Cytotoxicity in L929 fibroblasts and inhibition of herpes simplex virus type 1 Kupka by estuarine cyanobacteria extracts

Viviana R. Lopes[a, b], Michaela Schmidtke[c], M. Helena Fernandes[d], Rosário Martins[a, e, f], Vitor Vasconcelos[a,b, ,]

[a] CIIMAR/CIMAR, Centro Interdisciplinar de Investigação Marinha e Ambiental, University of Porto, Rua dos Bragas 289, 4050-123 Porto, Portugal

[b] Department of Biology, Faculty of Sciences, University of Porto, Rua Campo Alegre s/n, 4169-007 Porto, Portugal

[c] Department of Virology and Antiviral Therapy, Jena University Hospital, Hans-Knoell-Street 2, D-07745 Jena, Germany

[d] Laboratório de Farmacologia e Biocompatibilidade Celular, Faculdade de Medicina Dentária da Universidade do Porto, Rua Dr. Manuel Pereira da Silva, 4200-393 Porto, Portugal

[e] Escola Superior de Tecnologia da Saúde do Porto, Instituto Politécnico do Porto, Rua Valente Perfeito 322, 4400-330 Vila Nova de Gaia, Portugal

[f] IBMC–Institute for Molecular and Cell Biology, University of Porto, R.Campo Alegre 823, 4150-180 Porto, Portugal

Abstract

The cyanobacteria are known to be a rich source of metabolites with a variety of biological activities in different biological systems. In the present work, the bioactivity of aqueous and organic (methanolic and hexane) crude extracts of cyanobacteria isolated from estuarine ecosystems was studied using different bioassays. The assessment of DNA damage on the SOS gene repair region of mutant PQ37 strain of *Escherichia coli* was performed. Antiviral activity was evaluated against influenza virus, HRV-2, CVB3 and HSV-1 viruses using crystal violet dye uptake on HeLa, MDCK and GMK cell lines. Cytotoxicity evaluation was performed with L929 fibroblasts by MTT assay. Of a total of 18 cyanobacterial isolates studied, only the crude methanolic extract of LEGE 06078 proved to be genotoxic (IF > 1.5) in a dose-dependent manner and other four were putative candidates to induce DNA damage. Furthermore, the crude aqueous extract of LEGE 07085 showed anti- herpes type 1 activity (IC50 = 174.10 µg dry extract mL^{-1}) while not presenting any cytotoxic activity against GMK cell lines. Of the 54 cyanobacterial extracts tested, only the crude methanolic and hexane ones showed impair on metabolic activity of L929 fibroblasts after long exposure (48–72 h). The inhibition of HSV-1 and the strong cytotoxicity against L929 cells observed

emphasizes the importance of evaluating the impact of those estuarine cyanobacteria on aquatic ecosystem and on human health. The data also point out their potential application in HSV-1 treatment and pharmacological interest.

Keywords

Anti-HSV-1 activity; DNA damage; Estuarine cyanobacteria; Cytotoxicity

1. Introduction

Cyanobacteria are prokaryotic oxygenic photoautotroph organisms found in all kind of habitats. They are known worldwide for their ability to produce several types of toxins such as neurotoxins and hepatotoxins (Carmichael, 1994). However, on the last decades cyanobacteria have been also identified as a new and prolific source of bioactive compounds (Abed et al., 2009). The bioactive compounds produced include polyketides, amides, alkaloids, fatty acids, indoles and lipopeptides (Burja et al., 2001 and Patterson et al., 1994). Diverse biological activities have been surveyed and reported, ranging from antiviral, antialgal, antibacterial, antifungal to antitumour activity (Abed et al., 2009 and Bubik et al., 2008).

Previous studies have shown that the cyanotoxins and cyanobacterial extracts can be genotoxic and mutagenic, either with or without metabolic activation, but little information is available (Ding et al., 1999, Mankiewicz et al., 2002, Shen et al., 2002 and Zhan et al., 2004). Moreover, antiviral as well as cytotoxic activity of cyanobacterial extracts has been previously reported. Cyanobacteria belonging to the genera *Calothrix*, *Microcystis*, *Nodularia*, *Nostoc*, *Spirulina*, *Oscillatoria* and *Scytonema* have been described as producers of compounds with broad-spectrum antiviral properties. The inhibitory concentration (IC_{50}) of antiviral activity can range between 0.37 and 300.0 μg extract/ml. Spirulan-like substances, polysaccharides, carbohydrates and peptidic compounds have been reported as antiviral compounds against herpesviruses, paramyxoviruses, influenza viruses and human immunodeficiency virus type 1. Cyanobacteria have been also reported to cause cytotoxicity by induced apoptosis in mammalian cell lines as the human leukemia cell line, and fish cell lines (Hernandez-Corona et al., 2002, Kanekiyo et al., 2007, Mundt et al., 1997, Nowotny et al., 1997, Patterson et al., 1993, Rechter et al., 2006, Surakka et al., 2005, Teneva et al., 2003 and Zainuddin et al., 2002).

