ORIGINAL ARTICLE

MONOSACCHARIDES COMPOSITION AND CYTOSTATIC ACTIVITY OF POLYSACCHARIDE FRACTION OF *PHEMERANTHUS CONFERTIFLORUS* L.

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Abstract

Polysaccharides display several biological activities, such as antitumour, anti-oxidation, hypoglycaemic, antiviral and immunity enhancing properties. The polysaccharide fraction from *Phemeranthus confertiflorus* was extracted, hydrolysed and 8 monosaccharides were analysed and quantified by capillary electrophoresis. The polysaccharide fraction was further assessed for cytotoxicity on normal and tumour cell lines. The results obtained can promote *Phemeranthus* as an eligible plant with potential for human health, because of high monosaccharides content (predominant rhamnose) and promising cytostatic effect of polysaccharide fraction.

Rezumat

Polizaharidele prezintă diferite activități biologice, cum ar fi acțiune antitumorală, antioxidantă, hipoglicemică, antivirală sau capacitatea de creștere a imunității. Fracția polizaharidică din *Phemeranthus confertiflorus* a fost extrasă, hidrolizată și 8 monozaharide au fost cuantificate prin electroforeză capilară. În continuare, fracția polizaharidică a fost testată pe linii celulare normale și tumorale pentru evaluarea citotoxicității. Rezultatele obținute pot încuraja folosirea acestei plante în scopuri terapeutice datorită conținutului ridicat în zaharuri (în special ramnoză) și a efectului citostatic promițător al fracției polizaharidice.

Keywords: *Phemeranthus confertiflorus;* monosaccharides; cytostatic activity; capillary electrophoresis

Introduction

Polysaccharides from plants (and other natural sources) have received great interest due to their presumed physiological activities, such as anti-oxidative [3, 14], antitumor [11, 15], antiviral and immunomodulator activities [1, 6].

Phemeranthus confertiflorus was reported as a new alien species to Europe by Nagodă *et al.* [8], and in Romania it was recorded a single population in Bucharest, on the northern part of an area named Bucharest "*delta*". The species extended in the last years and is accompanied in its habitat mainly by *Portulaca pilosa* (Figure 1) and was collected from the arid, marginal area of the mentioned zone.

Initially, *Phemeranthus* was considered as belonging to *Portulacaceae* family. The species of this family are more investigated (especially *Portulaca oleracea* L.), and are considered medicinal and nutritional valuable. *Phemeranthus confertiflorus* belongs to *Montiaceae*, it is native to North America and is used as ornamental in rock gardens. *Phemeranthus* species are almost completely North American, with one exception for *P. punae*, found in northern Argentina. The centre of diversity of this genus is northern Mexico and the south-western United States [10].

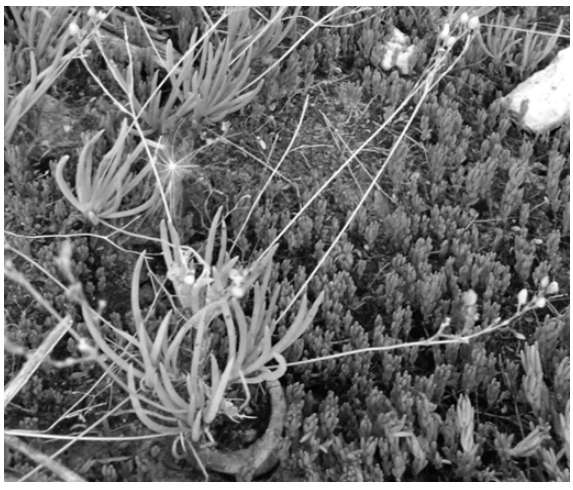

Figure 1.
Phemeranthus confertiflorus sorrounded by *Portulaca pilosa* in Bucharest "*delta*"

Phemeranthus confertiflorus is essentially unstudied. There are only some papers, previously mentioned, and others with brief considerations about occurrences of this species [5]. In our prior study, polyphenols, antioxidant activity and short-chain organic acids were characterized in *Phemeranthus* extracts (unpublished data). In this paper, the monosaccharides content of polysaccharide fraction extracted from *Phemeranthus* was investigated with the aim of exploring the potential of this species for health and nutrition. Capillary zone electrophoresis (CZE, known for high efficiency, minimal sample pre-treatment and low consumption of reagents [9, 13] was used for chemical composition of polysaccharide extract and the cytotoxicity and cytostatic activity of polysaccharide fraction extracted from the entire plant were evaluated on normal and tumour cells using MTT ((3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) and cell cycle analysis.

