

MICROCYSTIN CONGENERS CONTRIBUTE TO TOXICITY IN THE HALOPHILIC CYANOBACTERIUM *APHANOTHECE HALOPHYTICA*

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Abstract - *Aphanothece halophytica* is an extremely saline cyanobacterium. This study investigates the toxic nature of the organism and presents the first report of hepatotoxic cyclic heptapeptide microcystin analogs. The activity of the crude extract was investigated in mice. Results showed acute toxicity with mice death at about 4 h. Histopathological examination indicated massive alveolar hemorrhage and extensive congestion in liver cells. Increases in the levels of serum enzymes, i.e., AST (aspartate aminotransferase), ALT (alanine aminotransferase) and LDH (lactate dehydrogenase), provide further evidence of cell injury. An ELISA-based immunological detection kit confirmed the presence of microcystin analogs.

Key words: Cyanobacteria; *Aphanothece halophytica*; microcystin; serum enzymes; ELISA.

INTRODUCTION

Cyanobacteria are Gram-negative photosynthetic prokaryotes and inhabit diverse environments, being the most successful colonizers of hypersaline environments. Microcystins (MCs) are cyclic heptapeptides that are produced by different cyanobacteria genera such as *Microcystis*, *Anabaena*, *Planktothrix* and *Nostoc* (Spoof, 2004). There are over 80 different MCs (Hoeger et al., 2005), MC-LR being the most ubiquitous and extensively studied. Acute exposure to MC-LR can lead to severe liver damage, including massive intrahepatic hemorrhage, liver swelling, and even death (Pouria et al., 1998). MC toxicity is known to be associated with the inhibition of serine threonine protein phosphatases by interacting with catalytic subunits of these enzymes (Maynes et al., 2006).

Aphanothece halophytica is a unicellular halophilic cyanobacterium that can tolerate a wide range

of salinities of up to 0.2-3 M NaCl. Recently, it has been reported that oral administration of exopolysaccharide from *A. halophytica* (EPAH) significantly inhibited Sarcoma 180 growth and has shown protective and therapeutic effects on FM1-induced pneumonia in mice (Zheng *et al.*, 2005, 2006).

To date, the toxicity of the crude extract of hypersaline cyanobacterium *Aphanothece halophytica* has not been explored. This research aims to study its bioactive potential through histopathological analysis and serum enzyme activity in mammals, and presents the first report of the possibility of the toxic cyclic heptapeptide microcystin (MC) analog in the cyanobacterium.

MATERIALS AND METHODS

Aphanothece halophytica cells were grown photoautotrophically in BG11 medium supplemented with 18mM NaNO₃ and Turk Island medium. Cells were

grown in 250-ml flasks containing 100 ml medium, and aeration was provided by regular shaking. Illumination was provided by cool white fluorescent lamps at $95\mu\text{mol}/\text{m}^2/\text{s}$ with a 16:8 h light: dark cycle. *Aphanothece halophytica* 7418 (originally isolated by Dr. Y. Cohen from Solar Lake, Sinai 2) was maintained in the laboratory in axenic conditions and was used for the present study.

Cells were harvested by centrifugation at $8\ 000\times g$ after 45 days of growth and lyophilized. Extractions were carried out in methanol+TFA (0.01%) v/v (MT), (Krishnamurthy et al., 1986) and butanol:methanol:water (1:4:15) v/v (BMW), (Harada, 1996). The lyophilized cell mass was dissolved in the minimum quantity of extraction solvent and subjected to freeze/thaw cycles, after which they were centrifuged and the supernatant was collected. After three successive extractions, the supernatants were pooled and lyophilized to obtain the crude extract.

Evaluation of toxicity was carried out using male mice aged 6 weeks (Park strain, weighing approx. 25 g). Animals were housed at 20°C with a 12 h day/night cycle and given free access to food and water. Crude cyanobacterial extract prepared from BMW (1:4:15) and MT, at a concentration of 2 g/kg body weight was injected intraperitoneally. Animals were observed for symptoms of toxicity and survival time was recorded. 0.9% physiological saline was injected intraperitoneally to mice to serve as controls. These were killed after cervical dislocation in accordance with the animal ethical committee guidelines. All animals were dissected and the organs were removed for histological study. The experiments were performed in replicates. Specimens of liver, lung, brain, kidney, heart, spleen and intestine were dissected from the experimental animals. Organs were cut in small pieces and placed in Bouin's fluid (Bouin, 1897) for 22-24 h. Dehydration of tissues was carried out by repeated transfer to 70% ethanol, 90% ethanol and 100% ethanol. Tissues were embedded in paraffin and cut into blocks. Sections, 6 μm in thickness, were prepared with the help of a microtome and spread onto slides at 40°C . Staining was done with

Ehrlich's hematoxylin-eosin (Ehrlich, 1886). Paraffinized sections were rinsed with xylene and mounted with Canada balsam or DPX.

