

EXAMINATION OF EXOPOLYSACCHARIDES FROM
Porphyridium cruentum FOR ESTIMATION OF THEIR
POTENTIAL ANTITUMOUR ACTIVITY IN VITRO

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Abstract

Globally, research is being conducted focusing on new biologically active substances and technologies for their application in various sectors, in the hope that their biological and medical applications will be widely used in the next decade.

The introduction of therapeutic agents of biological origin isolated from certain species of bacteria and algae and their potential for cancer treatment is based on their lower toxicity and hence greater safety compared to synthetic pharmaceuticals. In this respect, the potential of microbial producers and microalgae is enormous. Among the biologically active components isolated from algae of the greatest importance for biology and medicine are complex polysaccharides, as well as some pigments.

In the present work, the antitumour properties of two fractions of polysaccharides from the red microalgae *Porphyridium cruentum* were observed by determining their effect on cell viability of four different cell lines.

New data from the study indicate that samples treated with *P. cruentum* polysaccharide have significant and dose-dependent antiproliferative and cytotoxic effects. Changes in nuclear morphology in MCF-7 tumour cells induced by polysaccharide treatment, including chromatin condensation, nucleus fragmentation, and apoptotic body formation, have been demonstrated. Polysaccharides show obvious pharmacological effects in the treatment of tumour cells without side effects.

Key words: polysaccharides, *Porphyridium cruentum*, antitumour activity, fluorescent microscopy

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Introduction. Being part of marine ecosystems, algae are one of the most abundant and potentially renewable resources, with high nutritional value and their polysaccharides have a variety of biological activities [1]. Algal polysaccharides are widely utilized for various biomedical applications considering the pharmaceutical potential, biodegradability and biocompatibility [2]. The unicellular marine microalga *Porphyridium cruentum* can synthesize and secrete sulphated polysaccharides [3], which are mostly released into the surrounding medium – extracellular highly sulphated acidic heteropolymers with a molecular weight in the range from 2×10^6 to 7×10^6 Da [4]. Algal exopolysaccharides arose interest in various fields in human life including health sector owing to their antioxidant, antidiabetic, immunoregulatory, antibacterial, antiviral, anti-inflammatory and anticancer effects, as valuable non-toxic natural agents [5–8]. Cancer, according to the World Health Organization has been listed as one of the world's five incurable diseases. Anticancer therapies in many cases show serious side effects. Different reports on polysaccharides from various natural origin indicated their potential as anti-metastatic cancer drug preparations [9]. It is generally believed that algal polysaccharides as natural metabolites, are less toxic and have fewer side effects than synthetic cancer drugs. The severity of cancer problem that has become a significant public health problem has caused further attention and research on the effective and safe cancer treatment methods.

The aim of the study was to explore new applications of sulphated polysaccharides from marine microalgae for safe application as antitumour agents by evaluating their in vitro cytotoxic activity in a panel of human cancer cell lines.

Materials and methods. Cell lines and culture conditions. Human tumour cell lines HeLa (cervical carcinoma), HT-29 (colorectal carcinoma), MCF-7 and MDA-MB-231 (hormone-dependent and hormone-independent breast adenocarcinoma) and mammary epithelial female cell line MCF-10A, were obtained from the American Type Cultures Collection (ATCC, USA) and used as model systems in the present study. Cell lines were grown in Dulbecco's modified Eagle medium (DMEM), supplemented with 10% fetal calf serum, 2 mM glutamine, and antibiotics penicillin 100 U mL^{-1} and streptomycin $100 \text{ } \mu\text{g mL}^{-1}$ at 37°C and 5% CO_2 .

Microalgal strain. The unicellular red microalga *Porphyridium cruentum*, strain VISCHER 1935/107 was obtained from the Trebon Collection, Czech Republic. Intensive cultivation was conducted in special vessels (200 mL) at 26°C , aeration with 2% CO_2 and Illumination 8000 lx.

Polysaccharides sample preparation. Intensively cultivated algal suspension (7 d) was centrifuged for 30 min (6000 g). The supernatant was precipitated with ethyl alcohol. Two fractions were visualized: 1 – on the surface-collected with a special needle; 2 – in the supernatant volume. It was centrifuged and the pellet with polysaccharide was collected. Both fractions were dissolved in distilled water using a magnetic stirrer. Dialysis took place for 48 h at 4°C . The polysaccharide underwent freeze drying before testing.

