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ORIGINAL ARTICLE

Antiproliferative, neurotoxic, genotoxic and mutagenic effects of toxic cyanobacterial extracts

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ABSTRACT
Cyanobacteria are the rich resource of various secondary metabolites including toxins with broad pharmaceutical significance. The aim of this work was to evaluate the antiproliferative, neurotoxic, genotoxic and mutagenic effects of cyanobacterial extracts containing Microcystin-LR (MCLR) *in vitro*. ELISA analysis results showed that MCLR contents of five cyanobacterial extracts were 2.07 ng/mL, 1.43 ng/mL, 1.41 ng/mL, 1.27 ng/mL, and 1.12 ng/mL for *Leptolyngbya* sp. SB1, *Phormidium* sp. SB4, *Oscillatoria earlei* SB5, *Phormidium* sp. SB2, Uncultured *cyanobacterium*, respectively. *Phormidium* sp. SB4 and *Phormidium* sp. SB2 extracts had the lowest neurotoxicity (86% and 79% cell viability, respectively) and *Oscillatoria earlei* SB5 extracts had the highest neurotoxicity (47% cell viability) on PC12 cell at 1000 µg/ml extract concentration. *Leptolyngbya* sp. SB1 and *Phormidium* sp. SB2 showed the highest antiproliferative effect (92% and 77% cell death) on HT29 cell. On the other hand, all concentrations of five toxic cyanobacterial extracts induced DNA damage between 3.0% and 1.3% of tail intensity and did not cause any direct mutagenic effect at the 1000 µg/plate cyanobacterial extracts. These results suggest that cyanobacteria-derived MCLR is a promising candidate for development of effective agents against colon cancer.

KEY WORDS: Cyanobacteria; Microcystin-LR; HT29 cells; HeLa cells; PC12 cells

Introduction

Cyanobacteria have been known as harmful to a large number of living organisms because of the production of toxic compounds, of which microcystines (MCs) commonly occur in freshwater blooms worldwide (Falconer & Humpage 2005; Dietrich *et al.,* 2008). The cyanobacterial toxins can be grouped by toxic effects on humans and animals as irritant toxins, dermatotoxins, neurotoxins, cytotoxins, and hepatotoxins (Kerbrat *et al.,* 2010; Nan *et al.,* 2011). The microcystines (MCs) are potent hepatotoxic cyclic heptapeptides produced by some of the cyanobacterial genera such as *Microcystis, Anabaena, Leptolyngbya, Oscillatoria, Phormidium* and *Planktothrix* (Izaguirre *et al.,* 2007; Frazao *et al.,* 2010; Wood *et al.,* 2010a; Shishido *et al.,* 2013). Several studies showed that Microcystin-LR (MCLR) is produced by some of the *Leptolyngbya* sp. (Gantar *et al.,* 2009), *Phormidium* sp. (Teneva *et al.,* 2005; Shishido *et al.,* 2013), *Oscillatoria tenius* (El Herry *et al.,* 2008) and *Oscillatoria agardii* (Luukkainen *et al.,* 1993) strains. To date, a large number of variants of MCs (e.g. MCLR, MCYR, MCRR, MCLA, MCLW, MCLF) have been identified (Lürling & Faassen 2013; Palagama *et al.,* 2017). MCLR is the most abundant variant of all MCs in natural waters (Dietrich & Hoeger, 2005). Intracellular toxicity of MCs is characterized by inhibition of serine/threonine protein phosphatase 1 (PP1) and PP2 catalytic subunits, glutathione depletion and production of reactive oxygen species (ROS) (Gupta *et al.,* 2003). Furthermore, inhibition of protein phosphatases 1 and 2A causes hyperphosphorylation and aggregation of tau protein leading to neuronal degenerative changes and apoptosis, similar as those observed in the brains of Alzheimer patients (Li *et al.,* 2012).

Previous studies have indicated that MCs are able to accumulate in the brains of some animals and cause neurotoxicity (Cazenave *et al.,* 2008; Metcalf *et al.,* 2009).

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Reduction in brain size was demonstrated in progeny of Swiss Albino mice subjected to cyanobacterial extract containing MCs (Falconer *et al.,* 1988). Meng *et al.* (2011) showed that MCLR lead to damage on cytoskeleton system via hyperphosphorylation of tau and the 27-kDa heat-shock protein (HSP27) in neuroendocrine PC12 cell line.