The test group was divided into three, with three animals in each group, for the evaluation of LD_{50} . Three different concentrations of the extract were prepared (0.6, 1.2 and 1.8 g/kg body weight) and were injected into the animals to determine the median lethal dose.

Blood was collected from the retro-orbital sinus of the experimental mice for serum enzyme analysis. The animals were administered four different concentrations of crude extract, at 0.6, 1.2, 1.8 and 2.4 g/kg body weight. (The selection of the doses administered was approximately 0.5 LD_{50} , LD_{50} , 1.5 LD_{50} and 2 LD_{50} , as determined previously). The entire procedure was carried out under general anesthesia. Blood was collected in glass tubes and left to clot. The clear serum was removed from the clot and stored at -20°C until further analysis. The experiment was carried out in triplicate. Total lactate dehydrogenase, aspartate aminotransferase and alanine aminotransferase were assayed by means of spectrophotometry through a commercially available enzyme assay kit (Coral Clinical Systems, Goa, India for AST and ALT and FAR Diagnostics, Verona, Italy for LDH).

The freeze-dried crude extract was dissolved in sterile water and used directly for microcystin analysis. ELISA was used for the determination of the concentration of total microcystin content. Antibody-coated plates, standards, substrates and all reagents were provided by the Envirologix microcystin plate kit (EP022, Envirologix, Inc., Portland Maine, USA). The assay was performed according to the manufacturer's instructions. The absorbance was measured at 450 nm by reading the plate on a Multiskan Ascent (Thermo Labsystems, USA).

HPLC analyses of microcystins were performed by the method of Lawton et al. (1994). The mobile phase was Milli-Q water (A) and acetonitrile (B), containing 0.05% trifluoroacetic acid. The linear gradient program for the mobile phase was 0 min 25%

B, 35 min 70% B, 37 min 70% B, 38 min 25% B, and 60 min 25% B. Samples were run at a flow rate of 1 ml/ min and separation was monitored with PDA detector in the range of 200-400 nm (Harada et al., 1999) with a resolution of 1.2 nm. The HPLC system consisted of a Waters 600 controller equipped with a 717 plus autosampler, a 2998 photodiode array detector and an inline degasser. The column used was a C18 Spherisorb S10 ODS column (20×250mm). Data acquisition was done using the Empower2 software. All solvents used were of HPLC grade and filtered through a 0.22- μ m membrane filter. About 0.25g of crude extract was dissolved in sterile distilled water and centrifuged at 15 000×g for 20 min. This was filtered through a 0.22- μ m membrane filter. The clear filtrate was injected on the HPLC column.

Analysis of variance (ANOVA) was performed with Dunnett's post test to compare mean values of each treatment group for significant differences to the untreated control group. Probit analysis was used for the estimation of the median lethal dose of the crude extract. Correlation coefficients were calculated by linear regression analyses. For all parameters, the criterion for significance was set at $p \leq 0.05$.

RESULTS AND DISCUSSION

Death of mice occurred within 4 h after exposure. Mice showed symptoms of restlessness, heavy breathing, and loss of movement coordination. The histopathology of lungs revealed extensive alveolar hemorrhage and infiltration within the septa, accompanied by massive congestion. Thickened septa were visible. Liver tissue showed marked congestion with focal hepatocytic necrosis and edema. Congested central vein and sinusoids were seen. Mild hemorrhage and congestion occurred in the kidney, while mild congestion was also seen in the spleen. No significant change was observed in brain, heart and intestines (Fig.1, a-l). The animals receiving BMW (1:4:15) extract showed a pronounced degree of injury to the organs as compared to the MT extract. Preliminary experiments with BMW and MT as extraction solvents have clearly established BMW as superior, with a greater yield of crude extract and significantly en-

hanced activity against mice. This finding reflects the intrinsic volatility of the polar metabolites as compared to the nonpolar metabolites present in the crude extract. Therefore, further experiments were performed with BMW (1:4:15) as extraction solvent.

The LD₅₀ was determined through probit analysis using Biostat 2008 software (Fig. 2). The LD₅₀ of crude extract was found to be 1.29 g/kg body weight. This serves as a useful index in defining acute toxicity, providing important insights into the mechanism of toxicity and the structure-activity relationship for a particular class of compound (Blazka and Hayes, 2007).