- The cyanobacteria ability to produce toxins as well as bioactive secondary metabolites makes these organisms potentially hazardous to aquatic ecosystem and to human health (Carmichael, 2001 and Martins et al., 2008). While toxicity of fresh and marine waters cyanobacterial metabolites has been well documented *in vitro* and *in vivo*, few surveys about the bioactivity of estuarine cyanobacteria have been published. Ecotoxicological effects of cyanobacteria of freshwaters and marine biotopes on animals, plants and humans have been reported. Toxigenicity has been mainly linked to the genera *Anabaena*, *Anabaenopsis*, *Hapalosiphon*, *Lyngbya*, *Microcystis*, *Nostoc*, *Synechoccocus* and *Planktothrix*. Neurotoxic and hepatotoxic effects as well

antialgal, immunosuppressive, antimycotic, antibacterial, antitumour and antiviral activity have been described (Gademann and Portmann, 2008, Hirata et al., 2003, Jaiswal et al., 2008, Martins et al., 2007, Martins et al., 2008, Mundt et al., 2001 and Wiegand and Pflugmacher, 2005). On the other hand, recent studies have already demonstrated that cyanobacteria isolated from estuaries induce toxic effects on marine invertebrates (Lopes et al., 2010).

- Considering the potential bioactivity of cyanobacteria from estuarine habitats, an assessment of genotoxicity, cytotoxic and antiviral activity of cyanobacteria was done. A battery of four *in vitro* assays was applied to assess specific potential biological activity. The DNA damage was evaluated using the SOS-Chromotest based on the induction of SOS-repair system of the strain *Escherichia coli* PQ 37 (Quillardet et al., 1982). The screening of antiviral activity was done by the evaluation of cytopathic effect using crystal violet dye uptake against coxsackie virus B3 strain, the influenza virus A strain Hong Kong/68, the herpes simplex virus type 1 strain and rhinovirus (Schmidtke et al., 2001). Cytotoxicity evaluation was also performed with L929 cell lines by MTT assay (Berridge and Tan, 1993, Freshney, 2000 and Li and Song, 2007).

2. Material and methods

2.1. Cyanobacterial cultures

- The sampling was performed during the low tide from both benthos and plankton on estuarine systems of North and Centre of Portugal specifically, Minho, Douro and Vouga (42° 15′ to 40° 15′ north latitude and 8° 54′ to 8° 38′ west longitude). Eighteen cyanobacterial strains were isolated, belonging to six genera *Cyanobium*, *Leptolyngbya*, *Microcoleus*, *Nodularia*, *Nostoc* and *Synechocystis* (see Table 1). Isolation and culture were performed in Z8 medium (Kotai, 1972) supplemented with 10–35 mg NaCl ml^{-1} (P.A. Sigma–Aldrich®, Germany) and at 25 °C, light intensity of 20.8–27.4 × 10^{-6} E m^{-2} s^{-1} with a light/dark cycle of 14/10 h. All the cultures were unicyanobacterial and non-axenic but no visible bacterial contamination was present in any of the cultures. Cultures of the isolates at the exponential growth phase were collected by centrifugation or filtration and washed with bidistilled water, frozen at −20 °C and then freeze-dried.

Table 1.

Estuarine cyanobacterial isolates included in the study.

Lab code	Taxon	Lab code	Taxon
LEGE 06068	*Cyanobium* sp.	LEGE 07075	*Leptolyngbya* sp.
LEGE 06069	*Leptolyngbya* sp.	LEGE 07076	*Microcoleus* sp.
LEGE 06070	*Leptolyngbya* sp.	LEGE 06077	*Nostoc* sp.
LEGE 06071	*Nodularia* sp.	LEGE 07080	*Leptolyngbya* sp.
LEGE 06072	*Phormidium* sp.	LEGE 07083	*Synechocystis* sp.
LEGE 06078	*Phormidium* sp.	LEGE 07084	*Leptolyngbya* sp.
LEGE 06079	*Synechocystis* sp.	LEGE 07085	*Leptolyngbya* sp.
LEGE 07073	*Synechocystis* sp.	LEGE 07091	*Leptolyngbya* sp.
LEGE 07074	*Leptolyngbya* sp.	LEGE 07092	*Microcoleus* sp.

