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ANTIPROLIFERATIVE AND CYTOTOXIC EFFECTS OF EXOPOLISACCHARIDES FROM *ARTHRONEMA AFRICANUM* ON PANEL OF HUMAN CELL LINES

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Abstract

Exopolysaccharides (EPS) isolated from the cyanobacteria *Arthronema africanum* were examined for antiproliferative activity. The effects of EPS on the human cancer cell lines of lung (A549), cervical (HeLa), colon (HCT), hepatocellular (HepG-2), and mammary (MDA-MB-231) carcinoma were determined using NRU-test. Non-tumour cell line HaCaT was used as control to assess the selectivity towards the cancer cells. EPS influence on the distribution of cells in different phases of the cell cycle was analyzed using flow cytometry. Acridine orange/ethidium bromide and DAPI staining were carried out to determine the ability of EPS to induce apoptosis. EPS-treatment caused decrease in cell viability of all types of cancer cells, with the strong inhibition against HeLa cells. The significantly lower cytotoxicity in non-tumour HaCaT cells demonstrated the selectivity of the cytotoxicity effects. EPS did not induce any changes in the cell cycle distribution. The fluorescence microscopy analysis revealed cytomorphological changes associated with the induction of apoptotic cell death after EPS treatment. The presented results show significant antiproliferative activity of the studied EPS and demonstrate that *Arthronema africanum* is a promising source of bioactive compounds with potential for use in cancer therapy.

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Introduction. Natural products are an "inexhaustible" source of new drug candidates for the treatment of various diseases. Their role is illustrated by the fact that 60% of approved drugs on the pharmaceutical market include natural products and their synthetic derivatives [1]. In recent years, there has been increasing attention to the search for natural compounds in microalgae due to their specific structure and biological activity. Cyanobacteria have been regarded as one of the most promising groups of organisms capable of producing secondary metabolites with diverse beneficial biological activities [2].

Cyanobacterium *Arthronema africanum* (Synechococcales) is a relatively new, almost unexplored strain, inhabiting extreme environments. The specific morphological and physiological characteristics of the strain determine its high metabolic plasticity and ability to survive severe stresses [3]. The pigment and lipid content, fatty acid profile of *A. africanum* strain and the influence of light and temperature on these parameters were analyzed [4]. The investigation of biological properties of *A. africanum* includes only a few studies describing its antioxidant, antimicrobial and antitumour activity [5,6]. A study on antitumour activity has focused on the inhibiting properties of C-phycocyanin isolated from biomass of *A. africanum* on Graffi myeloid tumour growth in vitro and in vivo [6]. *A. africanum* has not been fully studied regarding its antiproliferative and cytotoxic properties, however, exopolysaccharides isolated from different microalga and cyanobacteria were found to possess cytotoxicity against tumour cells [6].

The aim of the present study was to investigate exopolysaccharides from *A. africanum* for in vitro antiproliferative activity on a panel of human tumour cell lines.

Materials and methods. ***Cell culture.*** Human tumour cell lines A549 (lung carcinoma), HCT (colorectal carcinoma), HepG2 (hepatocellular carcinoma), HeLa (cervical carcinoma), and MDA-MB-231 (breast carcinoma), and the non-tumour cell line HaCaT (human keratinocyte) were cultured in Dulbecco's modified Eagle medium (DMEM), supplemented with 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin at 37 °C, 5% CO_2 and 90% relative humidity.

Microalgal strain. The blue-green alga *A. africanum* strain Lukavský 1981/01 was obtained from the CCALA (Trebon Collection of Autotrophic Organisms, Czech Republic) and maintained in the collection of the Institute of Plant Physiology and Genetics, Bulgarian Academy of Sciences. The microalgae were grown at 28 °C in a nutrient medium based on the media of Allen&Arnon and Zehnder, modified by Chaneva et al. [8], enriched with 2% CO_2, (v/v) under continuous unilateral lighting with cool-white fluorescent lamps at a photon flux density of 132 µmol m^{-2} s^{-1}.

Exopolysaccharide sample preparation. Cultures of *A. africanum* at the stationary phase of growth were centrifuged for 30 min at 5000× g. The supernatants, containing the soluble extracellular polysaccharides were precipitated with cooled 96% ethanol in a ratio of 1:2 (v/v) for 24 hours. The precipitate was collected, washed three times with 65% ethanol, dissolved in distilled water and then dialyzed (2.3 cm dialysis tubing, MW cut off 12.4 kDa) against distilled water at 4 °C with 2–3 changes of water, using a magnetic stirrer. The obtained polysaccharide solution was sterilized through a bacterial filter (0.45 µm), and dried by lyophylization.

