Cytotoxic effect of soil cyanobacterial extracts to mammal cell lines YAC-1 and WEHI

Cytotoxický efekt extraktů půdních sinic k liniím savčích buněk YAC-1 a WEHI

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Abstract

The cytotoxic effects of ten methanol extracts obtained from soil filamentous cyanobacteria (*Anabaena*, *Calothrix*, *Nodularia*, *Cylindrospermum*, *Tolypothrix* and *Trichormus*) were studied. Two different mammal cell lines (YAC-1, WEHI) were selected for cytotoxicity testing using the MTT test and flow-cytometry (*FC*). For comparison, the brine shrimp assay (using *Artemia* as a testing organism) was performed. The composition of extracts was studied using HPLC-MS. Both MTT and FC found the cytotoxic effect in 6 of 10 tested cyanobacterial extracts, but these results did not correlate with those obtained by brine shrimp assay. In *Anabaena torulosa* and *Cylindrospermum* sp. the most severe damage was recorded by both methods. In cells treated by the extract of *Anabaena torulosa*, 100% inhibition was found already after 60-minute exposure. Induction of necrosis was revealed by *FC*, since all tested cells were marked by propidium iodide indicating disruption of the cellular membrane. Surprisingly, no effects associated with this extract were found for *Artemia*. In *Cylindrospermum* sp. both necrotic (80%) and apoptotic (20%) induction was found by *FC*. The presence of microcystins in these extracts was not proved by HPLC-MS, but several peaks with unknown molecular masses were observed. Thus, the production of new cytotoxins with cytoxicity comparable to microcystin can be expected.

Abbreviations: FC – flow cytometry, **m/z** – molecular mass divided by charge of molecule, **LB** – lyophilised biomass, **PBS**- physiological buffered solution

Introduction

Cyanobacteria are known to produce a wide variety of bioactive compounds including toxins. Cyanotoxins can be classified into two main groups according to the damage they cause and also the type of bioassay used to screen their toxicity – biotoxins and cytotoxins (CARMICHAEL 1992). First group includes hepatotoxic microcystins and neurotoxic anatoxins, which were deeply studied because of their occurrence in water bodies (CARMICHAEL 1986,1990,1992, FALCONER *et al.* 1994, MARŠÁLEK *et al.* 1996, SIVONEN *et al.* 1996,

CHORUS 2004). Cytotoxins are compounds with direct toxic or destructive effect to certain cells. Recently several compounds with various effects to mammal cell lines have been described. This group is very heterogeneous in chemical structure and also in the damage inflicted. A large group of cyclic or aliphatic peptides with protease inhibition activity, e.g. cyanopeptides, cyanopeptolins, micropeptins, microginins, or aeruginosins, were isolated from planktic *Microcystis*, *Anabaena*, *Planktothrix* strains (BANKER & CARMELI 1999, GOLAKOTI 2000; HARADA *et al.* 1993; ISHIDA *et al.* 1997, 1997, 1999, 2000, RESHEF & CARMELI 2001). But the presence of such compounds in soil cyanobacteria, e.g. *Nostoc*, *Scytonema*, was also confirmed (YANG *et al.* 1993, MATERN *et al.* 2001). Other well-known group of cyanobacterial cytotoxins is the scytophycins, first isolated from *Scytonema hofmanii.* The best-known member of this group of macrolytic lactone is tolytoxin that causes severe cytotoxic effect through the depolarisation of cell micro-filaments (PATTERSON & CARMELI 1992). Although scytophycins were mostly isolated from branched cyanobacteria like *Scytonema* and *Tolypothix,* there is also evidence that it can be present in other cyanobacteria, such as *Cylindrospermum muscicola* (JUNG *et al.* 1991).