2.2. Cyanobacterial extracts

The freeze-dried cells were extracted with 70% methanol (1 ml per 25 mg dw) with an ultrasonic probe Vibra cell50 (Sonics & Materials Inc., Danbury, USA) at 4 °C during 3 × 20 s, centrifuged at 9300*g* during 10 min and evaporated. The obtained residue was resuspended in sterile 0.85% saline solution with 10% DMSO (AldraSorb™ 99.8% Sigma–Aldrich®, Germany) and used for the genotoxic trial. Regarding the cytotoxic and cytophatic effect assays, a similar procedure was used with slight modifications. Three solvents with increasing polarity were applied, *n*-hexane (P.A. Merck, Germany), methanol (P.A. Merck, Germany) and bidistilled water. The obtained residues were resuspended in the same solvent used for the extraction, except for the hexane which was replaced by 1% DMSO (AldraSorb™ 99.8% Sigma–Aldrich®, Germany).

2.3. DNA damage assay

The genotoxic activity was assessed by the SOS Chromotest kit version 6.3 (EBIP, Canada) based on a bacterial colorimetric assay developed by Quillardet and Hofnung (1993) and Quillardet et al. (1982). In brief, it was used a strain *of Escherichia coli* genetically modified in which the SOS gene promoter has been linked to the *Lac* Z gene. DNA damage was evaluated by measuring the absorbance of beta-galactosidase (ß-gal) at 615 nm using a PowerWave™ Microplate Spectrophotometer (BioTek, USA). Since the analysis and synthesis of the ß-gal enzyme it is dependent upon the viability of bacteria, ATP activity was also tested using alkaline phosphatase (PAL) measuring its absorbance at 405 nm. A standard genotoxic solution was used containing 10 μg mL^{-1} of 4NQO in 10% DMSO saline. Six twofold dilutions from 0.78 to 25.00 mg mL^{-1} of the cyanobacterial extracts in 10% DMSO saline solution were analyzed in triplicate. The data obtained were expressed as induction factor (IF) defined as $(B\text{-}gal_c/PAL_c)/(B\text{-}hal_0/PAL_0)$, where 0 represents the negative control (sterile ultrapure water) and *c* concentration of the sample.

2.4. Cytotoxicity assays

L929 cell lines (ATCC CCL-1™) were cultured in α-MEM (Gibco, Invitrogen No. 11900-073) supplemented with 10% (v/v) fetal bovine serum (Gibco Invitrogen No. 10106-169), 1% penicillin and streptomycin solution (Gibco Invitrogen No. 15140-122) and 2.5 μg mL^{-1} of fungizone (Gibco Invitrogen No. 15290-026). Cells were seeded at 3×10^3 cells per well (96-well culture plates) and exposed to the extracts for 24, 48 and 72 h. Cytotoxicity of the extracts was evaluated by the 3-(4, 5-dimethylthiazole-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay.

Cultures were maintained at 37 °C in a humidified incubator with 5% CO_2 atmosphere for the incubation times mentioned above, without any medium change. The MTT assay was carried out according to Berridge and Tan (1993). Cultures were exposed to the extracts for 24, 48 and 72 h. At the end of each testing time, the culture supernatants were removed and MTT solution (0.5 mg mL^{-1}) was added to each well and the plates were incubated for 4 h at 37 °C. The MTT solution was removed and DMSO was added to dissolve formazan crystals. The absorbance at 550 nm was read on a PowerWave™ Microplate Spectrophotometer (BioTek, USA). The percentage of viability was calculated as (AT/AC)∗100, where *AT* and *AC* are the optical densities of treated and control cells, respectively.

2.5. Antiviral activity

HeLa Ohio (human cervix carcinoma; ATCC CCL-2™), GMK (green monkey kidney cells; Schaper and Brümmer, Salzgitter, Germany), and Madin-Darby canine kidney (MDCK) cells (Friedrich-Loeffler Institute, Riems, Germany) were used in the antiviral trials. The HeLa Ohio cells were grown in MEM/E medium (SIGMA No. M-0643), and the GMK and MDCK cells in Dulbecco's modified MEM/E (SIGMA No. D-7777) supplemented with 10% NCS (HeLa Ohio; Greiner No. 758010, Germany) or 10% FBS (GMK and MDCK; Greiner No. 758075, Germany), 100 U/ml penicillin as well as streptomycin (CCpro No. Z-13-M, Germany). The test medium used for the anti-coxsackie virus B3 and anti-herpes simplex virus type 1 tests contained only 2% of the appropriate serum. The medium applied in the anti-influenza virus-A assays was formulated with 3 μg ml^{-1} trypsin and did not contain serum. All cells were proved to be free of mycoplasma contamination before using. Cultures were maintained at 37 °C in a humidified incubator with 5% CO_2 atmosphere.

