume of $V \geq 5 \mu\text{l}$ pure methanol was inhibitory to bacterial growth. The plates were left to dry for 15 min and were incubated for 18 h at $35^{\circ} \pm 2^{\circ}C$. For all agents the diameters of zones of inhibition were measured to the nearest millimeter and for the positive controls the results were interpreted according to CLSI (2012) breakpoints. Each fraction of PLs, as well as the NLs as a whole, was tested *in vitro* for their ability to inhibit growth of the following eight reference or clinical isolates: *S. aureus* NCTC 6571, Methicillin-Resistant *S. aureus* (MRSA) 1629, Methicillin-Susceptible *S. aureus* (MSSA) 1646, *Enterococcus faecalis* ATCC 29212, Vancomycin-Resistant *E. faecalis* (VRE) 880, Vancomycin-Resistant *E. faecium* (VRE) 1291, *Escherichia coli* ATCC 25922, and *Pseudomonas aeruginosa* ATCC 27853.

After the initial evaluation of antibacterial activity, the Minimum Inhibitory Concentrations (MICs) were determined only for *T. calypsus* by broth microdilution method as recommended by CLSI. The tests were performed in sterile 96-well microtiter plates. Briefly, 50 μl of two-fold serial dilutions of examined samples was added to 50 μl microbial suspensions adjusted to yield approximately 5×10^5 CFU/ml. MIC was encountered as the lowest concentration of the examined sample that inhibits the visible microbial growth after 24 h incubation at $37^{\circ}C$. Negative controls (methanol) were included, too.

Table 1a. Results obtained by applying the Kirby-Bauer method on 10 µl and 20 µl of methanol extract solution of *Toxopsis calypsus* showing the inhibition zones (in mm) of each fraction (T1-T12) of Polar Lipids, of Polar Lipids (PL), Neutral Lipids (NL), and Total Lipids (TL) in relation to the inhibition zones of the negative control (Meth = methanol) and the positive controls (Gen = gentamicin, Amp = ampicillin, Fox = cefoxitin, Tet = tetracycline, Cip = ciprofloxacin) when tested against eight (8) reference or clinical isolates.

<i>T. calypsus</i> 10µl	Fractions (T1-T12) of Polar Lipids (PL)												Lipids					Controls (-) and (+)				
	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	T11	T12	PL	NL	TL	Meth	Cip	Fox	Amp	Gen		
<i>S. aureus</i> NTCC 6571	11	14		10/13	10/13	10/13	13	13	10/13	14	10	10	9/15	13	8	10				20		
<i>S. aureus</i> MRSA 1629	14/16	11/13	14/13	12/13	8	6/7	12	13/14	13/14	14	7	9	9	10	8	9	25	19				
<i>S. aureus</i> 1646	14/16	13	13	12	8	12	12/14	11/13	13	13	7	10	8	8	9	9	28	32				
<i>E. faecalis</i> ATCC 29212	12	12	12	14	12	12	13	13	13	13	8	8/9	11/12	13	7/11	9						
<i>E. faecalis</i> 880	12/13	13	13	13/14	11	12	12	13	13	12	7	9	12	9	9	9	6		27			
<i>E. faecium</i> 1291	14	13	14	13	14	13	14	14	13	13	9	9	13	13	9	9						
<i>E. coli</i> ATCC 25922	9	9	10	9	9	9	10	11	10	11	8	9	10	9	9	9				20		
<i>P. aeruginosa</i> ATCC 27853	9/10	10	10	9	8/9	9	9	9	10	9	9	10	9	9	9/10	9				20		

<i>T. calypsus</i> 20µl	Fractions (T1-T12) of Polar Lipids (PL) in mm												Lipids					Controls (-) and (+)				
	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	T11	T12	PL	NL	TL	Meth	Cip	Fox	Amp	Gen		
<i>S. aureus</i> NTCC 6571	16	18		17	17	17	18	18	19	18/19	13	14	15	18/19	11/16	14				20		
<i>S. aureus</i> MRSA 1629	17/18	16/18	17/18	15	15/16	15/16	18	18	16/19	16/18	10	13/14	10	12/13	10	12	25	19				
<i>S. aureus</i> 1646	15/17	16/17	18	17	16	16	17/19	17	17	10	9	13/14	11	11/13	11	12	28	32				
<i>E. faecalis</i> ATCC 29212	17	18	17/18	17	17	16/19	18	18	18	20	11	11/12	16/17	20	10/16	12						
<i>E. faecalis</i> 880	18/20	17/19	15/17	18	15,17	18	18	18	18	18	11	12/13	16/17	12	12/13	12	6		27			
<i>E. faecium</i> 1291	19	19/20	19	19	19	19	20	19	18/19	18/19	10	13	18/19	18/19	12	12	6					
<i>E. coli</i> ATCC 25922	13	12/13	14	12/13	14	14	14	13/14	13/15	14/15	13	12	11	14	12	14				20		
<i>P. aeruginosa</i> ATCC 27853	13/14	13/14	13	13	13	13	14	12/13	13	13	12	13	12/13	13	12/13	13				20		