Many studies have focused on the screening for important biotechnological compounds in cyanobacterial extracts. The main interest was to search for anti-tumor, antibiotic or antiviral compounds. Several compounds were observed to have a strong cytostatic activity or ability of selective inhibition of tumours. The most promising candidates were the class of cytotoxic depsipeptides (peptides containing an ester linkage). Cryptophycins A-F isolated from *Nostoc* strains, that are associated with this group, are lipophilic peptides including mono-chlorinated L-O-methyltyrosine (TRIMURTULU *et al.* 1994). Except for the above mentioned groups, several others like tanazoles (CARMELI *et al.* 1993), mirabimides (CARMELI *et al.* 1991), lyngbyabellins and hapalindoles (KLEIN *et al.* 1992, LUESCH *et al.* 2002) were isolated from soil or benthic cyanobacteria.

Although the cytotoxicity of some cyanobacterial compounds is well documented, the mechanisms of such an effect have not been properly described yet. Neither have all the mechanisms of the best-known cyanobacterial toxin, Microcystin-LR, been completely described. It is well known that microcystins and nodularins are capable of protein-phosphatase inhibition in mammal cell lines, which leads to increased protein phosphorylation (YOSHIZAVA *et al*. 1990) ended by necrotic cell death. However, some research groups have reported in recent years that microcystins are capable of initiating apoptosis in hepatocytes (BOTHA *et al.* 2004, DING & ONG 2003) characterized by apoptotical morphological changes including membrane blebbing, cell shrinkage, externalization of membrane phosphatidilserine and chromatin condensation (MCDERMOTT *et al.* 1998; DING *et al*. 2000; FLANDMARK *et al.* 1999). Nevertheless, the exact trigger of this process is still unclear. The induction of free radical formation and mitochondrial alternations are two major events found in microcystin-treated cultured rat hepatocytes (DING & ONG 2003, DING *et al.* 1998), but, it is still speculative if this process operates in the middle of apoptosis triggering.

The aim of our study was to compare cytotoxicity of 10 original cyanobacterial isolates on mammal cell lines and to evaluate the damage inflicted on cells. For comparison, toxicity on *Artemia* cultures was performed. To ensure that these effects are not correlated with the presence of microcystin, HPLC-MS analysis was performed.

Materials and Methods

Origin of strains and cultivation. Ten different strains of soil cyanobacteria (belonging to the genera *Anabaena*, *Calothrix, Cylindrospermum, Nodularia, Tolypothrix*, and *Trichormus*) were used in this study (see Table 1). The cultivation for extraction was carried out for 45 days in liquid BBM (BISCHOFF & BOLD 1963) medium in 250-ml Erlenmeyer's flasks percolated by air. Cyanobacteria were cultured under artificial light with an intensity of 35 μmol photons PhAR m^{-2} s^{-1} at 20 ± 2ºC.

Biomass harvesting and extraction. Biomass was harvested by centrifugation in 50-ml glass cuvettes (3000 rpm, 15 min.), stored at -40˚C and lyophilised. 200 μg of lyophilised biomass was transferred into 10-ml glass test tubes; 6 ml of absolute methanol was added and let for 24 hours to extract. Test tubes were centrifuged (3000 rpm, 15 min), the supernatant was transferred and evaporated under vacuum. The resulting solid extract was dissolved in 1 ml of absolute methanol.

Table 1: List of strains used in the study

Label	Exact name	Isolated in/by	Locality/country	Habitat
1A†	*Anabaena torulosa*	1994/Lukešová	Novosedly/Czech Rep.	salty meadow
3A†	*Anabaena sphaerica*	1995/Lukešová	unknown	unknown
4N†	*Nodularia* sp.	1990/Lukešová	Nadym(Siberia)/Russsia	salty sand
5T†	*Trichormus variabilis*	1988/Lukešová	Dlouhá Ves/Czech Rep.	agricultural field
6T†	*Trichormus variabilis*	1989/Lukešová	Dlouhá Ves/Czech Rep.	agricultural field
10C†	*Cylindrospermum* sp.	1998/Lukešová	Dlouhá Ves/Czech Rep.	agricultural field
11C†	*Cylindrospermum* sp.	1995/Lukešová	Manitoba/Canada	forest soil
14T†	*Tolypothrix* sp.	1995/Lukešová	Sokolov/Czech Rep.	coal-mining dump
15Ca*	*Calothrix parietina*	1977/Zehnder222a	Traunstein/Austria	stone wet wall
16Ca†	*Calothrix* sp.	1995/Lukešová	Sokolov/Czech Rep.	coal-mining dump