2.5.1. Viruses

Virus stocks of the coxsackie-virus B3 strain Nancy (CVB3; Institute of Poliomyelitis and Virus Encephalitides, Moscow, Russia), the influenza virus A/Hong Kong/68 (influenza virus; Schaper and Brümmer, Salzgitter, Germany), the herpes simplex virus type 1 strain Kupka (HSV-1; own strain collection) and rhinovirus type 2 (HRV-2; Institute of Virology, Vienna, Austria) were prepared in HeLa Ohio (CVB3 and HRV-2), MDCK (Influenza virus), and GMK cells (HSV-1). The titre of virus stocks was previously determined by plaque assay on the respective cell monolayer.

2.5.2. Cytopathic effect (CPE) inhibitory assay

The *in vitro* assay of anti-viral activity was performed applying the protocol of Schmidtke et al. (2001) and Makarov et al. (2005). Briefly, the CPE assay is based on the lysis of HeLa Ohio, MDCK, and GMK cells by CVB3 and HRV-2, influenza virus, and HSV-1 K1, respectively. The antiviral evaluation was carried out in 1-day-old semi confluent (HRV-2) or 2-day-old confluent (CVB3, influenza virus, and HSV-1) monolayers of the mentioned cell lines growing in 96-well flat-bottomed microtiter plates (Falcon 3075). After removal of the culture medium, 50 µl of extract solution or medium (cell and control virus) and a constant amount of virus in a volume of 50 µl (0.1 multiplicity of infection for CVB3 and influenza virus, 0.01 multiplicity of infection for HRV-2) or medium (cell control) were added to the cells. Six wells of non-infected and six wells of infected cells without the test extract were used as cell and virus control, respectively, on each plate. To calibrate the assay, the 50% and 100% plaque inhibitory concentrations of reference compounds (each three wells; guanidine hydrochloride for CVB3, pleconaril for HRV-2, amantadine for influenza virus, and foscarnet for HSV-1) were included as positive control in each microtiter plate. Plates were incubated for 24 h (CVB3 and influenza virus), 48 h HSV-1, or 72 h (HRV2) at 37 °C in a humidified atmosphere with 5% CO_2. At this time a complete cytopathic effect was observed microscopically in virus control wells. The cells were fixed and stained with crystal violet formalin solution. After dye extraction, the optical density of individual wells was quantified at 550/630 nm with a microplate reader. The percentage of antiviral activities of the tests extracts were calculated by ((mean OD of six cell controls-mean OD of six virus controls)/(mean OD of test extract-mean OD of six virus controls)) × 100. The 50% CPE inhibitory dose (IC_{50}) was calculated.

2.5.3. Cytotoxicity

The cytotoxicity measurement by the crystal violet dye assay was made simultaneously with the antiviral activity evaluation to guarantee that the potential virus-specific compound or mixture should have low or no effects on cellular metabolism and thus showing none toxicity against the host organism. The 50% cytotoxic concentration (CC_{50}) of the cyanobacterial extracts was determined on 2-day-old confluent HeLa Ohio, MDCK and GMK cell monolayers grown in the internal 60 wells of 96-well tissue culture plates (Falcon 3075) as described by Schmidtke et al. (2001). Briefly, cell monolayers were incubated with eight twofold serial dilutions of compounds for 72 h (37 °C, 5% CO_2). Then, the cells were fixed and stained with a crystal violet formalin solution. After dye extraction, the optical density of individual wells was quantified at 550/630 nm with a microplate reader. Cell viability of individual compound-treated wells was evaluated as the percentage of the mean value of optical density resulting from six mock-treated cell controls which was set 100%. The CC_{50} was defined as the compound concentration reducing the viability of untreated cell cultures by 50%. It was calculated from the logarithmic regression curves.

2.6. Chemical analysis – MALDI TOF-MS

Lyophilised samples of the cyanobacterial cultures, which showed bioactivity, were screened for common cyanotoxins and other peptides by MALDI-TOF MS. The procedure and conditions can be seen in Lopes et al. (2010).

2.7. Statistical analyses

Data were expressed as means ± standard deviation (SD). Significant differences were determined by one-way analysis of variance (ANOVA) at $p < 0.05$. The goodness of fit of the data to normal distribution was tested applying the Kolmogorov–Smirnov statistic. The statistical analysis was performed with SPSS software (version 17.0, USA). When the normality and homoscedasticity assumptions were not met, a non-parametric Kolmogorov–Smirnov test for two- independent samples was applied. A significant level of $p < 0.05$ was accepted. The CC_{50}, defined as the concentration that caused 50% maximum cytotoxic effect, was calculated by regression analysis of the dose–response curves for MTT and crystal violet assay.