For preparation of sample 3 and 4 microwaves (400 W) were used to degrade *P. cruentum* polysaccharides. For this purpose, polysaccharide solution of sample 1 (5 mg/mL) was pretreated for degradation in an automatic hermetical microwave device at two different degradation conditions – 2×3 min (sample 3) and 3 min (sample 4).

Sulphate content. Sulphate content of PSH was determined by the barium-gelatin method [12] after hydrolysis of the sample in 1 mol L⁻¹ HCl (5 h, 105 °C) using sodium sulphate (Na₂SO₄) as a standard.

MTT cell viability assay. Viability of tumour cell lines was assessed by the MTT assay [13]. MTT assay reading was performed using ELISA plate reader (TECAN, SunriseTM). Cell viability was calculated as follows: cell viability% = OD₅₇₀(experimental)/OD₅₇₀(control) × 100.

Fluorescent microscopy. Cells were cultured on 13-mm-diameter cover glasses in 24-well plates and treated for 48 h with polysaccharide samples (50 µg/mL). Native preparations of control and treated MCF-7 cells were stained with fluorescent dyes AO (5 µg/mL) and EtBr (5 µg/mL) in PBS. Staining with DNA binding dye 4',6-Diamidino-2'-phenylindole dihydrochloride (DAPI) was performed. Cells were fixed with methanol, incubated for 15 min in 1 µg/mL DAPI in methanol in the dark. Stained cell cultures were visualized under Leica DM 5000B.

Statistical analysis. One-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test were performed. Values of * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ were considered significant, using software package GraphPad Prism 5.

Results and discussion. Sulphated polysaccharides contain sulphated ester groups within their sugar residues, which are common components of a wide range of macro and microalgae. Reports exist on the antiproliferative activity in cancer cell lines in vitro of sulphated heteropolysaccharides, as well as inhibitory properties on tumour growth in mice [10]. However, fewer studies have been conducted on microalgal polysaccharides than on macroalgal polysaccharides [11].

Effects of polysaccharide samples on the cell viability and proliferation of human tumour cell lines. In vitro cytotoxic activity was estimated in a panel of human cancer cell lines. Sample 1 showed higher activity towards all tested cell lines and it was further subjected to treatment.

Polysaccharide samples 1, 2 and 4 induced statistically significant reduction of the cell viability of HeLa cervical carcinoma cells at all tested concentrations. The values of cell viability ranged between 88.71% and 79.77%. Treatment with sample 3 did not affect significantly the viability of the tested tumour cell line (Fig. 1A). The polysaccharides, applied in a concentration range from 10 to 100 µg/mL significantly reduced the viability and proliferation of colon carcinoma cells HT-29 (Fig. 1B). Sample 4 showed the highest activity with the lowest cell viability values 45.91% compared to the control. Sample 1 also induced