*- strain obtained from CCALA collection, Institute of Botany ASCR Třeboň
† - strains obtained from the collection of the Institute of Soil Biology ASCR, České Budějovice

Brine shrimp assay. Brine shrimp assay was performed as previously described (LINCOLN 1996). *Artemia* cultures were maintained in seawater prepared by diluting the commercial sea salt AQUA MEDIC (Aqua Medic GmbH, D-49143 Bissendorf, Germany). 0.1 g of *Artemia* eggs were inoculated to 100 ml of seawater and cultured at 28°C. Before testing, 100 µl of extract was evaporated and 1 ml of media with living *Artemia* (containing about 15-20 individuals) was added. The numbers of living (moving) and dead individuals were counted before the addition of extract and after 24 hours of exposure. Percentage inhibition was counted as ration of living to all individuals after 24-hour cultivation.

Mammal cell cultivation. Two mammal cell lines were selected for cytotoxicity testing – YAC-1 and WEHI. While YAC-1 is a lymphoma cell line (lymfoblasts) induced by Moloney leukemia virus, WEHI are semiadherent fibroblasts derived from mouse fibrosarcoma. Cells were cultured in RPMI 1640 medium (Sigma R-8005) (MOORE *et al.* 1967) with the addition of 5% foetal calf serum (PAA A15-04), 1% glutamine (Sigma G-5763) and 1% of antibiotic-antimycotic solution (Sigma A-7292) in plastic tissue culture flasks at 37ºC and 3.5% CO_2 concentration. Before the experiment the viability and abundance of the cells were estimated by colouring the suspension with Tripan blue and counting in Bürker chamber with a light microscope. For testing, the cell suspension was centrifuged (1000 rpm, 10 min., 4ºC), and adequate amount of fresh RPMI medium was added to obtain the concentration of 1.10^6 cells per ml. Fresh suspension was transferred into microplates and equal amount of RPMI medium with the addition of 2% of cyanobacterial extract was added.

MTT test. (MOSMANN 1983) 96-well microplates were used for MTT test. 100 µl of cell suspension with a concentration of 1.10^6 cells per ml was

transferred into each well. Wells at the margin of the microplate were filled with 200 µl of distilled water. Inner 60 wells were used for testing. Four wells of each column were used for the exposure of cells to the extract and two cells were used as a control (with the addition of RPMI with 1% concentration of methanol). The effects of five extract concentrations (2, 0.2, 0.02, 0.002, 0.0002 mg LB.ml^{-1}) on the selected cell lines were tested. Microplates were incubated at 37°C and 3.5% CO_2 concentration for 12 hours. Then 10 µl of MTT solution (5mg.ml^{-1} in Hank's solution – see MARTIN 1994) was added and incubated for 4 hours. After incubation microplates were centrifuged (3000 rpm, 10 min.), and the supernatant was removed. 200 µl of DMSO was added and formazan crystals were dissolved using laboratory shaker (300-400 rpm). Test and background absorbency were measured at the wavelengths of 550 and 660 nm. The survival of cell lines was expressed as a ratio of test well absorbency to control well absorbency.

