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# ТОКСИЧНИ ЕФЕКТИ НА ЕКСТРАКТИ ОТ *PSEUDOANABAENA GALEATA* (CYANOPROKARYOTA) ВЪРХУ МИШКИ И КЛЕТЪЧНИ КУЛТУРИ *IN VITRO*

**Иванка Тенева[1], Румен Младенов[1], Балик Джамбазов[2]***

[1]Катедра «Ботаника», ПУ «П. Хилендарски», 4000 Пловдив;
[2]Катедра «Биология на развитието», ПУ «П. Хилендарски», 4000 Пловдив.
*Автор за кореспонденция: E-mail: balik@uni-plovdiv.bg

# TOXIC EFFECTS OF EXTRACTS FROM *PSEUDOANABAENA GALEATA* (CYANOPROKARYOTA) IN MICE AND CELL CULTURES *IN VITRO*

**Ivanka Teneva[1], Rumen Mladenov[1], Balik Dzhambazov[2*]**

[1]Department of Botany, Plovdiv University, 4000 Plovdiv, Bulgaria; [2]Department of Developmental Biology, Plovdiv University, 4000 Plovdiv, Bulgaria
*Corresponding author: E-mail: balik@uni-plovdiv.bg

**Key words:** *Cyanoprokaryota*, *Pseudoanabaena galeata*, toxins, *in vitro*, mouse bioassay.

**Abstract**
The freshwater cyanoprokaryote *Pseudanabaena galeata* has not been studied so far with respect to toxin production and potentially resulting public health and environmental effects. Therefore, the aim of this study was to investigate *Pseudanabaena galeata* for production of intracellular and/or extracellular compounds with cytotoxic potential. We tested the toxicity by a traditional *in vivo* mouse bioassay as well as *in vitro* assays using different cell cultures. After five days exposure of mice to cyanoprokaryotic extract, histopathological alterations in the liver and kidneys were observed. No animals died after exposure to the extract. Both, extract and medium in which *Pseudanabaena galeata* had been grown, showed cytotoxic effects on the used cell cultures *in vitro* in a dose-dependent manner. The presence of cyanotoxins as saxitoxins, anatoxin-a and microcystins/nodularins, was confirmed by ELISA and qualitative HPLC analyses. Thus, the freshwater *Pseudanabaena* species should be considered as a potential risk of public health and they may also play an important role on the transfer of cyanotoxins through food chain.

## INTRODUCTION

Blue-green algae (Cyanoprokaryota, Cyanobacteria, Cyanophyta) are prokaryotic organisms with a cosmopolitan distribution. They are an integral part of the freshwater and marine phytoplankton, and often have a dominant presence. Certain natural conditions (no wind, temperature between 15 and 30°C, pH between 6 and 9, and relatively rich in nitrogen and phosphorus environment) provoke an increased proliferation of this group of organisms, making them a dominant population among the phototrophic organisms in the respective water basin, which could cause formation of

the so called "algal blooms" (Carmichael et al., 1994). In these cases, Cyanoprokaryota may occur as producers of toxins causing intoxication in animals and humans. Cyanotoxins are three main groups: hepatotoxins (microcystins, nodularins, cylindrospermopsin), neurotoxins (saxitoxins, anatoxins) and dermatotoxins (lyngbyatoxin, aplyziatoxin). Causing direct or indirect intoxication (accumulation in fish and mussels), these toxins were identified as potential hazards to the animals and human health (Carmichael & Falconer, 1993; Pouria et al., 1998) and during the last 30 years have been triggered the interest of researchers worldwide. The most well studied and most commonly cited as producers of Cyanotoxins are: *Microcystis aeruginosa*, *Aphanisomenon flos-aquae*, *Anabaena flos-aquae, Cylindrospermopsis raciborskii, Planktothrix agardhii, Lyngbya majuscula, Nodularia* and *Oscillatoria* (Chorus & Bartram, 1999).
Despite the intensive work of many research groups, data about Cyanoprokaryota producing toxins are still incomplete. Poorly investigated in this aspect are the species of genus *Pseudoanabaena*. Marsalek et al. (2003) reported *Pseudoanabaena limnetica* as a producer of microcystins. First observation of microcystins in Tunisian inland waters was correlated with the dominance of the genera *Oscillatoria* and *Pseudoanabaena* (Herry et al., 2007). Oufdou et al. (2001) reported *Pseudoanabaena sp*. as sources of substances with antibacterial and antifungal activities.
The freshwater species *Pseudoanabaena galeata* has not been studied so far in terms of its toxic potential. Therefore, the aim of our study was to investigate this species as a producer of intracellular and extracellular substances with cytotoxic potential using biological (*in vivo* mousse bioassay and *in vitro* assays), immunobiological (ELISA) and chemical (HPLC) methods. Our results defined *Pseudoanabaena galeata* as a species producing both, hepatotoxins and neurotoxins. This is the first report of such bioactivity of *Pseudoanabaena galeata*.