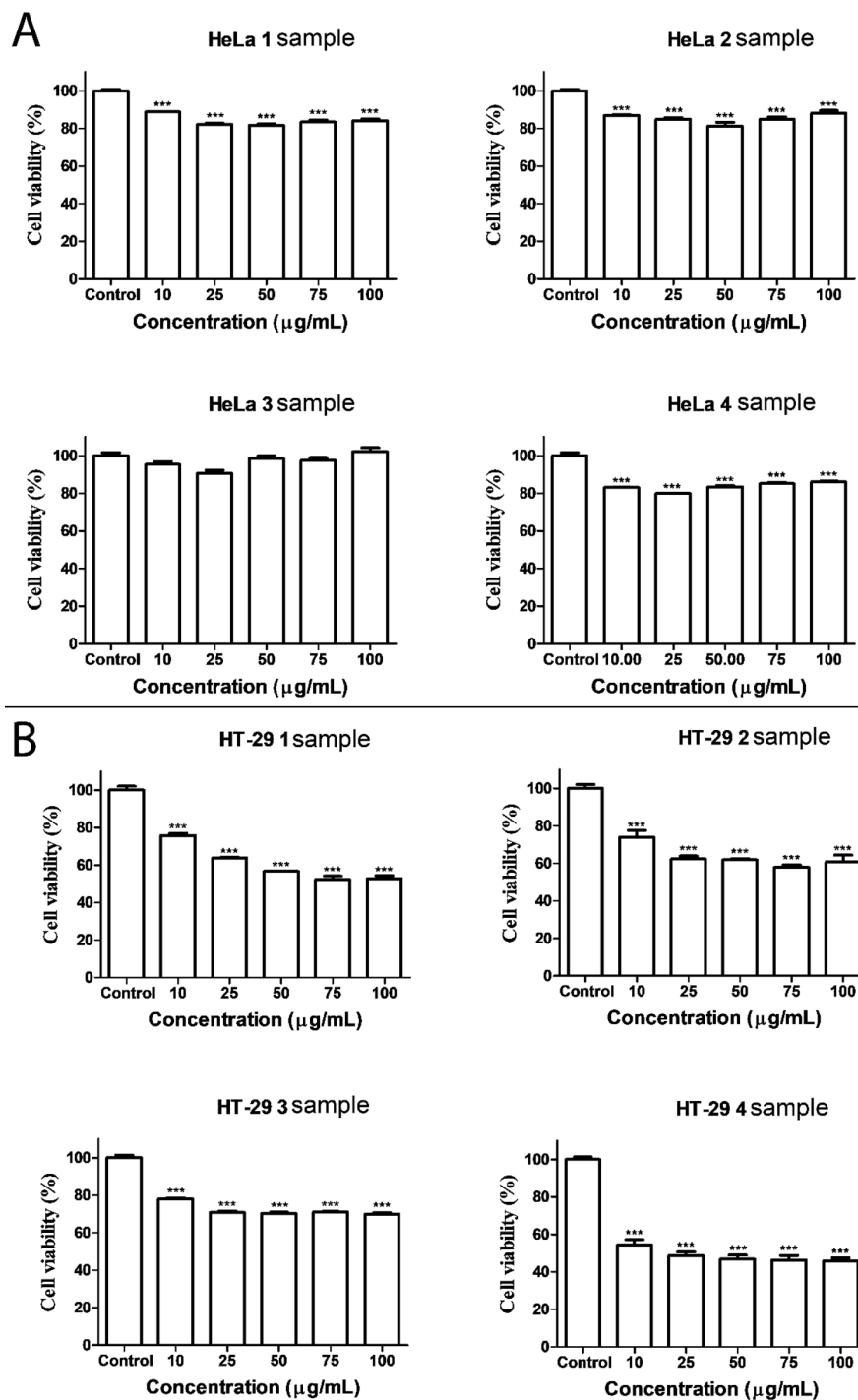


Fig. 1. Cell viability after treatment with the different samples (1–4) of the polysaccharide from the red microalga *Porphyridium cruentum* on HeLa (A) and HT-29 (B) cell lines