While most of the researches have been based on toxicity of cyanobacterial metabolites, some studies have revealed that some cyanobacterial species produced anti-inflammatory and anticarcinogenic compounds (Singh *et al.,* 2011). In 2008, the International Agency for Research on Cancer (IARC) estimated that 12.4 million were new cancer cases and about 7.6 million people died due to this disease worldwide (Boyle & Levin, 2008). Synthetic drugs used for several cancer therapies influence quickly dividing normal cells in the patient body causing certain other major irreversible adverse effects (Nooter & Herweijer, 1991; Naumovski *et al.,* 1992). Therefore, the investigation of different natural products for finding a new pharmacological agent is valuable both as a source of potential chemotherapeutic drugs and as a measure of safety (Nascimento *et al.,* 2013). Cytotoxicity, genotoxicity and mutagenicity studies can also help in assessing the effect and safety of natural products as new pharmacological agents (Romero-Jimenez *et al.,* 2005).

From the pharmacological point of view, MCs can be qualified as potential candidate in cancer drug development. MCs cause cellular damage following uptake through organic anion transporting polypeptides (OATP) and there are certain OATPs expressed prominently in cancer tissue as compared to normal tissue (Monks *et al.,* 2007; Sainis *et al.,* 2010). Recently, Kounnis *et al.* (2015) reported that MCLR showed cytotoxic activity against BxPC-3 and MIA PACA-2 pancreatic cancer cell lines expressing the OATP1B1 and OATP1B3 membrane transporters. Also Meickle *et al.,* (2009) have confirmed toxic compounds from *Lyngbya* sp. to show effective cytotoxic activity to KB carcinoma cells, HT29 colorectal adenocarcinoma cells and IMR-32 neuroblastoma cells.

The present study was carried out to evaluate the neurotoxic, and antiproliferative effects of five toxic cyanobacterial extracts which belong to *Leptolyngbya* sp., *Oscillatoria earlei, Phormidium* sp., *Phormidium* sp. and *Uncultured cyanobacterium* strains on ngF-differentiated PC12 cell, both human cervical adenocarcinoma cell line (HeLa) and human colorectal adenocarcinoma cell lines (HT29). The extracts were also investigated for their genotoxic and mutagenic effects to determine the possible mechanisms of cell death elicited by the extracts. This paper is the first report evaluating the genotoxic, mutagenic, neurotoxic and anticancer effects of the cyanobacterial extracts containing MCLR.

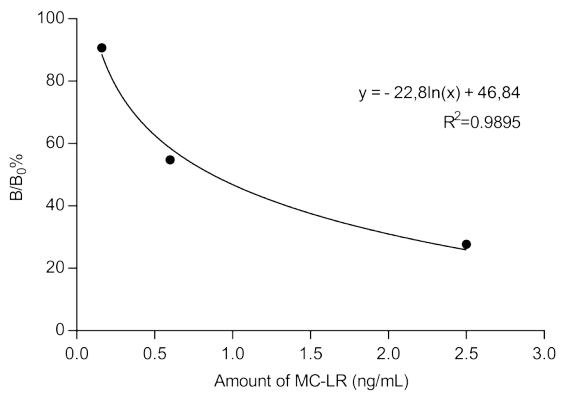

Figure 1. Logarithmic curve of Microcystin-LR standards (0.16, 0.60 and 2.5 ng/ml)

Materials and methods

Preparation of cyanobacterial extracts

The total biomass of 20 cyanobacterial isolates cultured in 500 ml of BG11 liquid medium (Rippka *et al.,* 1979) under continuous illumination (75 µM photons m^{-2} s^{-1}), at 25 °C for 30 days, were collected and rinsed twice with de-ionized distilled water and then dried at 60 °C. The dried algal biomass was powdered and 200 mg of dry algal powder was suspended in 10 ml of methanol. The suspension was kept at 50 °C in an incubator for three days. During this period, samples were taken away to ultrasonicate (20 KHZ) in cold water bath for one minute, twice a day. After three days, methanol extracts were centrifuged at 5000 rpm for 15 min at 5 °C, and the pellet was removed. One ml of supernatant was transferred to another tube for ELISA analysis. The methanol was evaporated from the remaining supernatant and the residue of cyanobacterial extracts and used for assays of neurotoxic and antiproliferative effect, genotoxicity, and mutagenicity.