## MATERIALS AND METHODS

### *Algal culture and extract preparation*

*Pseudoanabaena galeata* (Böcher) – kept in PACC (Plovdiv Algal Culture Collection) under No 5411 has been grown intensively under sterile conditions using a Z-nutrient medium. The culture was synchronized by altering light/dark periods of 16/8 hours. The temperature was 33°C and 22°C during the light and dark period, respectively. The intensity of light during the light period was 224 μmol photon $s^{-1}$ $m^{-2}$ (Lux 12000)**.** The culture medium was aerated with 100 liters of air per hour per one liter of medium, adding 1% $CO_2$ during the light cycle. The period of cultivation was 14 days. Extract of the blue-green alga was obtained according to the method of Krishnamurthy et al. (1986) with slight modifications. Briefly, *Pseudoanabaena galeata* was removed from the Z-medium and weighed, then frozen and thawed, and extracted twice (3 h and overnight) with water-methanol-butanol solution (15:4:1, v:v:v, analytical grade) at 22°C while stirring. The extract was centrifuged at 10000 rpm for 30 min. The supernatant of the extract was pooled and organic solvents removed via speed-vac centrifugation (SAVANT, Instruments Inc. Farmingdate, NY, USA) at 37°C for 2 h The resulting extract was sterilized by filtration through a 0.22 μm Millipore filter and prepared to give equivalent final concentrations of 150 mg/ml (wet weight/volume) suspended algal matter.
To investigate whether *Pseudoanabaena galeata* release toxic products into culture environment, the nutrient solution in which the alga was cultivated during the 14 days was filtered through a 0.22 μm Millipore filter. The final equivalent concentration of suspended algal matter per mL culture medium was 20 mg/ml (wet weight/volume). This algal medium was tested for cytotoxicity *in vitro.*

### *Toxicity of the Pseudoanabaena extract in vivo*

*Mouse bioassay*

A total of 6 male DBA/1 mice (19-22 g) were used for the experiment (three mice per group). All mice were kept in a climate-controlled environment with 12 h light/dark cycles in polystyrene

cages containing wood shavings. Mice were fed standard rodent chow and water *ad libitum* in a specific pathogen-free environment. Mice were injected i.p. with 0.5 mL test solution containing equivalent final concentrations per mouse of 15 mg suspended cyanoprokaryotic matter (682-790 mg/kg mouse). In order to obtain this test solution, the algal extract was diluted 1:4 with phosphate buffered saline (PBS). Control mice were injected with 0.5 ml PBS. The animals were observed for 24 h after treatment. Behavioral symptoms, weight and survival times were recorded.

*Liver and kidney histology*
All animals were subjected to histological examination of the liver and kidneys for pathology. After termination of the experiment, the liver and kidney slices were processed for light microscopy according to standard procedures. Briefly, the tissue samples were fixed in 4% buffered formalin for 24h, dehydrated in a graded series of alcohol, cleared in xylene, and embedded in paraffin wax. Multiple sections from each block were prepared at 5 μm thickness and stained with hematoxylin and eosin (McManus & Mowry, 1965).