Materials and Methods

Reagents

The standard monosaccharides were mainly purchased from Fluka (Switzerland): L(-) fucose, D(+) xylose, L(+) rhamnose, L(+) mannose, and from Merck (Germany): L(+) arabinose, D(+) galactose, D(-) ribose and D(+) glucose.

Ultra-pure water, 0.1 M and 1 M sodium hydroxide solutions were purchased from Agilent Technologies (Germany). Solvents (Merck, Germany) and solutions were filtered on 0.2 μm membranes (Millipore, Bedford, MA, USA) and degassed prior to use. Stock solutions for each standard were stored at 4°C. Working solutions were prepared daily by diluting the stock solutions in background electrolyte (BGE).

Samples preparation

The plant was collected in July 2015 from the so-called Bucharest "*delta*". *Phemeranthus confertiflorus* (Greene) Hershkovitz (voucher ID BUC 404604) was identified by specialists from the Botanic Garden, University of Bucharest. The plants were dried up to 10% from initial weight in a Memmert oven with aeration and then were finely ground using a Grindomix GM200 grinder.

10 g of crushed plant material were subjected to extraction with hexane and chloroform to remove fat materials and pigments. After the supernatants were removed, plant materials were subjected to extraction of polysaccharides. The dry plant material (10 g) was immersed in 100 mL deionized water for 10 h and then was heated at 100ºC for 1.5 h. The resulting extract was filtered through paper and the plant material was subjected to extraction in the same way again. Next, the extraction solutions were pooled, concentrated and precipitated by adding ethanol to a final concentration of 80% v/v. The mixture was left overnight (10 h) at 4ºC. The precipitate was collected after centrifugation (6000 rpm, 10 min) and reconstituted with 50 mL de-ionized water and completely deproteinated by several rinses with Sevag solution (chloroform/butyl alcohol v/v 4:1) [12]. By adding ethanol to a final concentration of 80% v/v at 4ºC for 10 h again, the supernatant was concentrated, precipitated, centrifuged and subsequently dried at 37ºC.

Carbohydrates analysis

The polysaccharide extract (20 mg) was dissolved in 1.5 M sulphuric acid solution (2 mL). The hydrolysis of the polysaccharide was performed at 100ºC, and then samples were cooled to room temperature and neutralized with 1 mL 6.0 M NaOH solution.

For 1-phenyl-3-methyl-5-pyrazolone (PMP*)* derivatization, to each test tube, an aliquot of 200 μL of mixed working standards solution, or the hydrolysed polysaccharide sample, 100 μL 0.5 M PMP methanol solution and 100 μL 0.3 M NaOH solution were added. The mixture was left to react for 30 min at 70ºC, then was cooled to room temperature and neutralized with 100 μL 0.3 M HCl solution. The final volume of mixture was brought to 1 mL by adding 500 μL distilled water. The excess PMP reagent was removed with 10 mL chloroform. The upper phase was collected and filtered through a 0.45 μm membrane and degassed by sonication [4].

The separation of the derivatized carbohydrates 1-phenyl-3-methyl-5-pyrazolone (PMP) was obtained using an Agilent CE instrument (software Chem Station) with diode array detector (DAD) and CE standard bare fused-silica capillary (Agilent Tech., Germany) with an internal diameter of 50 μm and effective length of 72 cm for all the electrophoretic separations. The method used was based on Chen *et al.* [2] with some modification and was previously developed and validated (unpublished data). The BGE used for separation was 90 mM boric acid adjusted to pH = 9.95 with 1 M NaOH solution. The applied voltage was 30 kV and the detection was fixed to 245 nm.

Cytotoxicity studies

Cytotoxicity of polysaccharide fraction of *Phemeranthus confertiflorus* was evaluated with MTT ((3-[4,5-di-methylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) Cell Proliferation Assay, a quantitative, convenient method based on reduction of MTT (yellow) to formazan (purple) [7]. MTT assessments were done after 24 h and after 72 h on two cell lines: a stabilized cell line, derived from mouse fibroblast cells (NCTC clone 929) and a tumour stabilized cell line of human colorectal adenocarcinoma cells (Caco-2). The normal and tumour cells were cultured in Minimum Essential Medium (MEM) containing 10% foetal bovine serum (FBS) and 1% antibiotics (penicillin,

streptomycin and neomycin), at 37ºC, in 5% CO_2 humidified atmosphere.

