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Biological activity of extracts from the leaves and roots of Jovibarba sobolifera (Sims.) Opiz.

Aktywność biologiczna ekstraktów otrzymanych z liści i korzenia *Jovibarba sobolifera* (Sims.) *Opiz.*

INTRODUCTION

Jovibarba sobolifera (Sims.) Opiz (Hen and Chickens Houseleek) belongs to the *Crassulaceae* family comprising 1250–1500 species [22]. Similarly like the others species of *Jovibarba* genus, *J. sobolifera* is the plant creating globular or hemispherical barren rosettes. It's leaves are widest above the middle, often with red apex [17,20]. It is found in Europe, in most mountains of southern Europe, and also in northern Africa and the Caucasus. In Poland *Jovibarba* Opiz grows mainly in the Sudety Mountains and the Carpathians. Species of this genus are succulent herbs and generally occur in dry and exposed habitats [17].

Species from *Jovibarba* Opiz were previously included in the *Sempervivum* L. genus, and research studies on *Jovibarba sobolifera* have been stimulated by the biological activity of plants from *Sempervivum* L. genus.

The plants from *Crassulaceae* family have been the scientists' point of interest for a number of years. *Sempervivum* species are well known plants in folk medicine for the treatment of ear inflammation [22,23]. Drinking tea prepared from leaves of *Sempervivum tectorum* is recommended for ulcer treatment [1]. Recently, it was found that *S. tectorum* extract has a lipid–lowering effect in rats and antioxidative properties [2,3].

News from literature referring to screening tests of water and ethanolic extracts from plants [5,7,19] and polysaccharides [6,11,12], which showed high cytostatic activity, suggested further research on fractions for antimitotic activities. Fractions of polysaccharides from rosette leaves and roots, and ethanolic extracts from fresh and dried leaves of *Jovibarba sobolifera* have been studied for this purpose. Cytostatic activity of the examined fractions was confirmed using the phytobiological Levan's test [15] and by use of test on Jurkat human T – lymphoblastic cell line (ECACC) with

trypan blue (0.4% aqueous solution of trypan blue) and Annexin V. Additionally, the evaluation of the number of cells in the early stage of apoptosis has been done.

MATERIALS AND METHODS

M a t e r i a l s . Rosette leaves and roots of *Jovibarba sobolifera* (Sims.) Opiz gathered in Józefów near Biłgoraj were used as the plant materials for research. The identity of the plant was confirmed by Professor Tadeusz Krzaczek and a voucher specimen is deposited in Chair and Department of Pharmaceutical Botany. During biological research RPMI 1640 (Sigma), antibiotics: penicillin (Polfa Tarchomin), streptomycin (Polfa Tarchomin), amphotericin B (Gibco, Carlsbad, USA); L-glutamine (Sigma), annexin V (Pharmingen, San Diego, USA) and buffer HEPES ph 7.4 composed of: 10 mM NaOH, 140 mM NaCl, 2.5 mM $CaCl_2$, were used.

A n a l y s i s o f p o l y s a c c h a r i d e s . The polysaccharides from dry and crushed leaves and roots were obtained using previously described method [13]. Plant materials were macerated with water for 24 h. The extracts obtained were filtered through filter paper, evaporated to dryness and the residues were dissolved in water. Then pure ethanol was added in order to precipitate mucilage.

Two fractions of the crude polysaccharides from leaves – Fr. I (36.70 g) and Fr. II (35.10 g), and one fraction of polysaccharides from roots – Fr. R (4.85 g) were obtained. All polysaccharide fractions were hydrolyzed allowing determination of the qualitative monosaccharide composition. Polysaccharide fractions were dissolved in 10 ml of 2N sulphuric acid and supplemented with 2 drops of 96% ethanol. The mixtures were heated with a reflux condenser for 10 h. After cooling, the hydrolysates were neutralized with $Ba(OH)_2$ to pH 7. The precipitates were discarded and the filtrates were evaporated to dryness in a water bath [24]. The residues were dissolved in 10 ml of methanol.