***Animal cell cultures and exposure conditions***
Two different primary mouse cell cultures were used for the cytotoxicity tests: kidney cells and endothelial cells. Mouse cells were cultured in 75 cm2 flasks in Dulbecco's Modified Eagle's Medium (DMEM, Gibco™, Paisley, Scotland, UK), supplemented with 10% (v/v) heat inactivated fetal calf serum (FCS, PAA Laboratories GmbH, Linz, Austria), 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma, Steinheim, Germany), at 37oC with 5% CO2 in air and high humidity. Cell viability was measured with the trypan blue exclusion test (Berg et al., 1972).
Prior to exposure, cells were plated in 96-well tissue culture plates at a density of $1.5x10^4$ per 200 μL DMEM medium with 10% FCS. After 24 h of attachment, the medium was removed and replaced by the exposure medium. Cells were exposed to three concentrations of the algal extract – 3.75 mg/ml (2.5% of extracts), 7.5 mg/ml (5% of extracts) and 15 mg/ml (10% of extracts), for 24 or 48 h prior to analysis of cytotoxicity by the MTT assay. The same volume of Millipore water was used as a control.
In addition to exposure to the algal extract, the cells were also exposed to varying concentrations of medium in which *Pseudoanabaena galeata* has been grown for 14 days. The cells were treated with algal medium at final concentrations of 2.5%, 5% and 10% under the conditions mentioned above. A similar concentration of Z-medium was used as appropriate control.

***Cytotoxicity assay (MTT test )***
The MTT (3-(4',5'-dimethylthiazol-2'-yl)-2,5-diphenyltetrazolium bromide, Sigma, St. Louis, MO, USA) assay was carried out in accordance with Edmondson et al. (1988). After the desired time of contact with algal substances (24 or 48 h), 20 μl of a 0.5% (w/v) solution of MTT in PBS were added directly to each well and incubated at 37°C for 4 h in dark. After incubation, the medium with the dye was aspirated and plates inverted to drain unreduced MTT, and 100 μL of DMSO was added to each well in order to facilitate solubilization of the formazan product. The plates were shaken, and absorbance was read at 570 nm.

***High performance liquid chromatography (HPLC) analysis***
Chromatography was performed with an ÄKTA™ explorer 100 Air system (Amersham Pharmacia Biotech AB, Uppsala, Sweden) using an UNICORN V4.00 software. The analytical column was a Discovery® $C_{18}$ (5x4 mm I.D., 5 μm) from Supelco (Bellefonte, PA, USA). The mobile phase consisted of a mixture of solvent A (10 mM ammonium acetate, pH=5.5) and solvent B (10 mM ammonium acetate-acetonitrile, 80:20, v/v) as follows: 0% of B at 0 min, 100 % of B at 45 min to 65 min using a linear gradient. Flow-rate was 0.8 ml/min and UV detection was performed at 238 nm. All runs were carried out at room temperature. The column was reequilibrated with 8 ml of the solvent A between runs. Each standard was run separately (AnTx-a 5 μg/ml, MC-LR 5 μg/ml,

STX 40.5 pg/ml, 200 µl injection volume) and thereafter a mixture of all standards with the same concentrations in 200 µl was run again. 200 µl of the sample were injected for HPLC analysis. Toxins and their concentrations in the sample were determined by comparing retention times and peak areas for each toxin with those of the standards.

***ELISA***

*Saxitoxins*

The samples were analyzed by the Ridascreen™ saxitoxin ELISA kit (R-Biopharm, Darmstadt, Germany). This is a competitive ELISA for the quantitative analysis of saxitoxin and related toxins based on the competition between the free toxins from samples or standards and an enzyme-conjugated saxitoxin for the same antibody. The mean lower detection limit of the Ridascreen™ saxitoxin assay is about 0.010 ppb.

*Microcystins*

*A*nalysis of samples was performed using the Microcystin Plate kit (EnviroLogix Inc., Portland, USA.). As for the saxitoxin ELISA, this a quantitative, competitive immunosorbent assay. The limit of detection of the EnviroLogix Microcystin Plate kit is 0.05 ppb.

**RESULTS**

***Toxicity of the Pseudoanabaena extract in vivo***

After five days exposure of mice to cyanoprokaryotic growth medium or extract, histopathological alterations in the liver (L) and kidneys (K) were observed (Fig.1), but the animals did not die. The liver histology from treated mice showed granulovacuolar degeneration and mitosis, inflammatory cellular infiltration, obscured cell borders, congestion, hemorrhage, and necrosis. Histological alterations in the renal tissue of treated mice included hemorrhage, inflammation between tubules, necrosis and destruction of tubular cells and atrophy of glomerulus.