Cell suspensions were obtained by sub confluent culture trypsinization and were seeded into 24-well plates; each well was seeded with 5 x 10^4 cells/mL, for 24 h and after standing overnight the culture medium was replaced with medium with different concentrations of polysaccharide fraction extracted from *Phemeranthus.* The cell viability on normal cell line (NCTC) was assessed with samples concentrations between 50 and 750 µg/mL, and for tumour cell line were used concentrations between 50 and 375 µg/mL. The supernatant was replaced after 24 h, respectively 72 h of exposure of cells to polysaccharide extracts and the cells were washed with 0.2 M, PBS pH 7.4, and then, to each well, 500 µg/mL MTT solution (0.25 mg/mL) was added. After 3 h of incubation in a humidified 5% CO_2/95% air atmosphere, at 37ºC, the MTT solution was removed and the formazan crystals formed in living cells were solubilised with an equal volume of isopropanol. After 15 min of incubation at 37ºC and gentle stirring the absorbance was measured at 570 nm with a Tecan Sunrise (Tecan, Austria) spectrometer. Cell viability was expressed as a percentage of control treated cells with different concentrations of analysed samples, all of them being analysed in triplicates.

C*ellular morphology*: the cells were seeded in the culture plates with 12 wells at a cell density of 5 x 10^4 cells/mL. At 24 hours after seeding, the cells were incubated in the presence of samples of interest at various concentrations. Cellular morphology was evidenced using haematoxylin-eosin staining. Cells cultured for 72 hours in the presence of various concentrations of polysaccharide extracts were washed with PBS and then fixed with Bouin fixative for 10 minutes. After the fixative removal, the cells were stained with haematoxylin for 7 minutes, were washed with distilled water and then stained with eosin for 5 minutes. Later, after washing the cells with distilled water, they have been examined with the light microscope Carl Zeiss Axio Observer D1.

The analysis of cell cycle by flow cytometry is a method used for the study of cell proliferation, which highlights the possible changes of DNA content in a studied culture. These changes may be due to the genotoxic agents which could damage DNA, or to some compounds that alter normal cell cycle development. The observed changes in the dynamics of the cell cycle, between cell fractions in the phases G1, S and G2, are based on quantifying DNA content in cellular samples cultivated after 72 hours of treatment with polysaccharide extracts. Flow cytometry experiments that allowed the cell cycle analysis were performed on tumour cell line Caco-2, derived from a human colorectal adenocarcinoma.

For cell cycle analysis, cell line belonging to Caco-2 was seeded in the culture plates, 6 well plates at a cell density of 8 x 10^4 cells/mL and were maintained in culture medium MEM (supplemented with 10% foetal bovine serum and 1% antibiotics) at 37ºC in humidified atmosphere with 5% CO_2. At 24 hours after seeding, the cells were incubated in the presence of polysaccharide extracts at different concentrations.

After 72 hours of cultivation in the presence of the extracts, the cells were tripsinizated, washed twice with PBS and fixed in 70% ethanol overnight at 4ºC. The cells were then washed with PBS and incubated with RNase A (0.5 mg/mL) at 37ºC for 30 minutes. Then the cells were labelled by incubation with propidium iodide (100 µg/mL) for 30 minutes at 4ºC. Cell cycle analysis was performed using a Becton Dickinson LSR II flow cytometer, and cellular DNA content was quantified using ModFit LT 3.0 software.

Statistical analysis

All the assays were carried out in triplicate in three different samples, at each level (dry plant, extracts, electrophoresis and cytotoxicity assays) and the results are expressed as mean values ± standard deviation (SD). The other statistical data were obtained with MaxStat version 3.60.

Results and Discussion

Monosaccharides content

Phemeranthus is related with *Portulaca* and the species are found together in their habitats. Because polysaccharides from *Portulaca oleracea* presented several biological effects, the polysaccharide fraction of *Phemeranthus* was evaluated for monosaccharides content in the attempt of correlation of the chemical composition and antitumor effect. 8 PMP derivatized monosaccharides were quantified (Figure 2) from polysaccharide fraction using a CZE method previously validated (unpublished data).

In Table I are presented the records obtained after the calculation of concentrations for each derivatized monosaccharide. The results are presented in mg monosaccharide per g (dry weight) polysaccharide extract and as monosaccharide molar ratios.