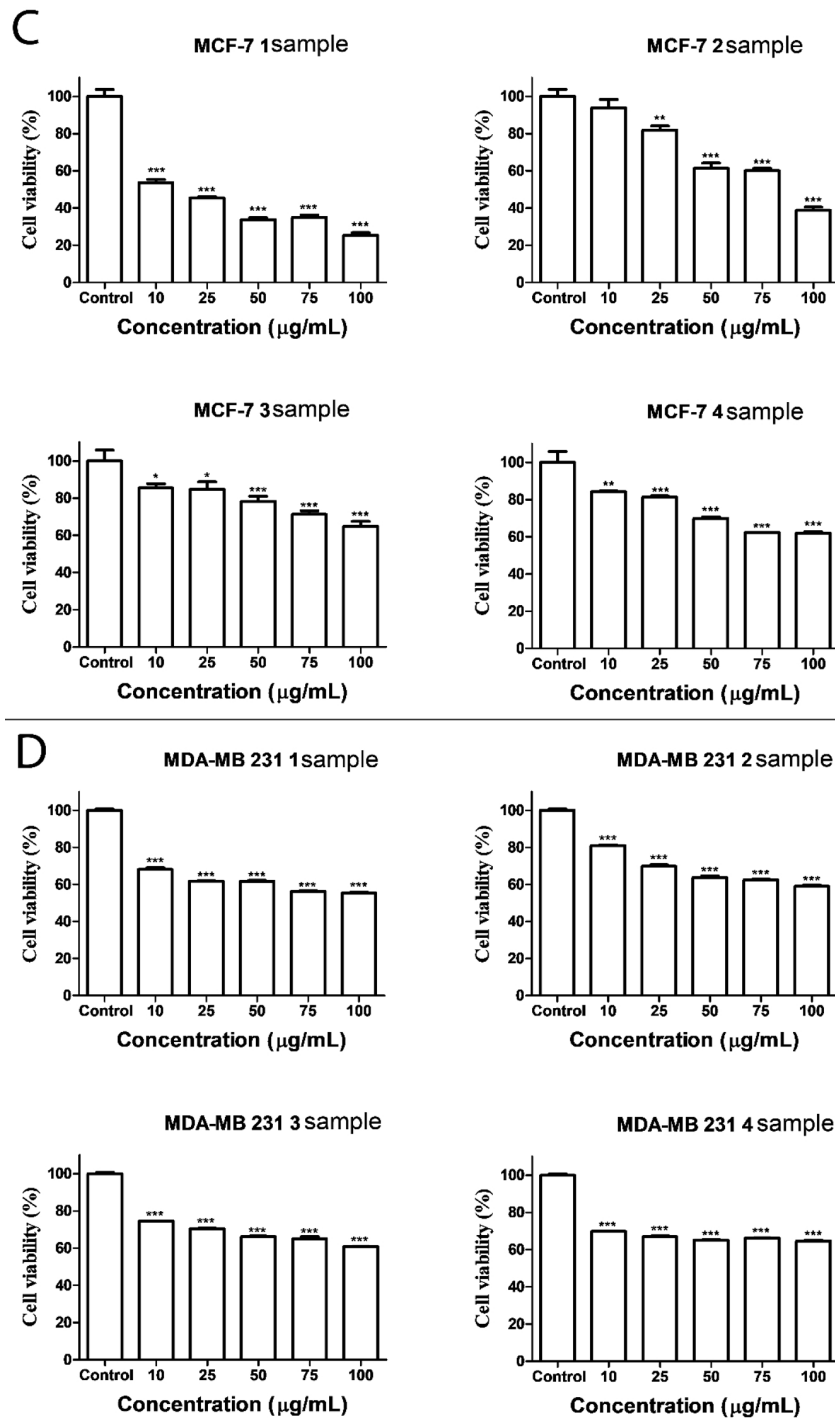


Fig. 2. Cell viability after treatment with the different samples (1–4) of the polysaccharide from the red microalga *Porphyridium cruentum* on MCF-7 (C) and MDA-MB-231 (D) cell lines

significant reduction of tumour cell viability with the most clearly pronounced dose-dependence of the observed effects.

In MCF-7 cell line, all tested samples showed significant and dose-dependent antiproliferative and cytotoxic effects. Sample 1 was the most active and reduced tumour cell viability to values ranging from 53.65% to 25.37% (Fig. 2C). The triple negative mammary adenocarcinoma cell line MDA-MB-231 appeared to be less sensitive to the cytotoxic and antiproliferative effects of the polysaccharides as compared to the estrogen-dependent breast adenocarcinoma cell line MCF-7 (Fig. 2C, D).

The lowest value of cell viability (55.34%) was established after treatment with 100 µg/mL of sample 1.

Application of microwaves was expected to degrade fraction 1 into shorter oligosaccharides and thus to improve their biological activity, as longer chain polysaccharides, suggest high viscosity and poor solubility so the polysaccharides are difficult to pass through membranes and enter the interior of the cell [14]. Such a phenomenon was observed in this study. The pretreated polysaccharide exhibited higher cytotoxic activity towards the tested cell lines after 3 min pretreatment with microwaves. Probably, the longer treatment with microwaves (2 × 3 min), did not lead to increased but decreased bioactivity (sample 3). This fact could be explained by structure disruption. Three minutes exposition appeared to be enough for stimulating the cytotoxic effect, either by obtaining shorter oligosaccharide structures or releasing sulphate groups. After determination of the sulphate content, the highest value was obtained for sample 4 – 9.21%, followed by sample 1 – 7.2%, which could well be explained by the importance of sulphates for exhibition of bioactivities. When chemically modified sulphated polysaccharide had been tested it showed certain inhibitory effect on HepG-2 cells, and the inhibition was significantly enhanced compared with that of the *Sargassum fusiforme* polysaccharide [15].