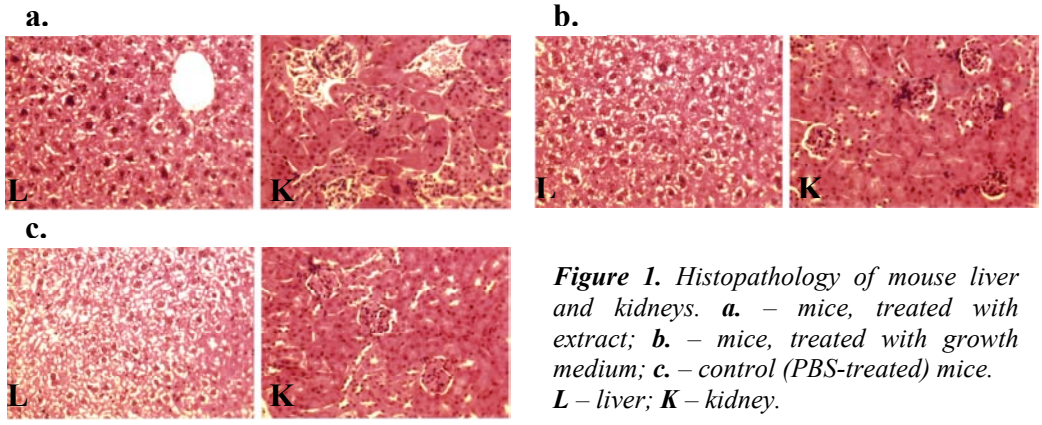


***Figure 1.*** *Histopathology of mouse liver and kidneys.* ***a.*** *– mice, treated with extract;* ***b.*** *– mice, treated with growth medium;* ***c.*** *– control (PBS-treated) mice.* ***L*** *– liver;* ***K*** *– kidney.*

***In vitro toxicity of Pseudoanabaena galeata extract and growth medium***

To investigate the cytotoxicity of the extract and growth medium *in vitro*, freshly established mouse primary cultures from different tissues (endothelial and kidney cells) were used.

After treatment of the cells with varying concentrations of *Pseudoanabaena* extract distinct responses were detected depending from the origin of the cells and time of exposure (Fig. 2). The cell viability (as measured by MTT) was weakly affected in almost all cell cultures after 24 h of exposure. A greatest cytotoxic effect (from 50% to 60 %) was observed for both cell lines 48 h after treatment with 10% of the extract and 10% of the growth medium (Fig. 2). Both, extract and

cyanoprokaryotic growth medium, showed cytotoxic effects on the used cell cultures in a dose-dependent manner. Kidney cells are more sensitive compared to the endothelial cells.