Flow cytometry. For *FC* measurements the cells were cultured in 24-well microplates. Into each well 500 µl of the suspension with 1.10^6 of cells per ml were transferred and 500 µl of RPMI, with the required extract concentration, was added. After 1h cultivation the suspension was transferred into 5-ml glass test tubes, washed twice with 5 ml of PBS, and centrifuged (10 min. at 1000 rpm in 4 ºC). Then the supernatant was removed and the pellets were resuspended in 100 µl Anexin-V-flos incubation buffer (10 mM Hepes, 140 mM NaCl and 5 mM $CaCl_2$). 2 µl of Anexin-V-Flos (Roche Cat. No. 1 828 618) and 5 µl of propidium iodide (Sigma P4170) at a concentration of 500 µg.ml^{-1} was added and incubated for 20 min. Afterwards, 1 ml of flow-cytometry buffer (PBS with the addition of 1% foetal calf serum) was added. The suspension was measured at EPICS XL-MCL flow-cytometer, 100 000 counts were taken into each measurement.

HPLC- MS measurement. The compositions of extracts were analysed with HP 1100 Agilent mass spectrometer HP 100 MSD SL-Ion trap. The extracts were subjected to analysis on reversed phase column (Zorbax XBD C8, 46 x 150 mm, 5 µm) at 35°C using gradient MeOH/H_2O + 0.1 HCOOH (30-100% Me for 30 min, 100% for 5 min) with a flow rate 0.6 ml.min^{-1}.

Results

Both MTT and FC tests found a cytotoxic effect in 6 of 10 tested cyanobacterial extracts. At this point both methods provided coherent results. A different level of cytotoxicity was found between the cell lines for some extracts. Also different responses to extract application were found in *Artemia* (Table 2), where only two extracts caused lethal effects. Data are summarised in Fig. 1.

The extracts of *Nodularia* (4N), *Tolypothrix* (14T), and *Calothrix* (15 and 16Ca) strains did not result in a decrease of viability in either of the extract

concentrations used. Only a slight decrease in absorbency (20%) for the MTT test was found in YAC-1 cells treated with 2 mg LB.ml^{-1} concentration of these extracts. In general, HPLC-MS spectra did not reveal many defined peaks (in retention times between 2 and 20 min.) indicating the presence of some peptidic compounds.

Table 2: Results of brine shrimp assay

extract	(0) living	(0) dead	(24) living	(24) dead	inhibition (%)
1A	9	1	8	2	**11**
3A	8	1	8	1	**0**
4N	13	0	13	0	**0**
5T	13	3	7	9	**46**
6T	8	2	8	2	**0**
10C	8	2	7	3	**13**
11C	11	1	5	7	**55**
14T	12	1	11	2	**8**
15Ca	7	0	7	0	**0**
16Ca	10	1	9	2	**10**

In other four extracts significant cytotoxic effects were recorded, but only at concentrations of 2 mg LB.ml^{-1}, and a slight effect was observed (up to 30%) in lower concentrations. In the cells exposed for 12 hours to the extracts of *Anabaena sphaerica* 3A, *Trichormus variabilis* 5T, 6T and *Cylindrospermum* sp.10C, the viability was between 20 to 60 %, but the response of YAC-1 and WEHI cell line differed significantly (see Fig. 1). The same results were obtained by flow-cytometry (Fig. 2). Cells treated by 3A, 5T, 6T and 10C in 1% concentration exhibited viability of 30.8, 50, 21.4 and 20% respectively. Only the *Trichormus variabilis* extract was found to have a lethal effect on *Artemia* (50% inhibition). The presence of smaller possibly peptidic molecules was observed by HPLC-MS. In *Anabaena sphaerica* 3A and *Cylindrospermum* sp. 10C unknown compounds were found. In both *Trichormus variabilis* strains, a molecular ion similar to Microginin 91-B (ISHIDA *et al.* 2000) was observed.