Table 1b. Results obtained by applying the Kirby-Bauer method on 10 µl and 20 µl of methanol extract solution *Phormidium melanochroun* showing the inhibition zones (in mm) of each fraction (P1-P10) of Polar Lipids, of Polar Lipids (PL), Neutral Lipids (NL), and Total lipids (TL) in relation to the inhibition zones of the negative control (Meth=methanol) and the positive controls (Gen = gentamicin, Amp = ampicillin, Fox = ceftioxin, Tet = tetracycline, Cip = ciprofloxacin) when tested against eight reference or clinical isolates.

	Fractions (P1-P10) of Polar Lipids (PL) in mm										Lipids					Controls (-) and (+)				
	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	PL	NL	TL	Meth	Cip	Fox	Amp	Gen	Str	
<i>P. melanochroun</i> 10µl																				
<i>S. aureus</i> NTCC 6571	8	8	11	8	8	8	5*	10	9	9	8	9/10	7	9				20		
<i>S. aureus</i> MRSA 1629	12/13	12	10/13	9	9	9	5*	10	9	9	8	9	9	9	24	18				
<i>S. aureus</i> 1646	11	12	12	9	9	9	5*	10	9	9	6/7	10	9	9	28	32				
<i>E. faecalis</i> ATCC 29212	12	12	13/14	11	12	10	5*	12	8	8	8	9	8	9	21					
<i>E. faecalis</i> 880	12/13	12/13	9	11	9	9	5*	12	9	10	8	8	8	9	6		27			
<i>E. faecium</i> 1291	14	13	13	13	13	13	5*	14/15	12	9	8	12/13	9	9	6			20	6	
<i>E. coli</i> ATCC 25922	9	19	9	9	9	9	5*	9	9	10	7	10	9	9						
<i>P. aeruginosa</i> ATCC 27853	9	9	9	9	9	9	5*	8	8	8	8/9	8,9	8	8/9					20	

	Fractions (P1-P10) of Polar Lipids (PL) in mm										Lipids					Controls (-) and (+)				
	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	PL	NL	TL	Meth	Cip	Fox	Amp	Gen	Str	
<i>P. melanochroun</i> 20µl																				
<i>S. aureus</i> NTCC 6571	17	17/18	18	16	17	17	5*	15	13	13	10	14	12	15				20		
<i>S. aureus</i> MRSA 1629	14/15	16	17/18	12,13	12/14	13/14	5*	12/13	12	12/13	11	12/13	10	12	24	18				
<i>S. aureus</i> 1646	17	15/16	15/18	12	13	12	5*	14	12	11/12	11	11/12	13	12	28	32				
<i>E. faecalis</i> ATCC 29212	16/19	17/19	18/19	15,17	15/17	15	5*	14	11/12	12	9	12/14	10	12/13	21					
<i>E. faecalis</i> 880	15	17	15	12	12	12	5*	14	11	13	11	12	12	12	6		27			
<i>E. faecium</i> 1291	18	21	19/20	17	18/19	18	5*	18	17	13	12	14	12	12	6				6	

RESULTS

Extraction of lipids from the Cyanobacteria *T. calypsus* and *P. melanochroun* yielded about 31 mg and 51 mg of total lipids (TLs), respectively. Polar lipids (PLs) were further fractioned by TLC, and after exposure in I₂ vapor a total of 12 and 10 bands were revealed for *T. calypsus* and *P. melanochroun*, respectively. The retention factors (Rfs) for each band of polar lipids compared to that of standards are shown in Table 2.

As examined by the Kirby Bauer Method, each fraction of PLs from both Cyanobacteria species, as well as TLs, PLs and NLs as a whole, yielded an inhibition halo against the examined Gram-positive bacteria, whereas none of the examined lipids was effective against the Gram-negative bacteria (Tables 1 a,b).