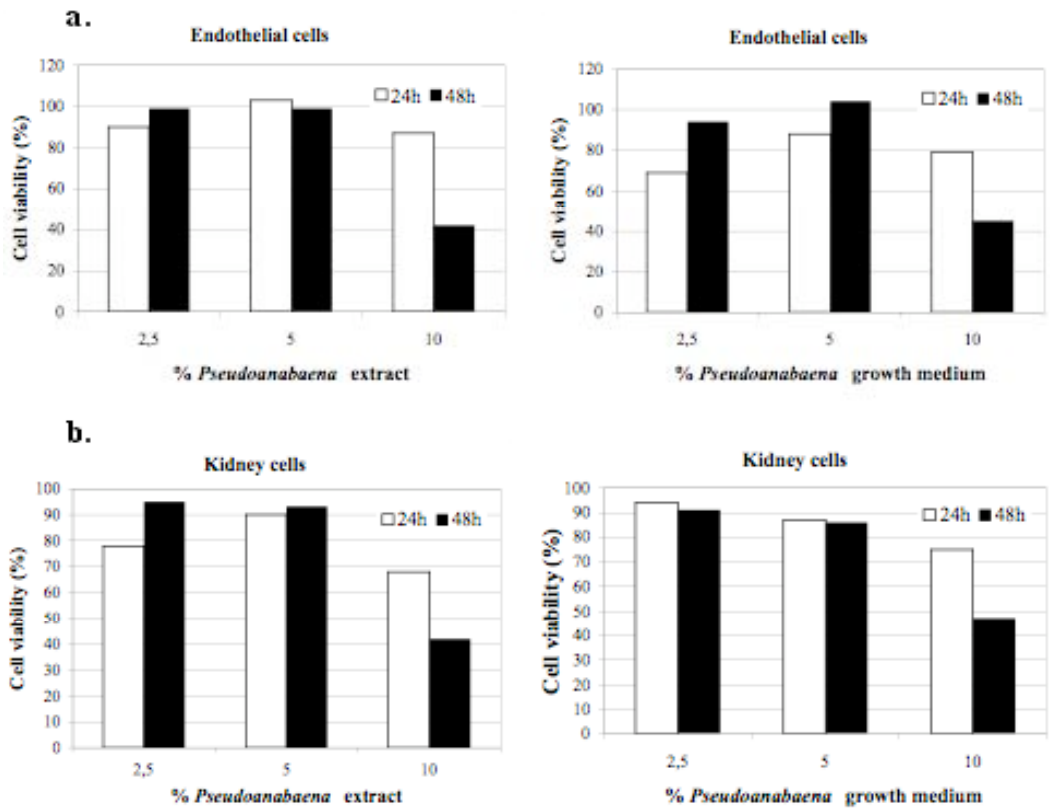


***Figure 2.*** *Viability of mouse cell cultures treated with Pseudoanabaena extract or growth media for 24 h (white bars) or 48 h (black bars) as determined by MTT assay.* ***a****. – endothelial cells;* ***b.*** *– kidney cells. The cultures were exposed to equivalent concentration of suspended algal matter of 3.75 mg/ml (2.5% of extracts), 7.5 mg/ml (5% of extracts) and 15 mg/ml (10% of extracts) or by diluting the culture medium with 2.5%, 5% and 10% of the Pseudoanabaena growth medium. An equivalent % of Millipore water or Z-medium (the medium in which the Pseudoanabaena had been grown) was added to the control cultures. Data are represented as mean values of triplicates.*

***HPLC analysis***

To further identify the toxic compounds, *Pseudoanabaena* extract and growth medium were analysed by HPLC using comparison of retention times to standards of cyanotoxins (Fig. 3). HPLC was arranged to detect cyanotoxins from different groups (e.g. anatoxin-a, saxitoxins, MC-LR) by one run under ones and the same conditions. Figure 3a shows the HPLC chromatogram of a standard mixture including AnTx-*a*, STX and MC-LR. *Pseudoanabaena* extract and growth medium showed distinct HPLC profiles (Fig. 3b, 3c). *Pseudoanabaena* extract (Fig 3b) shows peaks with retention time similar to the STXs (19.36 min) and to MCs (46.79). These peaks were not found in *Pseudoanabaena* growth medium (Fig. 3c). There were peaks with retention time 7.13 - 9.62 min (Fig. 3c) similar to the AnTox-*a*, which were not detected in the *Pseudoanabaena* extract.

The HPLC analysis confirmed the presence of cyanotoxins (even in low doses) in both, extract and growth medium of the investigated *Pseudoanabaena galeata*.