In other two extracts, the most severe damage to both cell lines was recorded. In cells treated with *Anabaena torulosa* 1A extract with the concentration of 2 mg LB.ml^{-1}, a viability of only 20% in both cell lines was estimated by MTT. Also in extract with concentration of 0.2 mg LB.ml^{-1}, 20% inhibition of YAC-1 was found (Fig. 1). The same treatments analysed by flow-cytometry show that the damage could be even greater. The viability of cells estimated by FC was 1.0 and 45% for 2 mg LB.ml^{-1} and 0.2 mg LB.ml^{-1} extract concentrations. Figs 2 and 3 show that a strong induction of necrosis occurred in

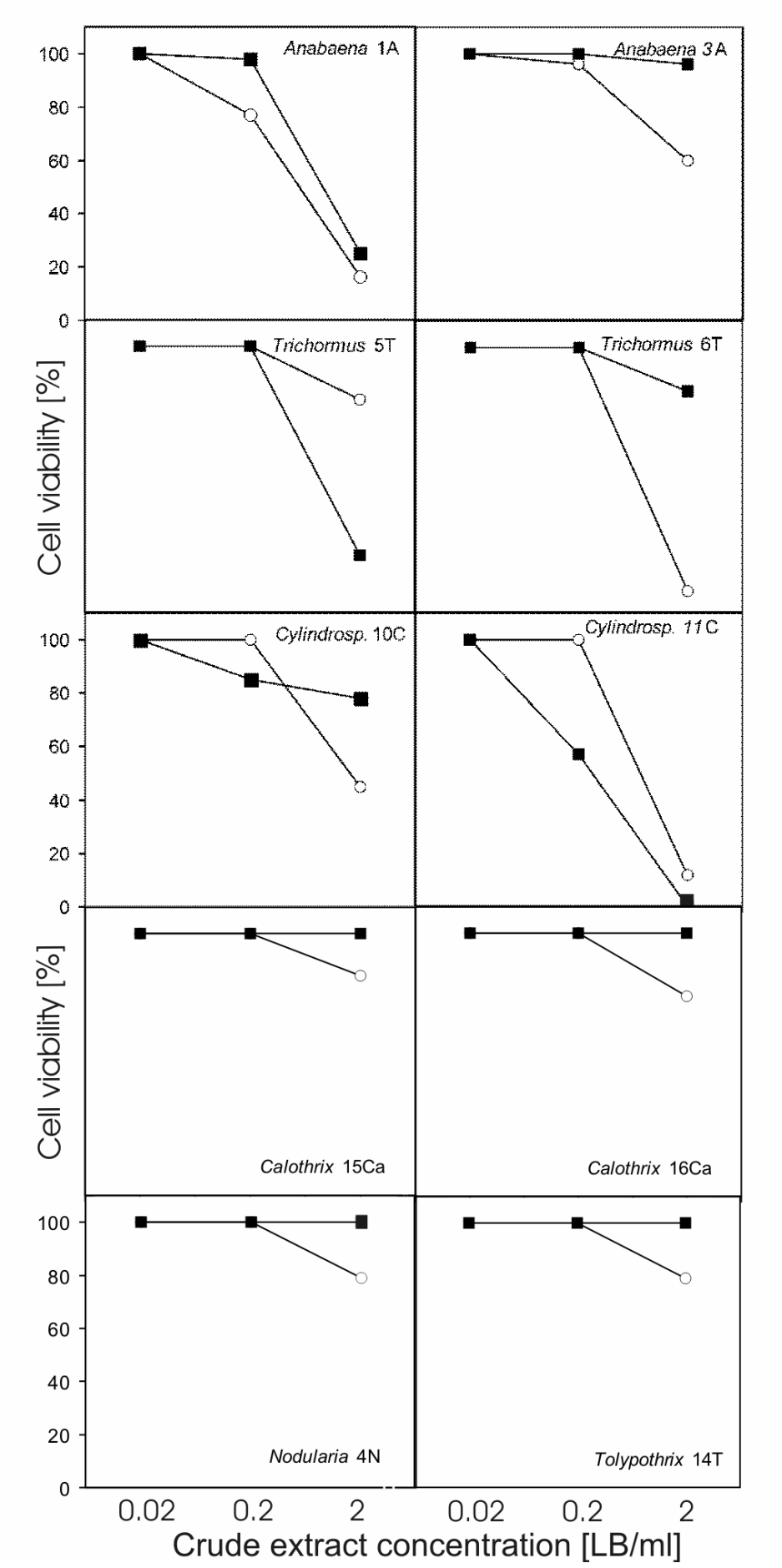

Fig. 1: Survival of two cell lines YAC-1 *(empty circles)* and WEHI *(filled squares)* assayed by MTT test after 12-hour exposure to three concentrations of cyanobacterial extract. Concentrations are shown as mg of lyophilized biomass per ml of medium.