As sample 1 showed higher activity towards all tested cell lines, its effect was tested to a mammary epithelial female cell line MCF-10A for comparison to the tumour cell lines (Fig. 3). Very slight decrease in cell viability was registered (7%).

The sulphate concentration of *P. cruentum* polysaccharides increased significantly after microwave digestion. Among the polysaccharides, sulphated polysaccharides are the bioactive macromolecules in which some hydroxyl groups are substituted with sulphate groups in their sugar residues, which are responsible in some cases for their antitumour properties. The results of some authors indicate the same as the higher antitumour activity of sulphated polysaccharides could be related to their molecular weights and sulphate contents [16].

Cytomorphological studies. Cancer cells escape normal apoptosis, continue to proliferate, interfere with normal tissues or organ, and cause body damage or even death. Therefore, inducing apoptosis has always been one of the exact

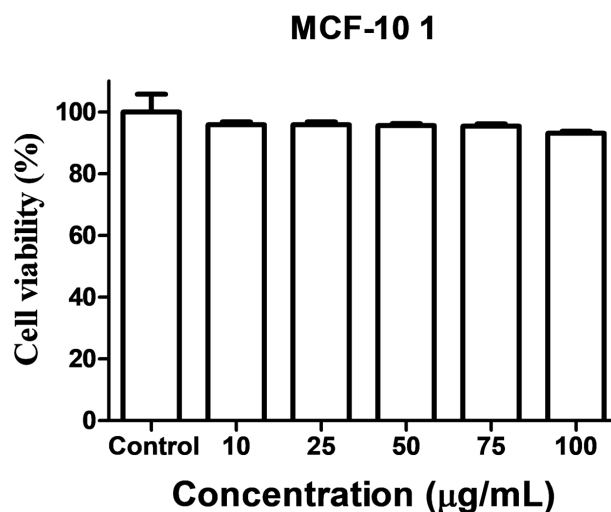


Fig. 3. Cell viability after treatment with polysaccharide from the red microalga *Porphyridium cruentum* on MCF-10A cell line

approaches to inhibit cancer [17]. The morphological alterations induced by treatment with 50 µg/mL polysaccharides in MCF-7 tumour cells were analyzed by fluorescent microscopy after staining with AO/EB. Acridine orange (AO) stains both live and dead cells, emitting strong green fluorescence, as a result of the intercalation in the double-strand DNA. As opposed to AO, EtBr can only enter dead and late apoptotic cells with poor membrane integrity, so it stains all dead cells to generate red fluorescence. The early apoptotic cells were characterized by bright green nuclear staining and chromatin condensation in a form of dense green regions and the late apoptotic cells are with orange nucleus with chromatin condensation. The controls (non-treated MCF-7 tumour cells) showed normal morphology and monolayer growth. The cells were with polygonal shape and uniform green staining (Fig. 4a). Incubation with polysaccharide samples induced changes in both growth and morphology of the MCF-7 cells. The cellular monolayer was damaged, cells with oval shape, with bright yellow-green nucleus with chromatin condensation, visible as dense green regions (early apoptotic cells) and cells with orange-red staining, condensation and margination of the chromatin or fragmented nucleus, forming apoptotic bodies (late apoptotic cells) were observed (Fig. 4b–e). Polysaccharides as natural products with anti-cancer activity that are widely present in microalgae, are the topic of many studies that had found they induce apoptosis of cancer cells being one of their important mechanisms [18]. DAPI staining was used for investigation of the apoptotic changes in the nucleus of the MCF-7 tumour cells treated with polysaccharide samples. The control (non-treated tumour cells) was with intact nuclei, with smooth edge and homogeneously distributed chromatin (Fig. 4a1). Cells at different phases of mitosis were found