3. Results

The genotoxic effects of the 18 extracts analyzed were evaluated using crude methanolic 70% (v/v) extracts, without metabolic activation and were expressed through its induction factors (IF). As shown in Table 2, LEGE 06078 showed genotoxic effects, presenting IF values higher than 1.5 and increasing significantly ($p < 0.05$) in a dose-dependent manner. The LEGE 06078 extract belongs to the *Phormidium* genus. Furthermore, 22% of the cyanobacterial extracts were potentially genotoxic since at least one of the two parameters evaluated, IF higher than 1.5 and response in a dose-dependent manner, were not verified. Some isolates showed an IF higher than 1.5 from a concentration of 6.25 mg mL^{-1} but not enough to consider genotoxic. It could be an artifact resulting of a high dose. These candidates belong mainly to Oscillatoriales, the exception being LEGE 06077, which belongs to Nostocales. The remaining isolates have no genotoxicity since the IF values were above 1.0 (not shown), independently of the concentration value.

Table 2.

SOS induction by crude (70% v/v) methanolic extracts of CB on *E. coli* strain without metabolic activation (S9). The strains not shown in the table had no significant effects. Mean values and standard deviation ($\dot{X}$; SD). Values typeset in bold indicate a positive genotoxicity response and in italics represent a potential genotoxicity.

Isolates code	[extract] (mg mL^{-1})	PA [a] ($\dot{X}$; SD)		β-GAL [a] ($\dot{X}$; SD)		IF [b]
Oscillatoriales						
LEGE 06070	**25.00**	0.166	0.055	0.031	0.016	*1.4*
	12.50	0.162	0.065	0.032	0.021	*1.5*
	6.25	0.213	0.024	0.070	0.044	***2.5***
	3.13	0.199	0.027	0.039	0.012	*1.4*
	1.56	0.190	0.030	0.037	0.010	*1.4*
	0.78	0.170	0.009	0.023	0.003	1.0
	0.00	0.152	0.016	0.020	0.003	1.0
LEGE 06072	**25.00**	0.211	0.008	0.052	0.003	*1.8*
	12.50	0.208	0.004	0.049	0.001	*1.7*
	6.25	0.207	0.002	0.051	0.004	*1.8*
	3.13	0.200	0.003	0.045	0.003	*1.6*
	1.56	0.211	0.010	0.044	0.000	*1.5*
	0.78	0.162	0.002	0.026	0.001	*1.1*
	0.00	0.136	0.017	0.019	0.002	1.0
LEGE 06078	**25.00**	0.210	0.009	0.056	0.004	**2.2***
	12.50	0.210	0.007	0.053	0.002	**2.1***
	6.25	0.212	0.003	0.055	0.002	**2.2***
	3.13	0.207	0.004	0.045	0.003	**1.9***
	1.56	0.209	0.004	0.045	0.004	**1.8***
	0.78	0.156	0.000	0.024	0.004	1.3*
	0.00	0.133	0.009	0.016	0.001	1.0

LEGE 07074	**25.00**	0.567	0.017	0.232	0.026	**2.2**
	12.50	0.759	0.028	0.220	0.022	*1.6*
	6.25	0.834	0.021	0.216	0.023	*1.4*
	3.13	0.958	0.029	0.195	0.034	1.1
	1.56	0.991	0.040	0.194	0.028	1.1
	0.78	1.080	0.005	0.149	0.039	0.8
	0.00	0.988	0.008	0.172	0.047	1.0
Nostocales						
LEGE 06077	**25.00**	0.643	0.034	0.235	0.017	***1.9***
	12.50	0.823	0.103	0.245	0.028	***1.6***
	6.25	0.894	0.138	0.229	0.021	*1.4*
	3.13	0.958	0.057	0.216	0.029	*1.2*
	1.56	1.153	0.205	0.208	0.040	1.0
	0.78	1.155	0.045	0.186	0.005	0.9
	0.00	0.951	0.074	0.180	0.008	1.0

a Galactosidase activity (β-Gal) and alkaline phosphatase activity (PA) were measured in optical densities.

b The induction factor (IF) is expressed as the ratio $R = \beta/p$ where β represents β-Gal activity and PA activity. The asterisk (•) indicates a significant difference between negative control (distilled water) and cyanobacterial cells, $P < 0.05$. Positive control for the assay without S9 mix: 4-NQO (10 μg ml^{-1}), IF = 4.8.

Cytotoxic effects of cyanobacterial crude extracts were analyzed in L929 mouse fibroblasts by MTT assay performed in cultures exposed for 24, 48 and 72 h. Generally, the extracts were found to be toxic against L929 cell lines. Although no signals of cytotoxicity at 24 h-exposure time were observed for L929 cells, marked cytotoxicity was observed with crude hexane and methanolic extracts for higher exposure times (48 and 72 h).