Among the Gram-positive bacteria, the reference and clinical isolates of enterococci were mostly affected since a greater number of fractions of PLs (including NLs and PLs as a whole) showed zones of inhibition. The highest zones of inhibition (20 mm) were observed:

(i) against *Enterococcus faecium* (VRE) by the fractions T2 and T7 of *T. calypsus*, and by the fraction P3 of *P. melanochroun*, at a total volume of 20 µl, and (ii) against *Enterococcus faecalis* (ATCC) by the fraction T9 of *T. calypsus* also at a total volume of 20 µl. One fraction of polar lipids (P7) from *P. melanochroun* showed no zone of inhibition eliminating the expected halo of pure methanol (see Table 1 a,b).

The results obtained by the broth microdilution method (MICs) for *T. calypsus* confirm those of the disk diffusion method (Table 3). Antibacterial activity of the 12 fractions of PLs (including NLs and PLs as a whole) extracted from *T. calypsus* was recorded against staphylococci and enterococci in MIC values 0.256 µg/ml and 0.512 µg/ml. Seven (7) fractions of PLs (T1-T3, T7-T10) showed the greatest MIC values against enterococci (0.256 µg/ml). Moreover, one of the above fractions (T10) showed antibacterial activity against *S. aureus* (MRSA) at MIC value of 0.256 µg/ml. The fractions T11 and T12 indicated no antibacterial activity at MIC ≥ 0.512 µg/ml.

Table 2. Retention factors (average ± standard deviation) for each fraction (T1–T12) of Polar Lipids of *Toxopsis calypsus* and for each fraction (P1–P10) of Polar Lipids of *Phormidium mealanochroun* compared to the retention factors given for the following standards: LPC = lysophosphatidyl-choline; SM = sphingomyelin; PC = phosphatidyl-choline; PE = phosphatidyl-ethanolamine; SULF = sulfatides; DGDG = digalactosyl-diglycerides; GALCER = galactosyl-cerebrosides; CERA = ceramides.

Retention Factors (Rf)				
<i>Toxopsis calypsus</i>		<i>Phormidium melanochroun</i>		Standards LPC = 0.08 SM = 0.17 PC = 0.28 PE = 0.57 SULF = 0.61 DGDG = 0.71 GALCER = 0.78 CERA = 0.93
T1	0.153 ± 0.009	P1	0.151 ± 0.024	
T2	0.202 ± 0.027	P2	0.196 ± 0.018	
T3	0.256 ± 0.020	P3	0.268 ± 0.015	
T4	0.304 ± 0.017	P4	0.355 ± 0.050	
T5	0.394 ± 0.033	P5	0.422 ± 0.040	
T6	0.450 ± 0.037	P6	0.514 ± 0.017	
T7	0.517 ± 0.034	P7	0.690 ± 0.032	
T8	2.217 ± 0.801	P8	0.844 ± 0.049	
T9	0.712 ± 0.035	P9	0.922 ± 0.038	
T10	0.804 ± 0.038	P10	0.959 ± 0.031	
T11	0.875 ± 0.031			
T12	0.918 ± 0.037			

Table 3. Minimum Inhibitory Concentration (MIC) values (in µg/ml) determined for each methanol fraction (T1-T12) of Polar Lipids, as well as of Polar Lipids (PL), Neutral Lipids (NL) and Total Lipids (TL) of *Toxopsis calypsus* when tested against eight reference or clinical isolates.

	<i>S. aureus</i> NTCC 6571	<i>S. aureus</i> MRSA 1629	<i>S. aureus</i> MSSA 1646	<i>E. faecalis</i> ATCC 29212	<i>E. faecalis</i> VRE 880	<i>E. faecium</i> VRE 1291	<i>E. coli</i> ATCC 25922	<i>P. aeruginosa</i> ATCC 27853
T1	0.512	0.512	0.512	0.256	0.256	0.256	-	-
T2	0.512	0.512	0.512	0.256	0.256	0.256	-	-
T3	0.512	0.512	0.512	0.256	0.256	0.256	-	-
T4	0.512	0.512	0.512	-	-	-	-	-
T5	0.512	0.512	0.512	-	-	-	-	-
T6	-	-	0.512	-	-	-	-	-
T7	0.512	0.512	0.512	0.256	0.256	0.256	-	-
T8	0.512	0.512	0.512	0.256	0.256	0.256	-	-
T9	0.512	0.512	0.512	0.256	0.256	0.256	-	-
T10	0.512	0.512	0.256	0.256	0.256	0.256	-	-
T11	-	-	-	-	-	-	-	-
T12	-	-	-	-	-	-	-	-
PL	-	-	-	0.512	0.512	0.512	-	-
NL	-	-	-	0.512	0.512	0.512	-	-
TL	-	-	-	0.512	0.512	0.512	-	-