The qualitative monosaccharide composition of the obtained solutions was determined using thin layer chromatography (TLC). TLC was performed on 100x100x0.1 mm cellulose plates (Merck, Darmstadt, Germany). The chromatograms were developed in DS horizontal chambers in mobile phases that are typically used for this group of compounds. In the case of monosaccharides analysis these were as follows: F_1: ethyl acetate-acetic acid-water (3:1:3 v/v/v); F_2: chloroform-methanol-acetic acid-water (7:3:1:0.5 v/v/v/v); F_3: n-butanol-pyridine-water (6:4:3 v/v/v); F_4: n-butanol-pyridine-water (6:4:7 v/v/v); F_5: pyridine-ethyl acetate-acetic acid–water (5:5:1:3 v/v/v/v); F_6: ethyl acetate-pyridine-water (12:5:4 v/v/v) after conditioning in the respective vapors for about 5 minutes [8–10,16].

The mobile phases F_1 and F_2 showed the best ability to separate the examined monosaccharides compounds.

Next, visualization by derivatization was performed by spraying the plates with acid solution of aniline phtalane [18] and 10% H_2SO_4. After derivatization the chromatograms were heated in 105˚C for 3 minutes and then they were observed in daylight.

The monosaccharides were identified on the basis of comparison of the colors and location of spots of analyzed compounds with those of authentic standards.

A l l i u m t e s t . Allium test was used for the evaluation of the biological activity of the isolated polysaccharide fractions [15]. 0.5% solutions of polysaccharides from leaves (Fr. I, II, A) and from

roots (R) that were prepared for examination. A testing material that was employed was the apical meristem of adventitious roots of *Allium cepa* L., incubated in tap water. After 5 days of growth at 20˚C the seedling roots were, on average, 1-2 cm long. The onions, after the growth of well developed roots of 1-2 cm in length, were longitudinally split into halves. Seedlings placed in tap water were used as the control and seedlings placed in examined solutions were used as the experimental. After 24 h the seedlings subjected to polysaccharides action were transferred to Carnoy's fixer. Then, microscopic squashed slides, coloured Feulgen's method earlier [4], were prepared. The slides from 10 seedlings were done for the experimental group. Inhibition of growth of roots and antimitotic activity were counted in percentages in relation to the control. Number of mitosis falling on 1000-1200 cells was counted for all slides. The results were statistically elaborated. The durability of inhibition activity of examined extracts was also checked. In this case, seedlings after 24 h of having been influenced by examined solution were displaced into the tap water for next 24 h.

Plant material, cells and Short Term Cultures. Ten percent-aqueous solutions of mucilage from leaves (Fr. LA) and from roots (Fr. RA), and 10% ethanolic extracts from fresh (Fr. LF) and dried (Fr. LD) leaves were examined. The polysaccharide fractions were tested at concentrations of 50, 100 and 150 µl solution in the 1000 µl suspension of cells; and 5, 10, 50 µl in the 1000 µl suspension of cells for ethanolic extracts.

Jurkat human T-lymphoblastic cell line (J45.01.93031145 ECACC/Sigma) were used to biological examinations. The cells at concentration 10^6 cells/mL were incubated in air atmosphere humidified 5% CO_2, for 24 h at 37˚C in an incubator (Biotech). The growing medium consists from: RPMI 1640 medium (Sigma, St. Louis, USA), 10% bovine calf serum (Sigma, St. Louis, USA), 2 mM L-glutamine and antibiotics [penicillin in concentration 100 U/mL, streptomycin in concentration 100 µg/mL (Polfa Tarchomin) and amphotericin B in concentration 2.5 µg/mL (Gibco, Carlsbad, USA)]. One day after seeding, the cells were exposed to the examined extracts, in the following concentrations: 1% and 1.5% for aquaeous fractions (Fr. LA and Fr. RA); 0.05% and 0.1% for ethanolic extracts of leaves (Fr. LF and Fr. LD). At the same time, a control test was conducted (without examined extracts addition). The final concentration of ethanol was thereby reduced to 1% in the assays. This concentration of ethanol did not affect cell viability at all. All tests were performed triplicate.

Cells were observed using a BX41 Olympus light/fluorescence microscope. Data was processed according to the MultiScan software.