The analysis of the 54 cyanobacterial extracts revealed 23 extracts with marked cytotoxicity activity at 72 h. The observed cytotoxicity of the extracts against L929 cell line, at 72 h incubation, expressed as cell viability percentage, is shown in Table 3. The aqueous extracts did not induce any cytotoxic effects on L929 cell line contrarily to the methanolic and *n*-hexane extracts. The methanolic extracts caused 15–85% loss of cell viability of L929 cells. From a total of 18 methanolic extracts, 12 caused moderate to potent cytotoxic effects, causing 15–50% inhibition of the cell viability of L929 cell line at 200.0 μg DW mL^{-1}. The occurrence of highest cytotoxicity (15–30%) was observed for the LEGE06077, 07073 and 07083 methanolic extracts which belong to the Nostocales and Chroococcales order, respectively. The *n*-hexane extracts did induce moderate cytotoxicity ranging from 30% to 70% of cell viability at the highest concentration of 200.0 μg DW mL^{-1}. From the 18 *n*-hexane extracts, 11 caused 50% of growth inhibition of L929 cell line at 200.0 μg DW mL^{-1}. These extracts fit mainly in the Oscillatoriales group similarly to methanolic extracts. Concerning to the lowest concentration, 3.1 μg DW mL^{-1}, a loss of cell viability between 0% and 15% was observed for the aqueous and *n*-hexane extracts. Besides, it was also observed a

slight stimulation of cell growth exposed to the aqueous extracts. At 3.1 µg DW mL^{-1} the methanolic extracts were the ones that induced the highest loss cell viability ranging from 8% to 50%. The one-way analysis (ANOVA) and Kruskal–Wallis statistical analysis revealed that *n*-hexane and methanol extracts are significantly different comparing with the control to *p*-value <0.05.

Table 3.

Cell viability of L929 cell line exposed to the maximum and minimum concentration of cyanobacterial extracts (CB) for 72 h. Results are expressed as percentage of control. H – *n*-hexane extracts; M – methanolic extracts; W – aqueous extracts. Bold values indicate results with cell viability above 50%.

	Cell viability (%) of L929 exposed to CB extracts					
Isolate	[H]		[M]		[W]	
code	200 µg DW mL^{-1}	3.1 µg DW mL^{-1}	200.0 µg DW mL^{-1}	3.1 µg DW mL^{-1}	200.0 µg DW mL^{-1}	3.1 µg DW mL^{-1}
Chroococcales						
LEGE 06068	**41.2**	93.4	71.1	80.4	61.4	94.3
LEGE 07073	54.9	87.6	**29.6**	59.9	81.8	124.4
LEGE 07083	49.9	86.3	**18.7**	48.7	91.2	115.4
LEGE 06079	**39.2**	89.6	**41.6**	76.6	92.4	93.3

Oscillatoriales						
LEGE 06069	51.2	87.0	53.7	72.8	81.0	105.1
LEGE 06070	**43.4**	95.9	**38.3**	71.7	60.9	112.6
LEGE 06072	**41.5**	95.9	**40.3**	84.2	85.3	101.8
LEGE 06078	66.1	89.9	61.6	92.9	89.3	96.1
LEGE 07074	53.6	104.8	54.8	78.5	87.6	109.2
LEGE 07075	**47.7**	97.9	73.2	91.7	98.4	114.3
LEGE 07076	**44.2**	100.3	**40.6**	85.0	90.6	103.8
LEGE 07080	**39.2**	93.2	**39.4**	85.7	68.8	112.7
LEGE 07084	**43.2**	99.3	51.0	72.3	83.9	104.2
LEGE 07085	52.3	104.0	**39.9**	83.0	103.7	108.2
LEGE 07091	**39.6**	97.1	**32.3**	73.0	96.2	112.3
LEGE 07092	**47.1**	107.5	**42.8**	79.9	92.7	120.5
Nostocales						
LEGE 06071	**34.3**	84.9	**46.8**	79.9	94.5	93.5
LEGE 06077	60.4	99.0	**15.7**	**47.4**	107.5	112.6

The CC50 values determined to *n*-hexane and methanolic extracts are expressed in Table 4. The strongest cytotoxicity (<45 µg mL^{-1}) was determined for methanolic extracts of LEGE 07073 and LEGE 07083 from the Chroococcales group and LEGE 06077 belonging to Nostocales group.

Table 4.

Cytotoxicity of CB hexane and methanolic extract on L929 cell line exposed 72 h. NCT – not cytotoxic in the tested dose range; H – *n*-hexane extracts; M – methanolic extracts; Bold values mean strong cytotoxicity.

	CC_{50} (μg mL^{-1})				
Isolate code	H	M		H	M
Chroococcales					
LEGE 06068	79.28	NTC	LEGE 07073	NTC	**40.86**
LEGE 06079	75.35	67.59	LEGE 07083	74.72	**25.80**
Oscillatoriales					
LEGE 06070	82.76	57.42	LEGE 07076	83.90	73.67
LEGE 06072	78.64	69.74	LEGE 07080	81.88	67.99
LEGE 06069	189.97	NTC	LEGE 07084	74.35	NTC
LEGE 06078	NCT	NTC	LEGE 07085	NTC	64.35
LEGE 07074	NTC	NTC	LEGE 07091	81.78	63.92
LEGE 07075	85.10	NTC	LEGE 07092	88.70	71.35
Nostocales					
LEGE 06071	72.47	68.07	LEGE 06077	NTC	**29.06**

It was also observed that cytotoxicity in L929 cells increases in a dose and time dependent manner as shown to the three extracts with highest CC_{50} values in Fig. 1. The ANOVA analysis also revealed that values of three mentioned extracts are significantly different (p-value <0.05) for the series of concentrations used.