ELISA analysis

Microcystin content was determined by an indirect competitive ELISA using an Envirologix QuantiPlate Test Kit for microcystins (Envirologix EP-022, Inc., Portland, Maine, USA), according to the manufacturer's instructions. Absorbance was read at 450 nm using a microplate reader. The microcystin contents measured in this paper were expressed as Microcystin-LR equivalents, and all the analysis was performed in two repetitions. In order to detect MCLR content, B_0% values for each sample and MCLR standards (0.16, 0.60 and 2.5 ng/ml) was calculated with the following formula B_0% = (average OD of calibrator or sample)/(average OD of negative control) × 100 and then a logarithmic curve was obtained using B_0% values of each MCLR standards (Figure 1).

DNA extraction, amplification and sequencing

DNA extraction was performed using CTAB method (Doyle & Doyle 1990) with minor modifications. PCR reactions were carried out in a total volume of 50 µl containing 100 ng of genomic DNA, 2× PCR master mix (Thermo Scientific, K0171) and 100 mM of cyanobacterial specific primers, Cya359F, Cya781Ra and Cya781Rb (Nübel *et al.,* 1997). Amplification was performed in a thermocycler using the following protocol; initial denaturation at 94 °C

for 5 min followed by 30 cycles of 1 min denaturation at 94 °C, 1 min at 60 °C and 1 min extension at 72 °C, and a final extension period of 7 min at 72 °C. PCR products were checked by 2% agarose gel electrophoresis with ethidium bromide staining and UV illumination, and then sequenced commercially using the CYA359F primer. The 16S rRNA gene sequences were compared to sequences in the NCBI database (http://www.ncbi.nlm.nih.gov/) using Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/BLAST/).

Cell cultures

PC12 cells (ATCC CRL-1721) are derived from a transplantable rat pheochromocytoma and represent a valuable model for neuronal differentiation. PC12 cells were cultured in Dulbecco's modified Eagle medium (DMEM) with 10% horse serum (HS), 10% fetal bovine serum (FBS), 100 IU/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine in a humidified CO_2 (5%) incubator at 37 °C (Ciofani *et al.,* 2010). Cells were differentiated for four days using 100ng/ml nerve growth factor (NGF) (Haq *et al.,* 2007). HeLa (human cervical cancer) (CCL-2™) and HT29 (human colon cancer) (HTB-38™) cell lines, the most commonly used for testing agents that may have antiproliferative effect, were purchased from ATCC. Cell lines were cultured in DMEM medium at 37 °C with 10% fetal bovine serum (FBS), 100 IU/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine under atmosphere of 95% air and 5% CO_2 in a 75 ml flask. The medium was changed every two days (Khanavi *et al.,* 2010; Priyadarsini *et al.,* 2010).

Neurotoxic and antiproliferative effect assays

For neurotoxic and antiproliferative effect testing, the cells were seeded in 96-well plates (10,000 cells per well) and incubated with increasing concentrations of cyanobacterial extracts (250, 500 and 1000 µg/ml) for 24 h. After incubations, cell viability was quantitatively measured with WST-1 assay (Cayman Chemical) (Lin *et al.,* 2010). Cell cultures were treated with 200 µl of culture medium added with 20 µl WST-1 mixture. The cells were incubated for 2 h at 37 °C in a CO_2 (5%) incubator and the absorbance of each sample was measured using microplate reader at wavelength of 450 nm (Epoch, BioTek) (Ciofani *et al.,* 2010). The neurotoxicity (according to % cell viability) was determined using the following formula: Cell Viability% = [Abs (sample)/Abs (control)] × 100 (Wen *et al.,* 2012). The antiproliferative effect was determined using the following formula: antiproliferative effect% = 1 – [Abs (sample)/Abs (control)] × 100 (Wang *et al.,* 2006).