NRU cell viability test. The cytotoxicity of exopolysaccharide sample on the human cancer cell lines was examined using NRU cell viability test as previously described [9]. Briefly, cells were plated in a 96-well plate at 1×10^4 cells/well and incubated under the standard conditions. Twenty-four hours later, cells were treated with EPS at concentrations from 31.25 to 2000 µg/ml for 72 h. Untreated tumour cells were used as negative controls. After treatment, Neutral Red medium was added to the plates for 3 h and then replaced with ethanol/acetic acid desorb solution. The absorption was measured on a microplate reader (TECAN, Grödig, Austria) at wavelength 540 nm.

FACS analysis of cell cycle. A FACS analysis was performed to assess the possible effect of EPS on the cell cycle progression. Briefly, HeLa cells were grown to about 80% confluence and were then treated with EPS at a concentration of 800 µg/ml. After 24 h of treatment, the medium was aspirated, the cells were washed twice with cold PBS, trypsinized and centrifuged at 1000 rpm for 10 min. The cell pellet was washed with PBS, and fixed with 70% ice-cold ethanol. Cells were stored at −20 °C for at least 12 hours. Before analysis, fixed cells were washed with PBS, treated with 20 µg/mL RNase A (Roche Diagnostics GmbH, Mannheim, Germany) for half an hour, stained with 20 µg/mL propidium iodide and analyzed by flow cytometer (Becton Dickinson). From each sample, 10 000 events were recorded, and the percentage of cells in different cell cycle was determined using FlowJoTM v10.8 software (BD Biosciences, San Jose, CA).

Fluorescent microscopy. Cells were cultured on 13 mm cover glasses in 24-well plates and treated for 48 h with EPS at a concentration of 800 µg/mL. To observe morphological changes, native preparations of control and treated HeLa cells were stained with fluorescent dyes AO (5 µg/mL) and EtBr (5 µg/mL) in PBS. Staining with DNA-binding dye 4′,6-Diamidine-2′-phenylindole dihydrochloride (DAPI) was performed to examine the nuclear morphology. For this purpose, the cells were fixed with methanol, incubated for 15 min at 37 °C with DAPI (1 µg/mL in methanol) in the dark. Stained cell culture samples were visualized under Leica DM 5000B fluorescence microscope.

Statistical analysis. The statistical analysis included application of One-way ANOVA followed by Bonferroni's post hoc test using the Prism software program, version 5 (GraphPad Inc., San Diego, CA, USA). $p < 0.05$ was accepted

as the lowest level of statistical significance. The inhibitory concentrations (IC_{50}) were calculated using nonlinear regression analysis (GraphPad Prism 4 Software). The results were presented as mean ± SD of three independent experiments.

Results. ***Evaluation of the cell viability by NRU assay.*** The effect of EPS on cell viability was measured on a panel cell lines, including lung non-small cancer cells (A549), colon adenocarcinoma (HCT), hepatocellular (HepG2), cervical adenocarcinoma (HeLa), hormone-independent breast adenocarcinoma (MDA-MB-231) cancer cells, and non-tumour human keratinocyte cell line (HaCaT) using NRU assay. EPS extract induced a significant reduction of cell viability in all cell lines tested (Fig. 1).

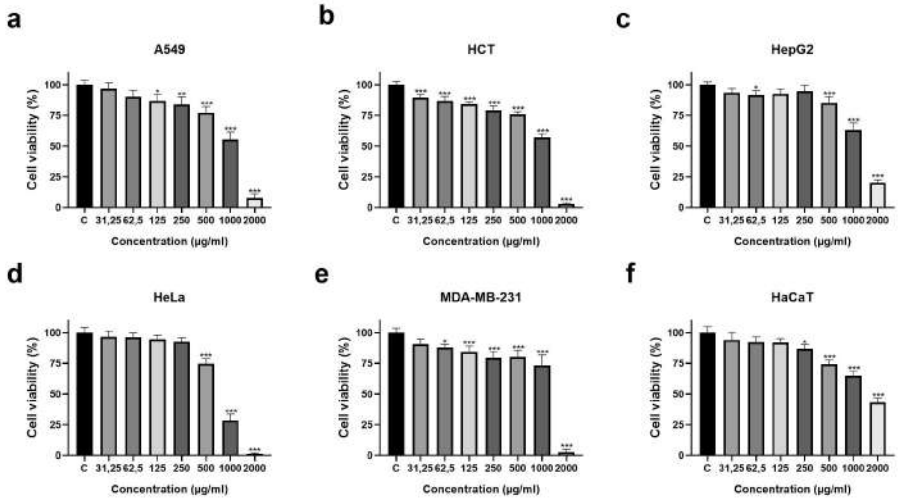

Fig. 1. Effect of the different concentrations of EPS on the viability of A549 (a), HCT (b), HepG2 (c), HeLa (d), MDA-MB-231 (e) human tumour and nontumour HaCaT (f) cells after 72 h of treatment, estimated by NRU assay. $^{***}p < 0.001$; $^{**}p < 0.01$; $^{*}p < 0.05$