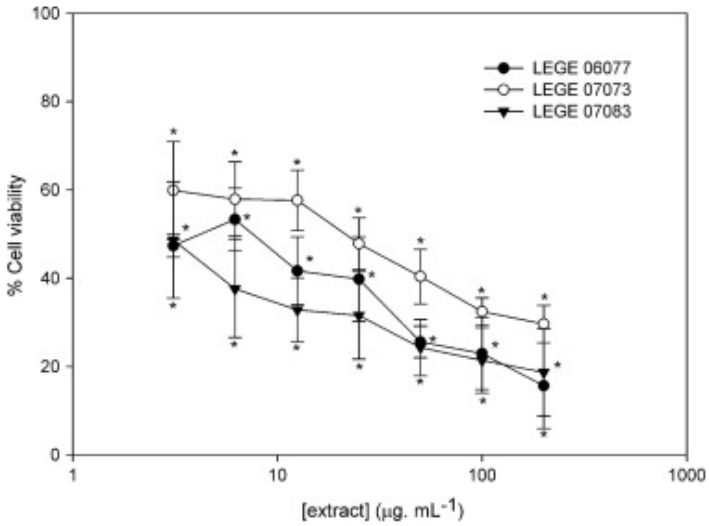

Fig. 1.

CB methanolic crude extracts-induced cytotoxicity profiles after 72 h-exposure time of L929 cell culture using MTT. Cell viability expressed as percentage of control. Results are given as mean and standard deviation of three replicates. The asterisk indicate statistical significance, $p < 0.05$.

The antiviral activity of CB extracts against influenza virus A, HSV-1, CVB3 and HRV-2 was determined by inhibition of viral cytopathic effect (CPE). With the exception of LEGE 07085, influenza virus, CVB3 and HSV-1 showed CPE inhibition less than 20%, which is considered low antiviral activity. LEGE 07085 showed anti-HSV-1 activity in GMK cells. The concentration required to reduce HSV-1 induced CPE by 50% was 174.10 μg mL^{-1}. No anti-HRV-2 activity was found. The same cell lines used to the antiviral activity assay were also used to evaluate cytotoxicity of the tested extracts. None of the extracts was cytotoxic in HeLa, MDCK, and GMK cells up to the maximum tested concentration of 200.00 μg mL^{-1} (data not shown).

4. Discussion

Cyanobacteria are known to be a rich source of bioactive compounds and for producing toxins harmful to a diversity of organisms such as mammals, fishes, invertebrates and algae. Our data have shown that estuarine cyanobacterial isolates are putative candidates to induce DNA damage on bacteria (*E. coli*) without metabolic activation. Of a total of 18 crude methanolic (70% v/v) extracts, 5% had significant genotoxic effects. The genotoxic extract LEGE 06078 belongs to *Phormidium* genus (Oscillatoriales). In addition, 22% of the extracts, belonging to the Oscillatoriales and Nostocales families, were considered as potential genotoxic candidates because they have shown IF values higher than 1.5 at higher concentrations values (12.50 μg mL^{-1}) but mostly without showing a dose-dependent response. Additionally, the absence of increased levels of alkaline phosphatase suggests that no *in vivo* cytotoxicity is observed. Nevertheless, decreased activity levels of this enzyme were observed in LEGE 07074 and 06077 extracts in a dose-dependent manner, which can affect the galactosidase functioning.

A study of Mankiewicz et al. (2002) had shown genotoxicity (without metabolic activation) by cyanobacterial extracts with the hepatotoxins microcystins. The IF values determined by Mankiewicz et al. (2002) range from 1.1 to 1.7 at 2 mg mL^{-1} which are slightly lower than the IF values of our data. Moreover, others studies with cyanobacteria, specifically benthic *Phormidium* strains from Baltic Sea, had shown the presence of apoptosis-inducing substances. Also, Shirahashi et al., 1993 and Teneva et al., 2005 and Surakka et al. (2005) had done a survey with freshwater *Phormidium* strains and showed that they are potential anti-tumour compounds producers. Nevertheless, the genus *Phormidium* of benthic estuarine cyanobacteria has far rarely been studied concerning toxin production and its potential effects on human and environmental health. Thus, our data are the first reporting genotoxicity induced by benthic *Phormidium* of estuarine ecosystems from temperate regions.