Genotoxicity assay

For this test, the blood samples were obtained from Gazi University Hospital Blood Center. Blood lymphocytes isolated from healthy donors (ages 25 to 30, healthy, not using any alcohol, non-smoker, not under any medication and no radiological examination within the prior 3 months) were used for determining genotoxic effects of cyanobacterial toxins. Human lymphocytes were isolated using Biocoll separating solution according to the procedures of Sierens *et al.,* (2001). In order to isolate human lymphocytes, the blood samples heparinized were mixed with Biocoll separating solution and then centrifuged at 3000 rpm at 25 °C for 20 min. After density gradient, lymphocyte cells were collected. The collected cells were washed with PBS. The genotoxicity was tested by exposure of human lymphocytes at various concentrations of extracts (250 µg/ml, 500 and 1000 µg/ml methanolic extract of cyanobacteria) at 37 °C for 1 h. A negative (phosphate buffer saline, PBS) and a positive control (50 µM H_2O_2 for 15 min.) were also maintained. The slides were left in ice-cold alkaline solution (10M NaOH, 0.2M EDTA, pH>13) for 20 min, and then electrophoresis was conducted at 25 V, 300 mA for 20 min. After electrophoresis, each slide was stained with 50 µl of 20 µg/ml ethidium bromide. The slides were viewed using a fluorescent microscope (Olympus) equipped with an excitation filter (546 nm) and a barrier filter (590 nm) at 400× magnification. The tail length (µm), tail moment and tail intensity (%) of 100 comets on each slide were examined, using specialized Image Analysis System (Comet Assay III, Perceptive Instruments Ltd., UK) (Cestari *et al.,* 2004). DNA tail damage% = 100 × (Tail DNA density/Cell DNA density) (Behravan *et al.,* 2011). The comet analysis was carried out with three parallels in two repetitions.

Mutagenicity testing

Ames test was employed as a standard plate incorporation assay with *Salmonella typhimurium* strain TA100 without S9. Sodium azide (SA) and dimethyl sulfoxide (DMSO) were used as positive control and negative control, respectively. 500 µl phosphate buffer, 100 µl methanol extracts for each concentration (500 and 1000 µg/ml), and 100 µl cell suspension (1–2 × 10^9 cells/ml) were added to 2 ml top agar (kept at 45 °C) and vortexed for 3 s. The mixture was overlaid on the minimal glucose agar plates, and plates were incubated at 37 °C for 72 h. After the incubation period, the number of revertant bacterial colonies on each plate was counted (Lupi *et al.,* 2009).

Statistical analysis

All data obtained from antiproliferative, neurotoxic, genotoxic and mutagenic effects analysis results were analyzed statistically by analysis of variance (ANOVA) and Tukey's post-test was used to identify statistical significance for $p<0.05$. Results are submitted as mean value ± standard deviation (n=5 for the WST-1 assays, n=3 for all other analyses) (Ciafoni *et al.,* 2010).

Results

Toxin-producing cyanobacterial isolates

The five cyanobacterial isolates were selected according to the toxin production among 20 isolates from various fresh waters in Turkey (data not shown). Toxin-producing isolates were identified based on their 16S rRNA gene region sequence. The sequences were first analyzed at NCBI (http://www.ncbi.nlm.nih.gov/) databases using BLAST

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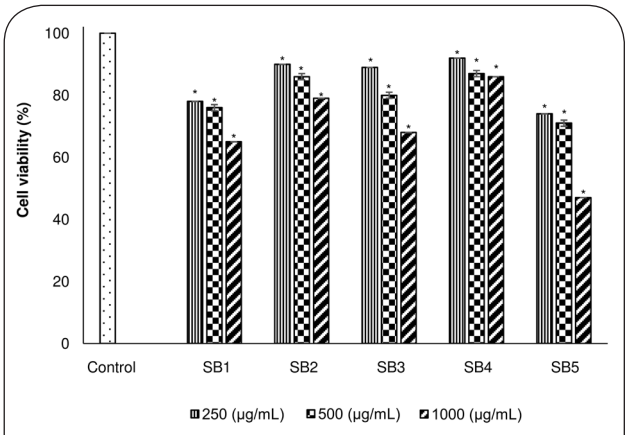

Figure 2. Neurotoxic effect of different concentrations (250, 500, 1000 µg/ml) of cyanobacterial extracts on ngF-differentiated PC12 cells (mean ± SD). Control; PC12 cells and medium. (n=5), *p<0.05; statistically different compared to control and each other.