Serum enzymes AST (aspartate aminotransferase), ALT (alanine aminotransferase) and LDH (lactate dehydrogenase) were analyzed with diagnostic kits, as liver and lungs were primarily affected. The concentration of each of the enzymes had increased markedly compared to the control. ALT showed an increase of 234%, AST was elevated by 45% while LDH registered a 96% increase in mice injected with the highest concentration of crude extract. The animals fed with a minimum dose of extract showed little alteration compared to the control. The ALT increase in animals treated with 0.6 g of crude extract was not significant. The levels of all three enzymes increased uniformly with the increase in concentration of the crude extract (Fig. 3).

Although the likely cause of death of the animals was extensive injury to lungs and liver, as suggested by histopathological observations, the highly elevated levels of the aminotransferases and LDH could also point to injury of other organs. Increased serum concentration of these enzymes is considered suggestive of hepatocellular damage (Rao et al., 2005, Billam et al., 2008), which is in agreement with the observed experimental observations. MCs display a broad spectrum of action that is not restricted to their hepatotoxic mechanism of phosphatase inhibition. Dörr et al. (2010) have stated that known MC variants are diverse not only in their species of origin but also in their functions and targets. This suggests its possible effects on other organs apart from

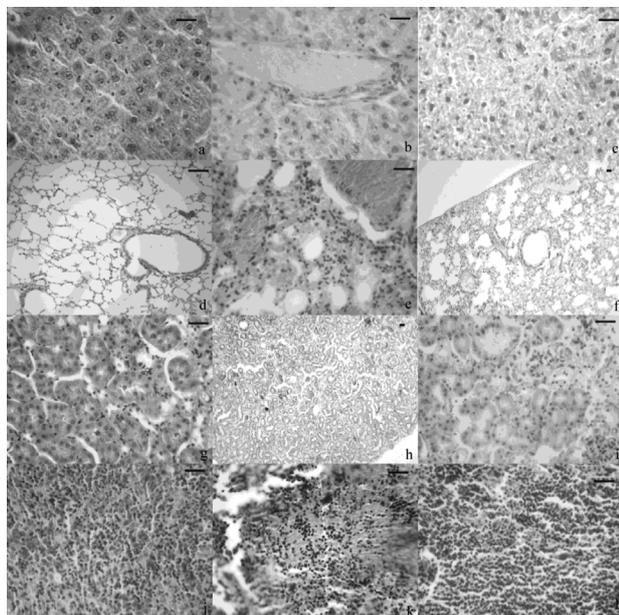


Fig. 1. a-c: photomicrographs of liver – control, BMW (butanol:methanol:water 1:4:15)-treated (congested central vein and sinusoids), (MT methanol+TFA 0.01%)-treated (focal hepatocytic necrosis). Scale bar: 40 μm .
d-f: photomicrographs of lung – control, BMW-treated (extensive hemorrhage with thickened septa), MT-treated (massive congestion and hemorrhage). Scale bar: 40 μm and 10 μm .
g-i: photomicrograph of kidney – control, BMW-treated (congestion and mild hemorrhage), MT-treated (mild congestion). Scale bar: 40 μm and 10 μm .
j-l: photomicrograph of spleen – control, BMW-treated, MT-treated. Mild congestion visible in both treatments. Scale bar: 40 μm .

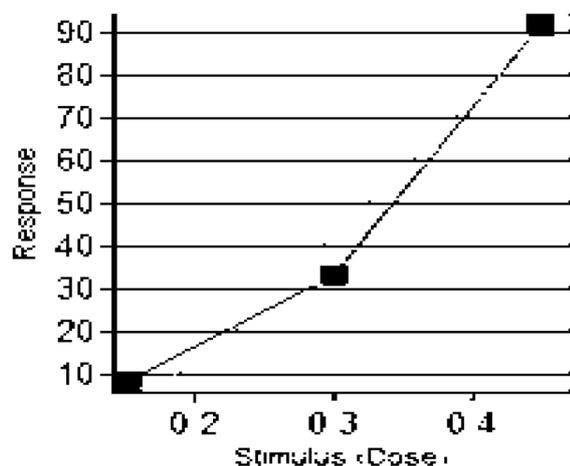


Fig. 2. Probit analysis for the estimation of LD50. $r^2 = 0.893$; $y = 7.33x - 1$.

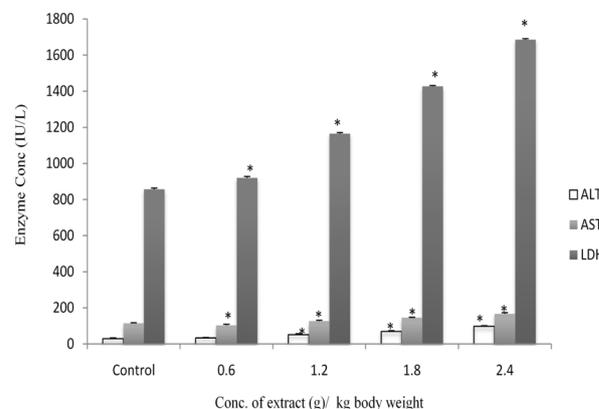


Fig. 3. Effects of *A. halophytica* extract on the levels of serum enzymes in rats.

the liver. Since the liver was one of the most affected organs, the presence of hepatotoxic microcystin or a microcystin-like toxin could not be ignored. Further confirmation of the presence of the toxin was carried out with HPLC and ELISA-based immunological quantification of microcystins.