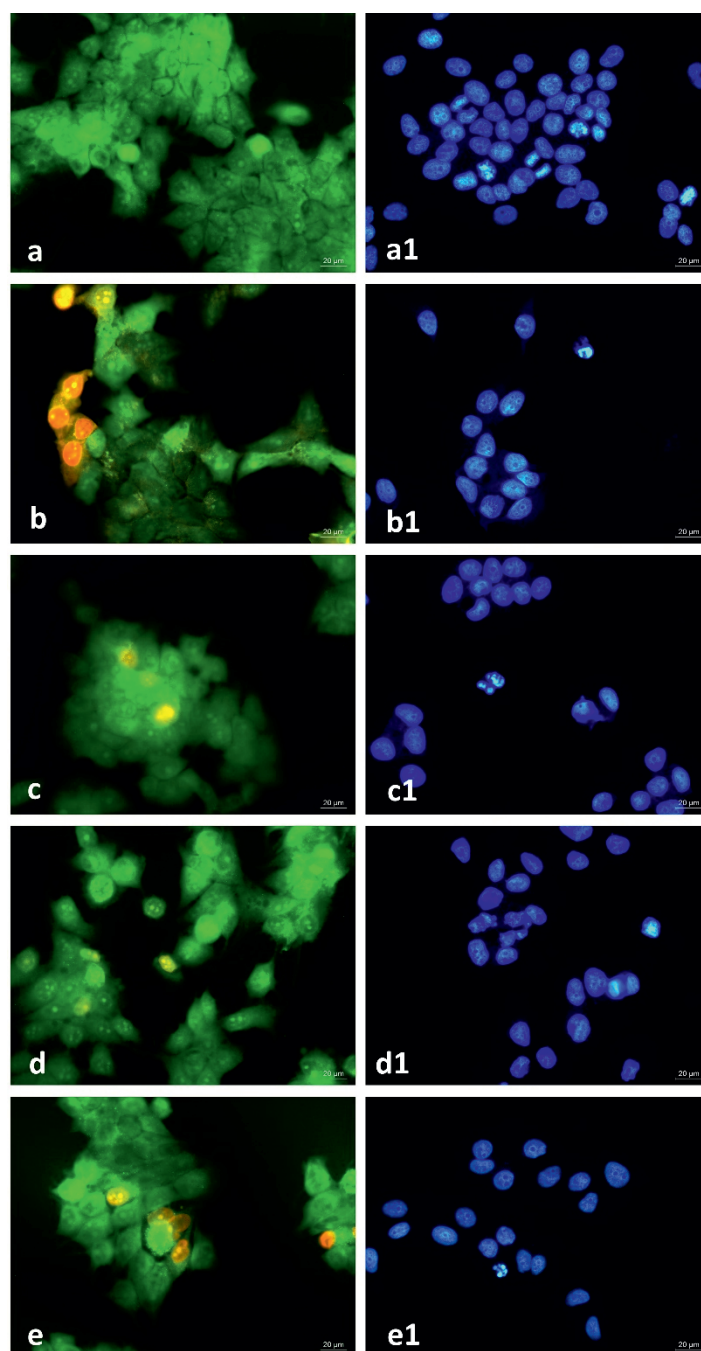


Fig. 4. Morphological changes in MCF-7 tumour cells treated with polysaccharides (50 µg/mL) for 48 h. a, a1 – control cells; b, b1 – cells treated with sample 1; c, c1 – cells treated with sample 2; d, d1 – cells treated with sample 3; e, e1 – cells treated with sample 4; a, b, c, d, e – AO/EB staining; a1, b1, c1, d1, e1 – DAPI staining

(Fig. 4a1). Changes in the nuclear morphology of MCF-7 tumour cells induced by the polysaccharide treatment included chromatin condensation (heterogeneously distributed chromatin with granular structure), fragmentation of the nucleus and formation of the apoptotic bodies (Fig. 4b1-e1).

Further investigations could elucidate the relation between structure of the polysaccharides and their bioactivities.

Conclusions. The new data obtained as a result of research show that samples treated with the *P. cruentum* polysaccharide (10–100 µg/mL) showed significant and dose-dependent antiproliferative and cytotoxic effect. Microwaves pretreatment led to increased sulphate content in the polysaccharide, thus decreasing tumour cell viability. Nuclear morphology changes in MCF-7 tumour cells induced by polysaccharide treatment, including chromatin condensation (heterogeneously distributed chromatin with granular structure), fragmentation of the nucleus and formation of apoptotic bodies were proved.

Polysaccharides have very obvious pharmacological effects in treating tumour cells with no side effects. We can conclude that polysaccharides can be used as an alternative in the treatment of cancer and to support traditional chemotherapy.

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