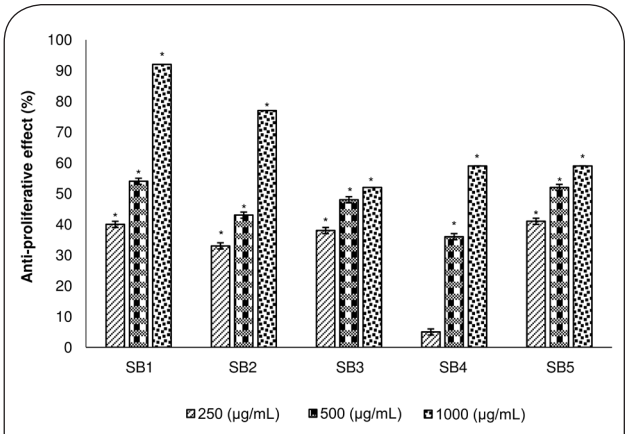

Figure 3. Antiproliferative effect of different concentrations (250, 500, 1000 µg/ml) of cyanobacterial extracts on HT29 cells (mean ± SD). Untreated cells were used as controls and control cells were taken as 100% viability (0% cytotoxicity). (n=5), *p<0.05; statistically significant difference compared to control.

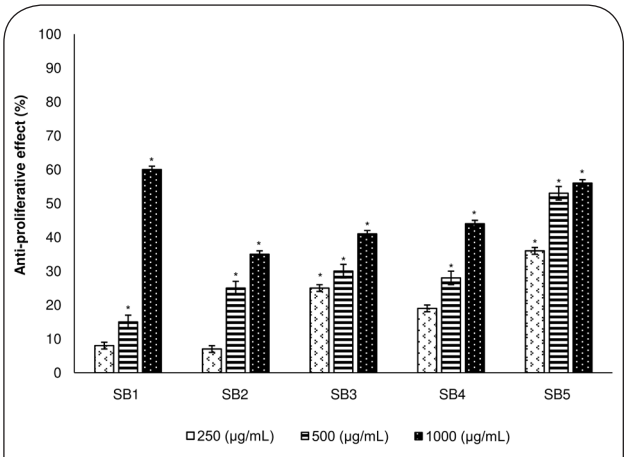

Figure 4. Antiproliferative effect of different concentrations (250, 500, 1000 µg/ml) of cyanobacterial extracts on HeLa cells (mean ± SD). Untreated cells were used as controls and control cells were taken as 100% viability (0% cytotoxicity). (n=5), *p<0.05; statistically significant difference compared to control.

(blastn) tool and corresponding sequences were downloaded. They displayed a similarity of ≥98% to a cultured cyanobacterium in the NCBI GenBank databases. Five toxic cyanobacterial isolates were identified as follows: *Leptolyngbya* sp., *Oscillatoria earlei, Phormidium* sp., *Phormidium* sp. and *Uncultured cyanobacterium* based on morphologic observations using light microscopy and 16S rRNA gene sequences. Accession numbers and similarity (%) of sequences are presented in Table 1. A total of 20 cyanobacterial isolates were analyzed by ELISA. Five of the 20 isolates produced Microcystine-LR. Microcystine contents of the five toxic strains are listed in Table 2. The minimum Microcystine-LR content was found in the strain *Uncultured cyanobacterium* SB3 (1.12 ng/ml) and the maximum in *Leptolyngbya* sp. SB1 (2.07 ng/ml).

Neurotoxic effect of cyanobacterial extracts

Cyanobacteria-derived Microcystine-LR (microcystine-leucine arginine) is a typically potent hepatotoxin, and also known to be neurotoxic (Meng *et al.,* 2011; Li *et al.,* 2015). In this study, the extracts of five cyanobacterial isolates contained MCLR indicated concentration-dependent neurotoxic effect. Toxic effects of cyanobacterial extracts showed to differ significantly (p<0.05) from each other and control. Neurotoxic effect of toxic cyanobacterial extracts (1000 µg/ml) were determined as *Phormidium* sp. SB4 (86%) < *Phormidium* sp. SB2 (79%) < *Uncultured cyanobacterium* SB3 (68%) < *Leptolyngbya* sp. SB1 (65%) < *Oscillatoria earlei* SB5 (47%), based on cell viability. The extract of *Oscillatoria earlei* SB5 showed the highest neurotoxic effect (47% cell viability), and the extract of *Phormidium* sp. SB4 showed the lowest neurotoxic effect (86% cell viability) on ngF-differentiated PC12 cells. The results obtained are given in Figure 2.