DISCUSSION

Natural products have been attributed to a few genera within Cyanobacteria, given that some of them were proved to be polyphyletic groups, e.g. genus *Lyngbya* was shown to be composed of several phylogenetically distant and unrelated lineages (Sharp et al., 2009; Engene et al., 2010, 2012, 2013; Komárek et al., 2013). Moreover, phylogenetic inferences of marine cyanobacterial strains responsible for over 100 bioactive secondary metabolites revealed an uneven taxonomic distribution, with a few groups being responsible for the vast majority of these molecules (Engene et al., 2013). These data suggest a high degree of novel biodiversity among natural product-producing strains that was previously overlooked by traditional morphology-based taxonomic approaches.

The two species selected for this study are new Cyanobacteria from Greek caves established by both the traditional and the molecular (polyphasic) approach (Lamprinou et al., 2012, 2013): a) *Phormidium melanochrouron* is an oscillatoriacean species characterized by a blackish thick mucilaginous sheath (autapomorphic character), and b) *Toxopsis calypsus* is a nostocacean species characterized by both isopolar and heteropolar life cycle (autapomorphic character). The observed out-competing behaviour of the former species towards other Cyanobacteria in our cultures and the fact that previous studies were focused on different antibacterial compounds extracted from genus *Phormidium* (e.g. Madhumathi et al., 2011; Vijaya Baskara Sethubathi & Prabu, 2012) making this genus a target in the search for a potential lipid antibacterial activity. On the other hand, the taxonomic position of the latter species (*T. calypsus*) among Nostocales has been crucial in the search for a similar activity of lipids, since the order Nostocales is known for intense antibacterial and antifungal activity and has been the focus of many relevant investigations with *Nostoc* and *Anabaena* being the most well studied genera (Mundt et al., 2001; Abdel-Raouf & Ibraheem, 2008; Asthana et al., 2009; Kausik et al., 2009).

Lipids and some free fatty acids from microalgae and Cyanobacteria are known to display antibacterial properties (Borowitzka, 1995; Desbois & Smith, 2010; Plaza et al., 2010; Najdenski et al., 2013). In our study, most of the lipids extracted from *P. melanochrouron* and *T. calypsus* demonstrated potential activity against the Gram-positive clinical and reference bacteria with pronounced effectiveness against the enterococci; on the contrary, no activity was observed against the Gram-negative bacteria (cf. Ramadan et al., 2008). Although the exact mechanism is rather unknown, lipids are supposed to be the responsible disrupting agents of the bacterial cellular membranes by penetrating into the thick peptidoglycan wall layer of the Gram-positive bacteria, but not affecting the thin peptidoglycan wall layer of the Gram-negative bacteria (Najdenski et al., 2013).

The MIC values of lipids from *Toxopsis calypsus* (0.256 µg/ml) were highly active against all tested enterococci in comparison with previously reported MIC values from crude extracts of other cyanobacterial

strains (ranging from 0.5 mg/ml to 512 mg/ml; e.g., Kaushik & Chauhan, 2008; Asthana et al., 2009; Kumar et al., 2012). These data and future similar research on *Phormidium melanochrouron*, accompanied by precise composition and characterization of these active compounds, are highly promising steps for developing effective antibiotics from cave Cyanobacteria in pharmaceutical industry.

Extreme habitats experiencing steady or fluctuating exposure to one or more environmental factors, i.e. salinity, osmolality, desiccation, solar irradiance, barometric pressure, pH, temperature, nutrient limitation (Seufferheld et al., 2008; Dapkevicius, 2013) are considered as one of the most promising sources of biotechnologically useful compounds. As a result, several studies have been devoted to screening secondary metabolites produced by microorganisms inhabiting such environments (e.g., Harvey, 2000; Nicolaus et al., 2010; Changet al., 2011; Singh & Gabani, 2011). Caves are considered as extreme environments in terms of nutrient limitation and insufficient light with rather understudied microorganisms; thus, caves are promising sources for successful natural product research, justifying their conservation and our effort of screening the isolated Cyanobacteria.

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