Trypan Blue assay. *In vitro* cytotoxicity assay was carried out using trypan blue assay. The cell lines in concentration $5x10^6$ cell/ml were treated with different concentrations of testing extracts and incubated 24 h at 37˚C in air atmosphere humidified 5% CO_2. At the end of this period, the medium from each plate was removed by aspiration. Then, the cells were washed with PBS (buffered isotonic salt solution) and centrifuged at an 800 rpm for 10 min, and then PBS was removed by aspiration. Then, 10 µl suspension cells were incubated for 5 min with 10 µl 0.4% trypan blue solution (Sigma). Samples were analyzed by Olympus BX41 light and fluorescence microscope for the presence of: nonviable cells, which were dark blue and viable cells that excluded the dye.

Annexin V Fluos assay. Living, apoptotic and necrotic cells were detected by Annexin V method. The Annexin V Assay (Pharmingen, San Diego, USA) was used to estimate the number

of cells in the early and late stages of apoptosis (according of manufacturer protocol). The 24-hrs cell cultures were centrifuged at 1000 rpm for 10 min at room temperature and the culture medium was removed. Then incubated for 5 min at room temperature in the buffer comprising 10 mM Hepes [N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid) hemisodium salt]/NaOH, pH 7.4; 140 mM NaCl; 2.5mM $CaCl_2$; Annexin V labelled with 0.65 μg/ml of FITC and propidium iodide (PI), at the concentration of 12 μg/ml (by manufacture protocols).

Thereafter, samples were analyzed by an Olympus BX41 light and fluorescence microscope for the presence of: viable cells annexin V negative, PI negative; early apoptotic cells – annexin V positive, PI negative; late apoptotic/secondary necrotic – annexin V positive, PI positive (by manufacturer procedure).

The extracts mediated apoptosis were expressed as the percentage of apoptotic cells/total cells. The amount of apoptotic cells/sample was determined as the % of annexin V positive cells per sample. Cell morphology was examined using a BX41 Olympus light and fluorescence microscope. Data was processed according to the MultiScan softwere. Apoptotic cells (compaction and margination of nuclear chromatin, cytoplasmic condensation, and membrane blebbing and cell shrinkage) were observed.

RESULTS AND DISCUSSION

A lot of experimental and clinical research has demonstrated that polysaccharides derived from high plants and mushrooms, exhibit a number of beneficial therapeutic properties, including immunostimulatory, antitumor, radioprotective and antiulceric properties. Plant polysaccharides are also used in clinical oncology to increase the effectiveness of chemotherapeutic preparations and reduce their side effects. Most polysaccharides derived from higher plants are relatively nontoxic and do not cause significant side effects, which is a major problem associated with immunomodulatory bacterial polysaccharides and synthetic compounds. That is why, plant polysaccharides are good candidates for therapeutics with immunomodulatory and antitumor action [21]. Mushroom polysaccharides have been considered to have anticancer activity. Research has proved the effects of a hot water polysaccharide extract of *Pleurotus ostreatus* on the proliferation of human colon cancer cells [14]. From *Xerocomus badius* fruit bodies polysaccharide fractions were isolated which showed mitostatic and mitodepressant activity testing using Allium test [24].

As the chemical composition of plants of *Crassulaceae* family has been more and more accurately recognized, their pharmacological examination has proceeded. It has been established that organic acids, present in big amounts, as well as phenolic compounds are jointly responsible for the medicinal properties of the plants. Phenolic compounds, mainly flavonoids, are present at ethanolic extracts from leaves of *J. sobolifera* (not published jet).

As a result of hydrolysis, it was found that all the polysaccharide fractions of *Jovibarba sobolifera* (Sims.) Opiz contain the following sugars: glucose, rhamnose and galactose. Fraction II from leaves contained arabinose and sedoheptulose also. Cytotoxic activity was performed for the fraction of polysaccharide from leaves (fraction I and II) and from roots (fraction R).