Antiproliferative effects of cyanobacterial extracts

The two different human cancer cell lines were tested for the in vitro antiproliferative bioactivity of the extracts. The cyanobacterial extracts showed an antiproliferative effect on HeLa and HT29 cells. The treated cells differed significantly (p<0.05) from control cells (untreated). All of cyanobacterial extracts exhibited higher antiproliferative effect against HT29 colon cancer cell line HeLa cervix cancer cell line. Furthermore, these five cyanobacterial extracts induced concentration-dependent antiproliferative effect through loss of viability. The best concentration in destroying the cancer cells was 1000 µg/ml of cyanobacterial extracts which had the highest antiproliferative effects compared to the other three concentrations. HT29 cells exposed to *Leptolyngbya* sp. SB1 and *Phormidium* sp. SB2 extracts showed 92% and 77% cell death, respectively, as presented in Figure 3. *Leptolyngbya* sp. SB1 and *Oscillatoria earlei* SB5 extracts showed a high antiproliferative effect on HeLa cells, 60% and 56% cell death, respectively, as given in Figure 4.

In vitro DNA damage of cyanobacterial extracts

The genotoxic effects of cyanobacterial extracts were determined with human lymphocyte cells by

Comet Assay. Positive control (50 µM H_2O_2) induced approximately 25% DNA damage. All concentrations of cyanobacterial extracts induced DNA damage between 3.0 and 1.3, according to the percentage of tail intensity (Table 3). Interestingly, the cyanobacterial extracts-treated lymphocyte cells were approximately 8 or 19 fold less DNA damaging than H_2O_2 treated (Figure 5). Although there was not enough tail density difference between negative control and cyanobacterial extracts-treated lymphocyte cells, it was found that as increasing concentrations of extracts, the DNA tail damage was also increasing. According to comet test results, if the ratio of tail length to head length is below 5% it is accepted that there is no DNA damage (Yen *et al.,* 2001) This showed that the cyanobacterial extracts do not induce significant DNA damage in comparison to positive controls treated with H_2O_2.

Mutagenicity of cyanobacterial extracts

The Ames test was utilized to determine the mutagenicity of cyanobacterial extracts (500 and 1000 µg/plate) in this study. In the Ames test, *Salmonella typhimurium* strains were used carrying different mutations in the operon coding for histidine, and TA100 tester strains served to detect mutagens causing base-pair substitutions (Huang *et al.,* 2007). The mutagenicity assays showed that none of the cyanobacterial extracts induced any significant increase in the number of revertant colonies, indicating the absence of any mutagenic activity on *Salmonella typhimurium* TA100 cells. The results obtained from studies on the mutagenic potential of cyanobacterial extracts are presented in Table 4.

Discussion

Previous studies have suggested that MCs have a cytotoxic effect and tumor-promoting activity mediated by inhibition of protein phosphatases 1 and 2A (Humpage *et al.,* 2000; Lone *et al.,* 2015). Moreover, Cyanobacteria-derived Microcystine-LR (MCLR) has been proposed as potential neurotoxic compound by several studies (Feurstein *et al.,* 2009, 2010; Wang *et al.,* 2009; Meng *et al.,* 2011; Hu *et al.,* 2016). On the other hand, in spite of its tumor-promoting activity, earlier studies have suggested that MCLR may be utilized for cancer treatment as an anticancer agent (Monks *et al.,* 2007; Obaidat *et al.,* 2012). MCLR uptake is mediated by various organic anion–transporting polypeptides (OATPs) (Komatsu *et al.,* 2007; Monks *et al.,* 2007). Monks *et al.,* (2007) demonstrated that MCs cause cytotoxicity in OATP1B1 and OATP1B3 expressing HeLa cells. This study ensured a potential clue for the usage of MCs as anticancer agents for tumors expressing the OATP transporters. Besides, Lankoff *et al.* (2003) reported that MCLR induce the formation of abnormal mitotic spindles similar to taxol which is an antimitotic compound used to treat cancers, especially breast and ovarian cancers.