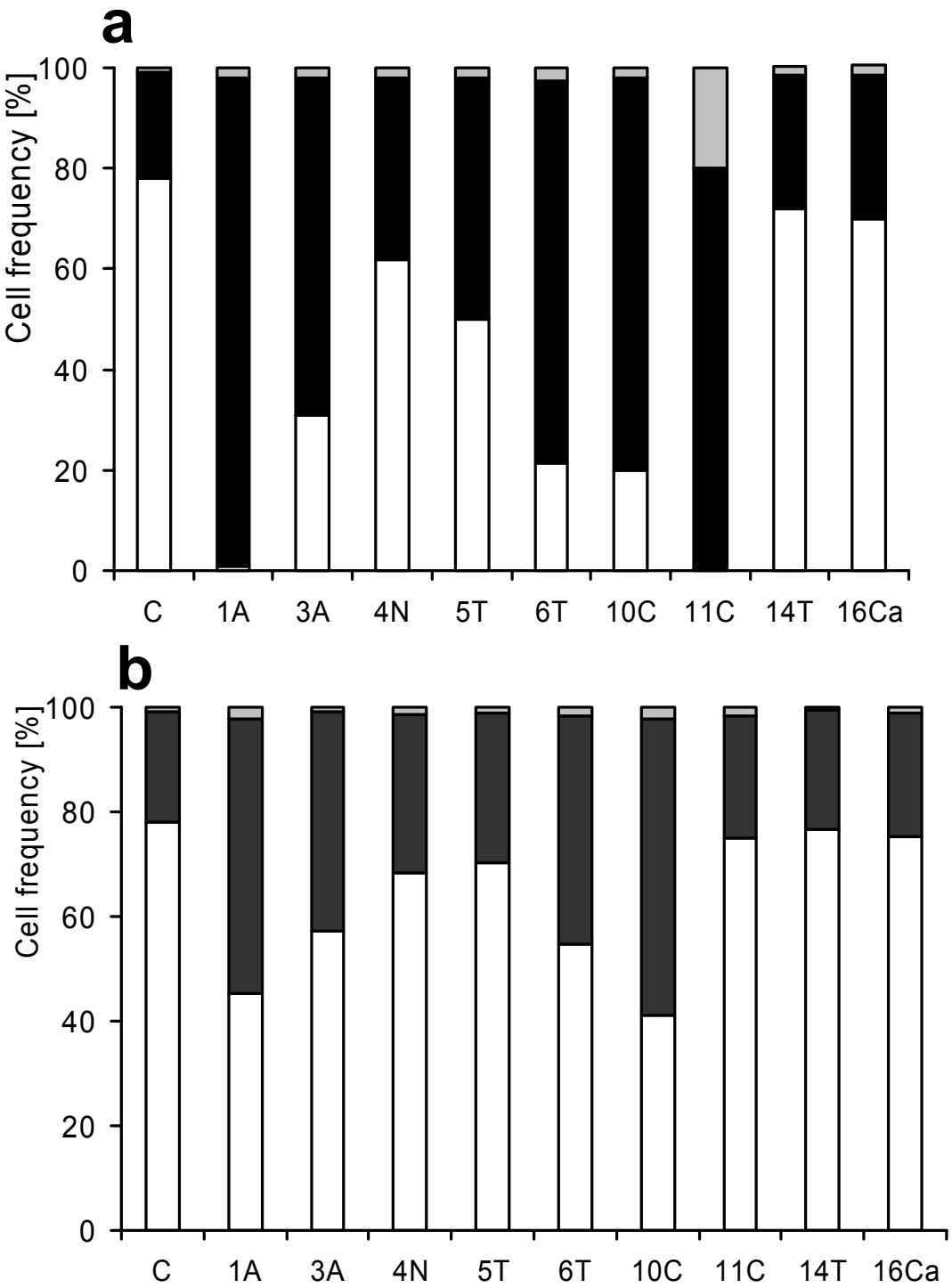

Fig. 2: a - Graph showing the frequency of living (*white area*), necrotic (*black area*) and apoptotic cells (*gray area*) in cultures of WEHI cell line treated with cyanobacterial extracts in concentration of 2 mg LB.ml^{-1}. b - Graph showing the frequency of living, necrotic and apoptotic cells in cultures of WEHI cell line treated with cyanobacterial extracts in concentration of 0.2 mg LB.ml^{-1}.