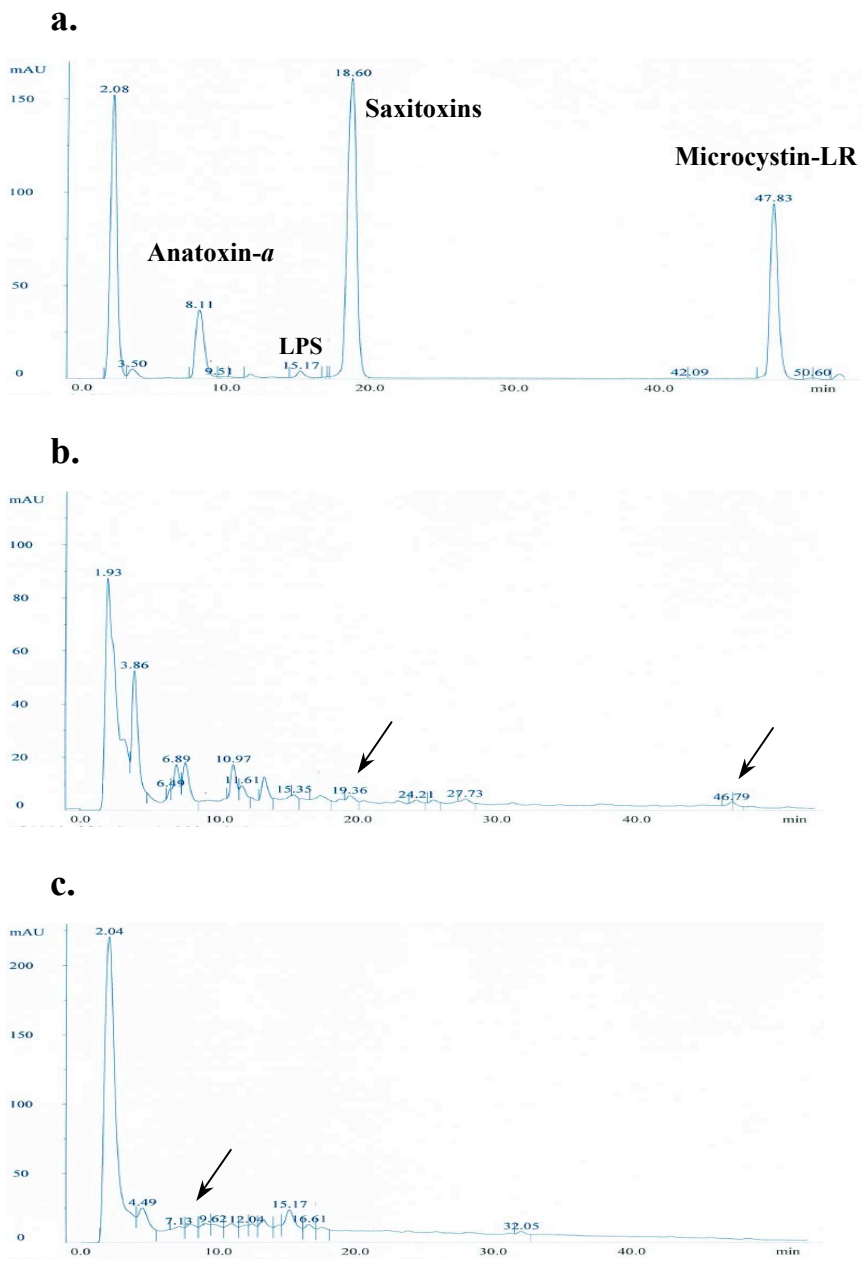


***Figure 3.*** *HPLC chromatograms of (**a.**) a mixture of standard cyanotoxins; (**b.**) the extract obtained from Pseudoanabaena galeata (**c.**) the growth medium of Pseudoanabaena galeata.*

***ELISA analysis***

To confirm the presence of cyanotoxins, we also tested the *Pseudoanabaena* extract and growth medium by commercially available ELISA kits for saxitoxins and microcystins. The saxitoxin ELISA assay, which has 10-30% cross-reactivity to decarbamoyl saxitoxin, gonyautotoxins II, III, B1, C1 and C2, showed that both, *Pseudoanabaena* extract and growth medium, contained some levels of these toxins (135 ppt and 7.5 ppt respectively). The microcystin/nodularin ELISA kit has

crossreactivity to microcystin LR, LA, RR, YR and nodularin. In tested samples, these toxins were detected only in the *Pseudoanabaena* extract with concentration 0.0625 ppb.
These data correlated with the data of HPLC analyses und confirmed the presence of neuro- and hepatotoxins in *Pseudoanabaena galeata* extract and neurotoxins in *Pseudoanabaena galeata* growth medium.
Results suggest that the freshwater *Pseudoanabaena* species should be considered as a potential risk for public health and they may also play an important role in the transfer of cyanotoxins through the food chains.

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