In the present study, we investigated antiproliferative, neurotoxic, genotoxic and mutagenic effects of cyanobacterial extracts containing MCLR on HeLa cells, HT29 cells, PC12 cells, human lymphocyte cells, and also *Salmonella typhimurium* TA100 cells. Our results demonstrated that MCLR content of five of the 20 cyanobacterial extracts were 2.07 ng/ml, 1.43 ng/ml, 1.41 ng/ml, 1.27 ng/ml, and 1.12 ng/ml for *Leptolyngbya* sp. SB1, *Phormidium* sp. SB4, *Oscillatoria earlei* SB5,

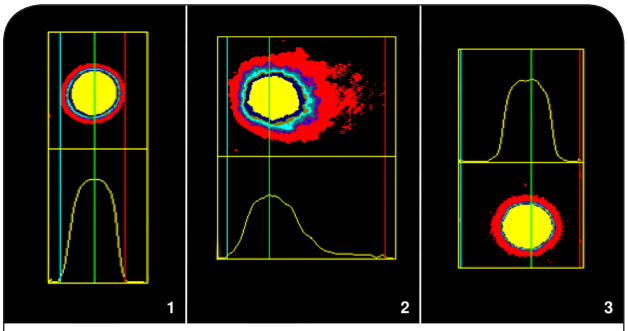

Figure 5. Fluorescence images of comet assay. DNA damage was detected in human lymphocyte cells exposed to (1) PBS (negative control), (2) 50 µM H_2O_2 (positive control), and (3) *Leptolyngbya* sp. SB1 extract (1000 µg/ml concentration).

Table 1. Sources and 16S rRNA-based identification of cyanobacterial isolates. The highest similarities to the sequences in GenBank and Accession numbers are shown.

Code	GenBank No	Cyanobacteria species	Homology (%)	Source
SB1	JX088103.1	*Leptolyngbya* sp. CAWBG532	98	Tatlarin/ Nevsehir
SB2	KU740243.1	*Phormidium* sp. CBC	99	Bölükören/ Nevsehir
SB3	KF856318.1	*Uncultured cyanobacterium* clone 4BF1C	99	Yalıntaş/ Nevsehir
SB4	KR872396.1	*Phormidium* sp. KO02	99	Bölükören/ Nevsehir
SB5	KF487296.1	*Oscillatoria earlei* S11	98	Tatlarin/ Nevsehir

Table 2. Microcystine-LR contents of cyanobacterial extracts based on ELISA assay.

Cyanobacterial extracts	B/B_0%*	Estimation of MCLR content (ng/ml)
Leptolyngbya sp. (SB1)	30.29	2.07
Phormidium sp. (SB2)	41.26	1.27
Uncultured cyanobacterium (SB3)	44.07	1.12
Phormidium sp. (SB4)	38.60	1.43
Oscillatoria earlei (SB5)	38.82	1.41

*: Mean optical density of test sample or control sample ÷ mean optical density of negative control × 100.

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Phormidium sp. SB2, *Uncultured cyanobacterium* SB3, respectively. *Oscillatoria earlei* SB5 and *Leptolyngbya* sp. SB1 extracts had the highest neurotoxic effect while *Phormidium* sp. SB4 and SB2 extracts had the lowest neurotoxic effect at 1000 µg/ml concentration. Although toxin content of *Oscillatoria earlei* SB5 extract (1.41 ng/ml) was lower than of *Leptolyngbya* sp. SB1 (2.07 ng/ml) and *Phormidium* sp. SB4 (1.43 ng/ml) extracts, neurotoxic effect of *Oscillatoria earlei* SB5 extract (47%) on PC12 cells was higher than *Leptolyngbya* sp.SB1 (65%) and *Phormidium* sp. SB4 extracts (86%) at 1000 µg /ml concentration based on cell viability. Furthermore, the neurotoxic effect of *Uncultured Cyanobacterium* SB3 extract (68%) defined as the lowest toxic isolate was higher than *Phormidium* sp. SB4 (86%) and *Phormidium* sp. SB2 (79%) extracts at 1000 µg /ml concentration. There was no direct correlation between MCLR toxin content and neurotoxic effect according to our results. In our opinion, the reason is that MCLRs obtained from five toxic isolates may have different chemical variants of MCLR such as [Leu^1], [Leu^1, Glu(OCH$_3$)^6], [Leu^1, Ser^7], [D-Asp^3], [Dha^7] [D-Asp^3, Z -Dhb^7] e.g. (Shimizu *et al.,* 2013; Qi *et al.,* 2014) and this variation may be changed at the neurotoxic effect of cyanobacterial extracts.