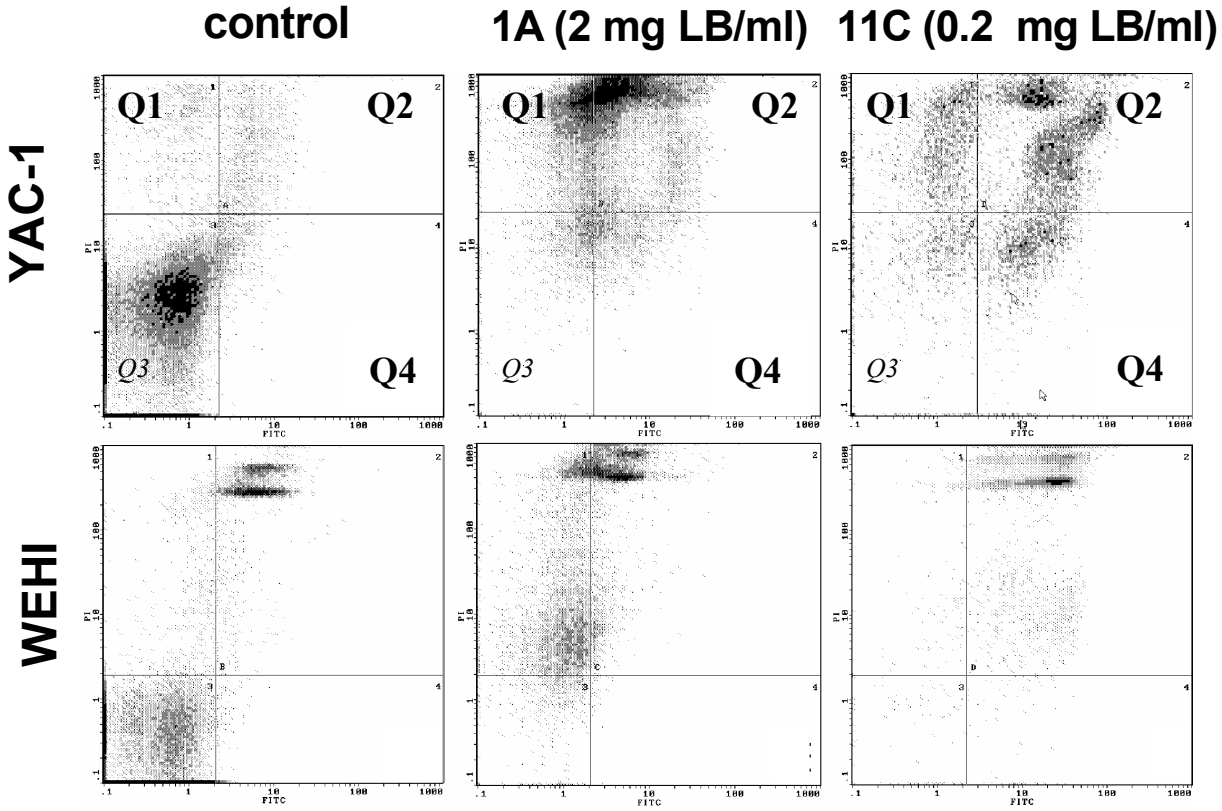

Fig. 3: Results of flow-cytometrical analysis of YAC-1 and WEHI cell lines treated with 2 mg LB.ml^{-1} and 0.2 mg LB.ml^{-1} extracts of *Anabaena torulosa* 1A and *Cylindrospermum* sp. 11C. Quadrants: Q1 – cell debrises, Q2 – necrotic cells, Q3 – living cells, Q4 – apoptotic cells.

the cell population. All tested cells were labelled by propidium-iodide (quadrant 1 and 2), but not with Anexin-V-flos (quadrant 4). This indicates that propidium iodide can penetrate cell membrane and no apoptotic stages were observed. Dyeing with tripan blue and observation under a light microscope confirmed these results; 98% of cells were coloured with tripan blue, which indicates the disruption of cell membrane. Results of the experiment, in which the measurements were done after only 60-minute exposure, indicate that the induction of necrosis by this extract can be extremely rapid. The cell population was damaged even after a very short exposure. In the extract of this strain unknown molecular ions (m/z) 642.7, 646.9, 610 and 625 were found. The observations of doubly charged ions in their spectra imply that there can be low molecule peptides, but whether these molecules are responsible for the necrosis induction remains unclear.

Different effects were observed in cells treated with *Cylindrospermum* sp. 11C extract. Strong cytotoxic effects were found in cells treated with 2 mg LB.ml^{-1} concentration by both methods – 100% inhibition for YAC-1 and 90% for WEHI cell lines (see Fig. 1). FC analysis revealed 100% inhibition of cell growth as well. Except for necrotic cells (80%) some cells were labelled by