All concentrations of EPS from *A. africanum* induced a statistically significant decrease in cell viability of HCT colorectal cancer cells and the effect was concentration-dependent. The maximum growth inhibition of 60.08% and 5.12% was obtained at concentrations of 1000 µg/mL and 2000 µg/mL, respectively (Fig. 1b). In A549, HepG2 and HeLa cancer cells, significant inhibition of proliferation ($p < 0.001$) was obtained only at the high concentrations of 500 µg/mL, 1000 µg/mL and 2000 µg/mL (Fig. 1a, c, d). Significant reduction in cell viability of MDA-MB-231 cancer cells was registered at six different concentrations and the maximum decrease in cell viability to 2.7% was found at a concentration of 2000 µg/mL (Fig. 1e). It was established that EPS at high concentrations also decreased the viability of non-tumour HaCaT cells, but the reported values were higher than those found for different types of tumour cells. We also calculated the half-maximal inhibitory concentrations (IC_{50}) for the individual cell

Table 1

IC_{50} values of EPS on HaCaT, MDA-MB-231, A549, HCT, HeLa, and HepG2 cancer cells. The results are expressed as mean ± SD

Cell lines	IC50 ± SD (µg/ml)
HaCaT	2021±76
MDA-MB-231	1323 ± 85
A549	1108 ± 110
HCT	1130 ± 49
HeLa	767.5 ± 49
HepG2	1298 ± 105

lines (Table 1). Based on these values, we constructed hierarchical rows of the cell sensitivity to EPS as follows: HeLa > A549 > HCT > HepG2 > MDA-MB-231. In this regard, HeLa cancer cells were the most sensitive to EPS-induced cytotoxicity with an IC_{50} of 767.5 ± 49. Moreover, the EPS exhibited a moderately selective antiproliferative effect and weak cytotoxicity towards the non-tumour HaCaT cells with an IC_{50} higher than 2000 µg/mL (Fig. 1f, Table 1).

FACS analysis of cell cycle. Induction of apoptosis and cell cycle arrest are a potential therapeutic approach for cancer treatment. In order to investigate whether EPS from *A. africanum* inhibits the proliferation of HeLa cells by regulating the cell cycle, HeLa cells were treated with 800 µg/ml of EPS from *A. africanum* for 24 h, and then were stained with PI, and their DNA content was measured by flow cytometry. FACS analysis demonstrated no changes in the cell cycle distribution of the EPS-treated cells (Fig. 2). These results implied that induction of cytotoxicity might occur through the mechanisms associated with apoptosis.

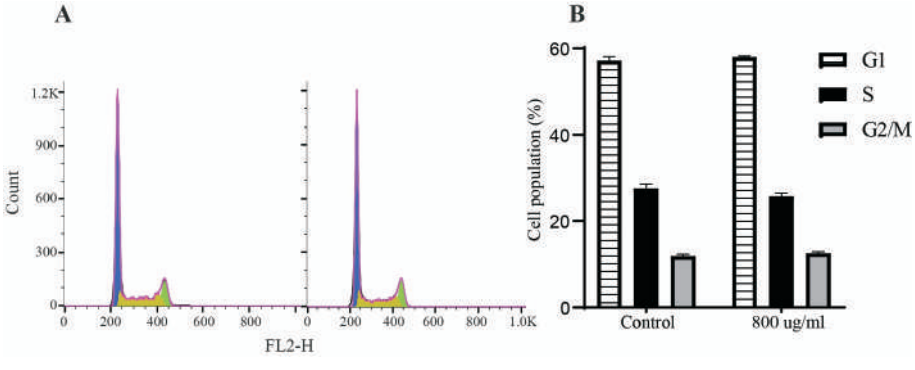

Fig. 2. Effects of EPS from *A. africanum* on cell cycle progression of HeLa cells. A – Representative histogram of control cells and cells treated with EPS (800 µg/ml); B – Quantification of the cells in the different phases of the cell cycle. Results are presented as mean ± SD from three independent experiments

Morphological changes of HeLa cells. Cytomorphological changes at the cell and nucleus level were examined under a fluorescent microscope after double staining with AO/EB. As shown in Fig. 3, Control HeLa cells were oval in shape, uniformly stained green and formed a monolayer. Cells in stages of mitosis were also seen (Fig. 3a). In contrast to the control, EPS-treated HeLa cells were reduced in number, with scarce monolayer growth, rounded in shape and with morphological characteristics of early (intense green and yellow fluorescence) and late (red-stained) apoptosis (Fig. 3b).The nuclear morphology of the treated cells was further analyzed after staining with DAPI. The nuclei of control cells were ordinary-sized, single, smooth-contoured and were characterized by homogenous blue staining (Fig. 3c).