The *in vitro* cytotoxicity tests are based on the concept that toxic chemicals or compounds can affect basic functions of cells which are common among all cells causing measurable cellular functional and structural damage. The choice of cell lines for *in vitro* cytotoxicity remains difficult as the type of cells available is very diverse (Bubik et al., 2008, Puerto et al., 2009, Surakka et al., 2005 and Takamatsu et al., 2004). Nevertheless, cytotoxicity evaluation with cell cultures allows a rapid, standardized and sensitive way to

screen a large number of extracts for the presence of biologically harmful molecules. Regarding this, permanent cell lines are easy to maintain in culture and include a homogeneous cell population, being well suited for screening proposes due to the high reproducibility behaviour.

The fibroblast cell line L929 is highly proliferative and is widely used in cytotoxicity testing, mainly regarding toxicity towards cellular viability and proliferation. Fibroblast cells are the most common cells of all types of connective tissues, being actively engaged in the synthesis and upkeep of the collagenous extracellular matrix, also modulating adjacent cell behaviour including migration, proliferation and differentiation. In this way, biological evaluation with fibroblast cell cultures might be regarded as general bioassay, providing reliable information concerning basal cytotoxicity (Freshney, 2000). Among the 54 estuarine cyanobacteria extracts (hexane, methanol and water) tested, only hexane and methanol extracts impaired the viability of L929 fibroblasts. The cytotoxic effects were observed with methanolic and *n*-hexane extracts in a dose- and time-dependent manner, being clear only for exposure times higher than 24 h. The aqueous extracts did not affect the cell viability independently of the dose or exposure-time. The data obtained also point out that methanolic extracts have marked cytotoxic effects, with CC_{50} values that are slightly lower than those found with the hexane extracts. Members of all the three cyanobacterial orders studied had cytotoxic effects, although the lowest cytotoxic values were observed for Chroococcales and Nostocales. Surakka et al. (2005) had reported high cytotoxicity to human leukemia cells induced by *Nostoc*, *Nodularia*, *Phormidium* and *Anabaena* benthic strains from littoral habitats of Baltic Sea. Those genera excepting the *Phormidium* genus belong to the Nostocales family, supporting our findings. Our results showed that none of 18 isolates used in this study produce microcystins, nodularin or saxitoxin. So, we can infer that the observed cytotoxicity may be due to bioactive compounds present in the cyanobacterial extracts not previously described as known cyanotoxins.

The 54 cyanobacterial extracts were also screened for antiviral activity. The aqueous extract, LEGE 07085, derived from *Leptolyngbya* isolate showed antiviral activity against HSV1 with an IC_{50} of 174.10 μg $mL^{-1}$with low or no toxic effects on GMK cells up to 200 μg mL^{-1}. Several reports have been published about antiviral activity on cyanobacteria. The results were found for cyanobacteria from fresh and marine waters belonging mainly to Oscillatoriales and Nostocales orders. The antiviral effects observed were against HSV-1 and 2, influenza A and HIV viruses induced mostly by methanolic extracts \(Hayashi et al., 1996, Hernandez-Corona et al., 2002, Kanekiyo et al., 2007, Kanekiyo et al., 2005, Nowotny et al., 1997, Ohta et al., 1998, Rechter et al., 2006 and Zainuddin et al., 2002). Nevertheless, Hayashi et al. (1996) reported anti-HSV activity (ED = 212 μg mL^{-1}) of an aqueous extract of *Nostoc* in HeLa cell lines. In addition, the previous reports showed that the antiviral compounds and extracts belonged to the polysaccharide, carbohydrates and peptide groups. This might indicate that the compounds present in the cyanobacteria extracts may have highly polar moiety backing up the data now obtained. Furthermore, all the estuarine cyanobacteria extracts showed no long-term cytotoxicity to HeLa Ohio, MDCK and GMK cell lines at concentrations of up to 200 mg mL^{-1}. Thus, the antiviral activity observed is induced by a potential virus-specific compound or mixture that has no effects on cellular metabolism.

In addition, the MALDI-TOF analysis showed the presence of pheophorbide a and pheophytin a pigments as well as several peaks ranging from 600 to 1400 m/z ratio in the crude LEGE 07085 extract. Ohta et al. (1998) reported novel anti-HSV1 substances got from a marine green alga, *Dunaliella primolecta*, characterized as pheophorbide a like compounds. Thus, we assume that the potential anti-HSV-1 compounds present on the aqueous LEGE 07085 extract may be a pheophorbide like compounds. This work reports that benthic and picoplanktonic estuarine cyanobacteria contain potentially genotoxic, cytotoxic and anti–herpes compounds. It will be important to study those extracts that had positive results with the battery of assays we used, specifically those compounds that inhibited the HSV1.

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