Table 3. DNA damage (tail length, tail moment, and tail intensity) in human lymphocyte cells exposed to cyanobacterial extracts according to Comet assay.

Agent	Conc. (µg/mL)	Tail length (µm)	Tail moment	Tail intensity(%)
*NC	–	12.0±1.2	0.3±0.0	0.5±0.2
**PC	–	50.4±0.6	24.0±0.2	25.0±1.7
Leptolyngbya sp. (SB1)	250	13.8±1.1	0.6±0.1	2.3±0.6
	500	19.5±1.0	0.8±0.1	2.7±0.2
	1000	20.8±0.0	1.0±0.1	3.0±0.1
Phormidium sp. (SB2)	250	25.4±0.4	0.9±0.2	1.3±0.1
	500	26.5±0.5	1.3±0.2	1.9±0.3
	1000	30.9±0.6	1.6±0.1	2.5±0.2
Uncultured cyanobacterium (SB3)	250	20.4±0.2	0.8±0.1	1.7±0.2
	500	25.2±0.4	1.3±0.2	2.0±0.0
	1000	29.5±0.6	1.8±0.2	2.4±0.2
Phormidium sp. (SB4)	250	16.0±0.3	0.7±0.2	1.4±0.3
	500	17.0±0.2	1.0±0.1	2.0±0.3
	1000	18.0±0.5	1.4±0.2	2.4±0.2
Oscillatoria earlei (SB5)	250	19.0±0.6	1.0±0.2	1.7±0.3
	500	24.0±0.8	1.4±0.2	2.2±0.3
	1000	30.8±0.7	1.8±0.1	2.9±0.4

*: Negative control (PBS), **: Positive control (50 µM H_2O_2), Mean ± SD (n=3), $p<0.05$; statistically significant difference compared to positive control.

Table 4. Mutagenic activities of cyanobacterial extracts on *Salmonella typhimurium* TA100.

Agant	Amount (µg/plate)	TA100 (revertant colony/plate)
*NC	10	244±7
**PC	10	967±6
Leptolyngbya sp. (SB1)	250	269±1
	500	328±11
Phormidium sp. (SB2)	250	272±3
	500	293±4
Uncultured cyanobacterium (SB3)	250	300±2
	500	321±6
Phormidium sp. (SB4)	250	293±1
	500	319±5
Oscillatoria earlei (SB5)	250	272±3
	500	297±1

*: Negative control (only DMSO), **: Positive control (Sodium azide), Mean±SD (n=3), $p<0.05$; statistically significant difference compared to positive control.

Because cyanobacterial extracts are potential agents for the development of biologically active molecules, it is also important to evaluate the genotoxic and mutagenic effects of these extracts. In this regard, the genotoxic effect of cyanobacterial extracts on human lymphocyte cells was assessed in this study and all concentrations of cyanobacterial extracts induced DNA damage according to the percentage of tail intensity. This showed that the cyanobacterial extracts do not induce DNA damage significantly. Also, we determined that cyanobacterial extracts did not show direct mutagenic effect on *Salmonella typhimurium* TA100 cells. Similarly to our results, several studies reported that cyanobacterial extracts containing cyanotoxins and pure MCLR did not induce reverse mutation in *S. typhimurium* strains (Wu *et al.,* 2006). In a contrary study, a strong mutagenic response which caused by microcystic cyanobacterial extract was shown using four *S. typhimurium* (TA97, TA98, TA100 and TA102) (Ding *et al.,* 1999).

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In summary, our results showed that both neurotoxicity of *Leptolyngbya* sp. SB1 and *Phormidium* sp. SB2 cyanobacterial extracts were slightly lower on PC12 cells and highly antiproliferative on HT29 cells as well as lower genotoxicity and mutagenity considering their potential pharmacological applications. Therefore we suggested that MCLR obtained from *Leptolyngbya* sp. SB1 and *Phormidium* sp. SB2 extracts may be used as an agent for treatment of colon cancer.

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