As it can see from Table I, rhamnose (Rha) is predominant compound in hydrolysed polysaccharide fraction, followed by glucose (Glu), arabinose (Ara) and xylose (Xyl). Fucose and ribose were not detected.

Further, the polysaccharide extract was evaluated for cytotoxicity on normal and tumour cells.

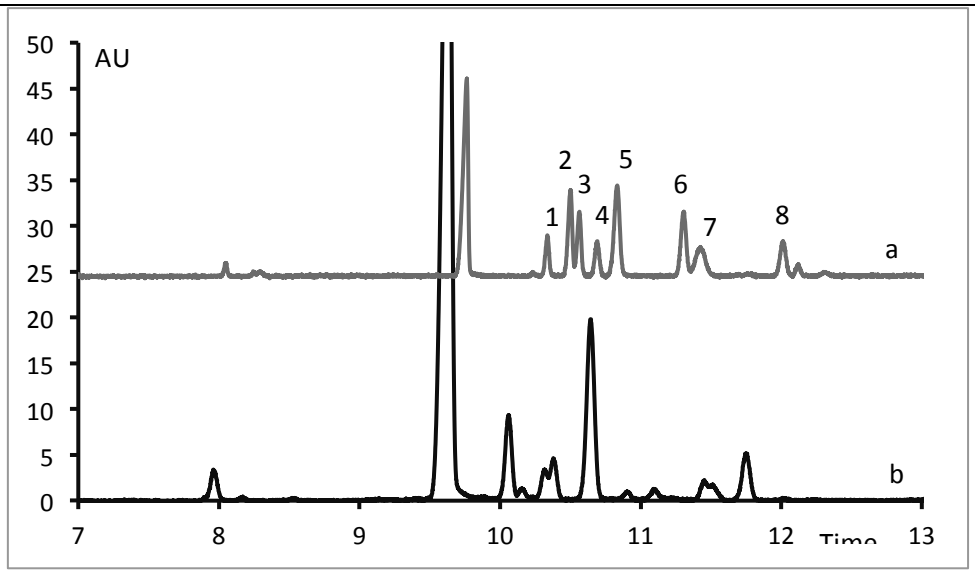

Figure 2.
Electrophoregrams of monosaccharide standards mixture (a): 1 – xylose; 2 – arabinose; 3 – glucose; 4 – rhamnose; 5 – ribose; 6 – mannose; 7 – fucose; 8 – galactose; and (b) monosaccharides composition of *Phemeranthus confertiflorus* polysaccharide extract

Table I
The quantified monosaccharides from polysaccharide fraction extracted from *Phemeranthus confertiflorus*

saccharide	Xylose	Arabinose	Glucose	Rhamnose	Mannose	Galactose
mg/g polysaccharide	44.79 ± 1.47	90.68 ± 2.99	179.62 ± 4.09	668.36 ± 4.16	4.96 ± 0.09	13.05 ± 0.7
Molar Ratio	1.1	2.2	3.6	13.3	0.1	0.3

Biocompatibility and cytostatic effect

The biocompatibility/cytotoxicity of the polysaccharide extract was assessed with MTT assay in the concentration range between 50 - 750 µg/mL, after 24 and 72 hours. The analysed samples showed a slight tendency to stimulate the proliferation of cells between 50 and 250 µg/mL of polysaccharide extract from *Phemeranthus* after 24 h of incubation. At 500 and 750 µg/mL the cell viability is around 70%, so the extracts are slight cytotoxic at these concentrations.

The cell viability has the tendency to decrease after 72 hours of incubation of normal cells with polysaccharide extracts, but the values remain over 80% (are not cytotoxic) for the concentration range between 50 and 375 µg/mL. Thus, the conclusion is that the tested samples are not cytotoxic in the range of concentration 50 - 375 µg/mL even after 72 hours of incubation.

The cytotoxic capacity of polysaccharide extracts was assessed on Caco-2 tumour cell line, at 24 and 72 hours, in the concentration domain between 50 and 375 µg/mL for which the normal cell line presented cell viability higher than 80%.

After 24 hours of incubation, the cell viability was maintained over 80% for all the range of concentration (Figure 3a). The situation was changed after 72 h, the cell viability decreasing significantly in direct relation with increasing of sample concentration (Figure 3b). The cytotoxicity of the 250 and 375 µg/mL polysaccharide extracts on tumour cells was much higher comparing with the lack of cytotoxicity obtained on normal cells.