HPLC analysis of crude extract of *A. halophytica* showed a retention time of 4.5 and 4.8 min with absorption maxima at 238 nm corresponding to that obtained for standard microcystin with a retention time at 4.6 min (Fig. 4). The limit of detection for the HPLC system as determined by standard deviations of the blank was found to be 0.02 $\mu\text{g l}^{-1}$. The quantification of microcystins was performed using ELISA; false-positive results were assessed by multiple determinations of blank. Measurements that were above the lowest assay levels (lowest calibrator 0.16 $\mu\text{g l}^{-1}$) were considered as false positives, while for false-negative determination, the concentration level was set at 0.25 $\mu\text{g l}^{-1}$. No false negatives were obtained, while the number of false positives was negligible. The total amount of microcystins detected was 3.99 $\mu\text{g g}^{-1}$ of lyophilized cells. The limit of detection (LOD) of this kit is 0.147 ppb and the limit of quantification (LOQ) of this kit was validated at 0.175 ppb as provided by the manufacturers. The Enviroligix Quantiplate kit for microcystins does not distinguish between the microcystin toxin variants, but detects their presence to differing degrees. The manufactur-

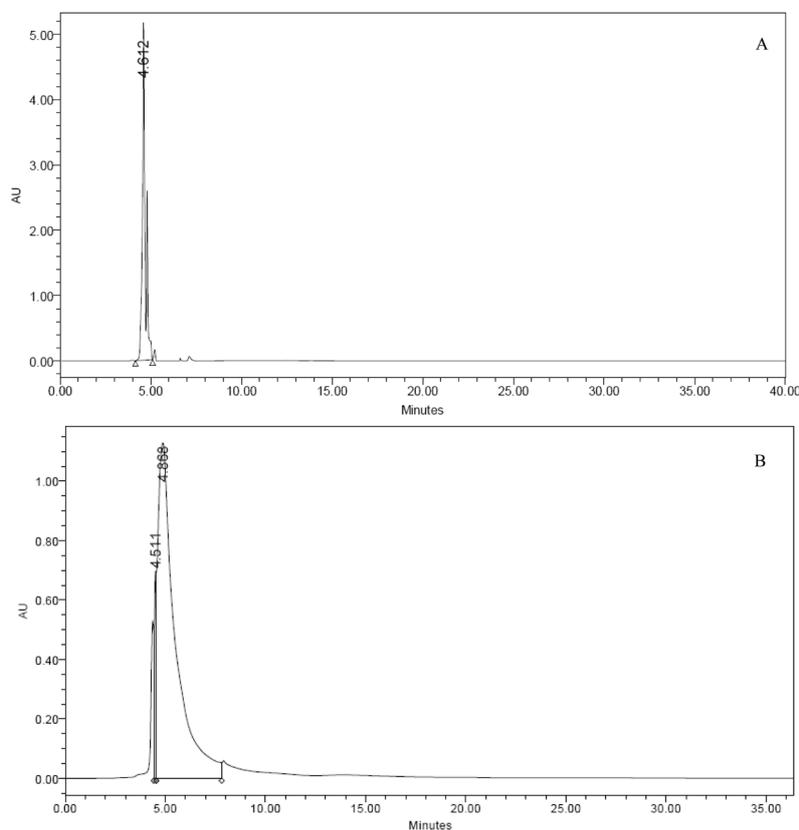


Fig. 4. HPLC chromatogram of standard microcystin (A) and crude extract of *A. halophytica* (B) extracted at 238 nm.

ers of the kit have provided a % B_0 value and the limits of detection for four microcystin toxin variants and nodularin toxin.

The presence of microcystins was verified by HPLC analysis. The detection of microcystins was based on their characteristic UV spectra (Lawton et al., 1994). As reported earlier by Bajpai et al. (2009), the retention time of standard microcystin was in accordance with that obtained for the *A. halophytica* extract.

One-way ANOVA followed by Dunnett's test showed that the three enzyme concentrations measured for the extract-treated animals were significant as compared to enzyme concentrations for the control.

In view of the above, it may be concluded that microcystin congeners contribute to the toxicity in

the halophilic *Aphanothece halophytica*, but other compounds present in the cyanobacterial crude extract could also be partly responsible for the toxic effects (Vishwakarma, 2013).

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