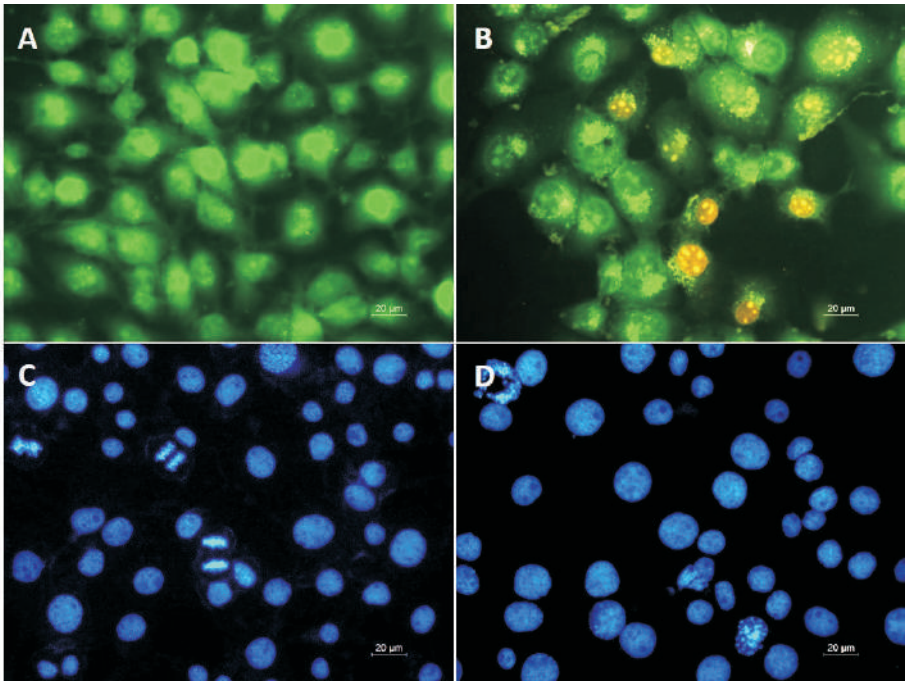

Fig. 3. Fluorescence micrographs of AO/EtBr (A, B) and DAPI (C, D) stained HeLa tumour cells incubated with 800 µg/mL *A. africanum* EPS for 48 h. A, C – control (untreated) cells; B, D – cells treated with EPSH; scale bar = 20 µm

DAPI staining of EPS-treated HeLa cancer cells showed nuclear changes characteristic of cell apoptosis, such as chromatin condensation and nuclear fragmentation, as well as the formation of small, spherical or ovoid cytoplasmic fragments, some of which contain pyknotic remnants of nuclei (Fig. 3d). The obtained results indicated that tested EPS of *A. africanum* could induce apoptosis in HeLa cancer cells.

Discussion. Cyanobacteria and algae can be easily cultivated and valuable compounds with wide biomedical applications can be extracted from the obtained

biomass. Generally, EPS mainly consist of glucose, galactose, fucose, xylose, arabinose, rhamnose, mannose, fructose, and/or sugar derivatives. However, the potential biological activity of EPS can vary depending on their molecular weight and sulphate content, which is important for their effect on cancer cells [10]. Earlier reports show that the EPS from cyanobacteria and algae have antitumour activity in the human colon cancer model [11,12]. Moreover, crude polysaccharide extracts from *Chlorella pyrenoidosa* exhibited antitumour activity against human lung carcinoma cell line A549 [13]. An antiproliferative effect of EPSs from *P. marinum* on mammary carcinoma cells was found, and its efficiency increased with the decrease in the molar mass [14]. The homopolysaccharide from *G. impudicum* inhibited tumour cell growth both in vitro and in vivo [15]. The EPS of *Tribonema* sp. was found to induce concentration-dependent anticancer activity on HepG2 cells with an inhibition rate of 66.8% for 250 µg/mL. Moreover, this anticancer effect seems to be due to induction of cell apoptosis, rather than affecting the cell cycle and mitosis [16]. Antitumour activity of EPS from *A. africanum* was studied and reported for the first time. Our studies have shown that EPS derived from *A. africanum* have a cytotoxic effect on tumour cells with different tissue origin. We investigated the possible mechanisms of cytotoxicity and found that the observed effects were not related to a delay in cell proliferation but rather were a consequence of direct targeting of apoptosis.

Conclusion. Our results show that EPS from *A. africanum* possess high potential to inhibit the proliferation of five human cancer cell lines, more pronounced for HeLa cervical cancer, as well as to induce cell death via the apoptosis pathway. Furthermore, the antiproliferative effects of EPS appear to be specific to tumour cells, as non-tumour cells show a weak response to it. Molecular mechanisms underlying EPS-induced apoptosis in cervical cancer, genomic and proteomic responses remain to be elucidated in more detail.

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