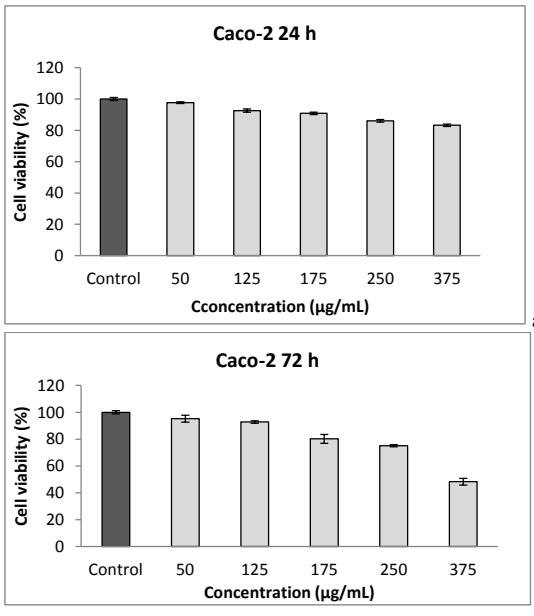

Figure 3.
Cell viability of Caco-2 line cell after incubation with polysaccharide fraction extracted from *Phemeranthus confertiflorus,* at different concentrations and periods of time (a – 24 h, b – 72 h)

This aspect can be seen on light microscopy images of tumour cell line Caco-2 treated with polysaccharide extracts after 72 h of cultivation (Figure 4). The inhibition effect of cell viability could be observed both on decreasing of cell density and on atypical morphology of Caco-2 cells, which presented granulated cytoplasm, poly-nucleus and rounded shape.

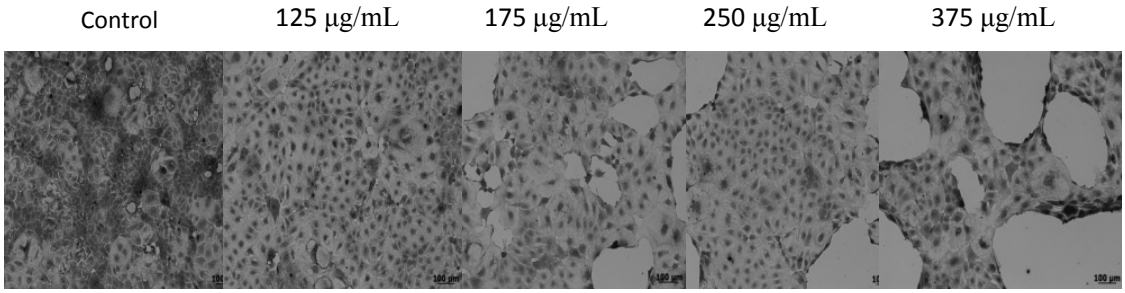

Figure 4.
Light microscopy images of tumour cell line Caco-2 treated with polysaccharide extracts after 72 h of cultivation (haematoxylin and eosin staining)

The analysis of cell cycle for tumour cell line Caco-2 treated with different concentrations of polysaccharide extract for 72 h was based on the content of DNA in each phase of interphase G1, S and G2. In the control sample of untreated tumour cells was observed a dissimilar cell distribution, namely 64% for G1, 26% for S and 10% for G2. After the incubation of cells during 72 hours with poly-saccharide extract in concentrations between 125 µg/mL and 375 µg/mL, the number of cells from G1 phase decreased from 51.56% at 125 µg/mL to 44.43% for 375 µg/mL. These values indicate a delay of cell cycle for Caco-2 cells treated with plant extracts and, implicit, a decrease of cell proliferation in direct ratio with the increase of concentration of poly-saccharide extract.

Conclusions

The polysaccharide fraction extracted from *Phemeranthus cofertiflorus* contained mainly Rha, Glu, Ara and Xyl, presented no toxicity on normal cells and promising cytotoxicity on tumour cells even at moderate concentrations of the plant extract. We can notice that the cytostatic effect of *Phemeranthus* polysaccharide extract was higher than those observed by us on *Portulaca oleracea* and *Portulaca pilosa* extracts [16].
After this study, *Phemeranthus confertiflorus* could be appreciated as favourable for pharmacological properties such as cytostatic activity, which is associated with its content in saccharides, probably the high content of rhamnose and glucose.

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Conflict of interests

The authors declared no conflict of interests.

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