Anexin-V-flos indicating a membrane turn-off during process of apoptosis (20%) – (see Fig. 2 and 3-quadrant 4). Similarly to the previous strain, also in the extract of *Cylindrospermum* sp. 11C unknown compounds were found. Several well defined molecular ions of higher m/z values (1118, 1134, 1152, 1162 and 1146.6) were obtained, suggesting the presence of unknown compounds.

Discussion

Both FC and MTT analyses using mammal cell lines provided similar results in the testing of cyanobacterial cytotoxicity; these results were not comparable with the results of brine shrimp assay. Such a fact, already recorded before (PICCARDI *et al.* 2000, MIAN *et al.* 2003), indicates that brine shrimp assay is not suitable for determining cytotoxicity of cyanobacterial extracts universally as it is used in many screening works (SOLIS *et al.* 1993) .

At least two different soil cyanobacterial extracts were found to be significantly cytotoxic, in both different effects and effective concentration levels were observed. Moreover, the cytotoxic effect (the 100% inhibition of *Anabaena torulosa* 1A and *Cylindrospermum* sp. 11C extract) is comparable to the results obtained in cells treated with microcystin LR (an equal concentration of the active compound is estimated) (BOTHA *et al*. 2004). Several recent works have demonstrated that Microcystin and *Microcystis* extracts can induce apoptosis in mammal cells through changing the mitochondrial membrane potential. This process results in oxidative damage (DING *et al.* 1998, 1998, 2000, DING & ONG 2003, BOTHA *et al.* 2004). Our data also show that other compounds are capable of inducing apoptotic changes in mammal cells. However, confirmation of the above-mentioned hypotheses requires isolation and a full description of the metabolites' structure and composition.

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