On the ground of Levan's test it was stated that polysaccharides inhibited linear growth and caused distempers in mitotic fissions. The morphological observations showed that seedlings of *Allium cepa* L. incubated in examined solutions did not change the color but had inconsiderable

influence on turgor and caused its flabbiness. Half percent-fraction I from leaves was most active. This solution inhibited linear growth of roots in 75% and mitotic fission in 85.63%. The microscopic observations proved that in slides of this group, binuclear cells appeared, which testifies to disturbance in cytokinesis. Polysaccharide fraction A from leaves also characterized high cytostatic activity and it inhibited linear growth of roots in 62.79% and mitotic fission in 71.22%. The microscopic slides showed transformed pictures of fission stages. The occurrence of distempers included shortened and thicker chromosomes in prophase. The results of Levan's test are shown in Table 1. There was noted a correlation between inhibition of the longitudinal root growth and the suppression of mitotic activity. All observed fractions, except of mucilage from radix (Fr. R), caused inhibition of mitosis in excess of the inhibition of linear growth.

Table 1. The influence of polysaccharides from *J. sobolifera* on linear growth (a) and mitotic division (b) on roots of *Allium cepa* L.

Polysaccharide fraction [0.5%]	Growth of roots [cm]	Mitotic index (IM)	Inhibition percentage	
			a	b
Fr. I	0.24	1.35±0.31	75%	85.63%
FR. II	0.37	8.7±0.2	36%	40.22%
Fr. R	0.79	3.83±1.00	59.82%	50.19%
Fr. A	0.54	2.63±0.97	62.79%	71.22%

Explanations: Fr. I, II and A – fractions of polysaccharides from leaves, Fr. R – fraction of polysaccharides from roots of *J. sobolifera*

The results of test with trypan blue have shown that ethanolic extracts had the most intensive activity. IC_{50} (inhibitory concentration, 50%) values were determined at 0.05% for both extracts. Fraction LF had more cytotoxity effect. For aqueous extracts IC_{50} values were determined for 1% fraction LA and for 1.5% fraction RA. It had been stated that mucilage from leaves had stronger cytotoxic activity than mucus from roots.

In the apoptosis courses examination with annexin V, morphological changes of cells were observed: the change in external layer of cell membrane – asymmetry of lipid part of membrane loss, atrophy of consignment functions and breaking of structural integrality of cell membrane, make it possible to distinguish apoptotic from living cells.

After 24 h stimulation of Jurkat cell line with examined fractions, extracts LA and LF proved to be more active. Changes in Jurkat cell line in the early stage of apoptosis after extract LF setting have been presented in Fig. 3. The cells in this figure showed green fluorescence which is characteristic for changes of spatial conformation of the plasma membrane. In Figure 4 we observed red fluorescence of propidium iodide which is characteristic for late stage of apoptosis. The cells on figures 3 and 4 showed characteristic plasma membrane blebbing.

The results of trypan blue and annexin V tests were shown in Fig. 1, 2 and Tab. 2, 3.

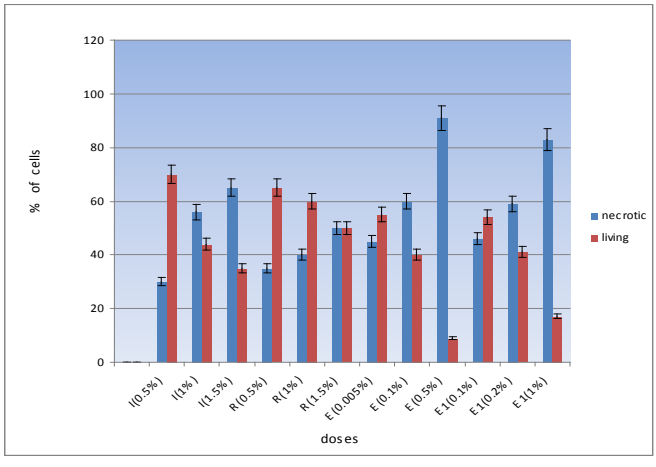


Fig. 1. The vability effect of the selected extracts from *J. sobolifera* after 24 h incubation at examined concentrations on Jurkat cell line (trypan blue assay). Explanations: I – aqueous solution of polysaccharides (Fr. LA) from leaves, R – aqueous solution of polysaccharides (Fr. RA) from roots, E – ethanolic extract from fresh leaves (Fr. LF), E1 – ethanolic extract from dry leaves (Fr. LD)

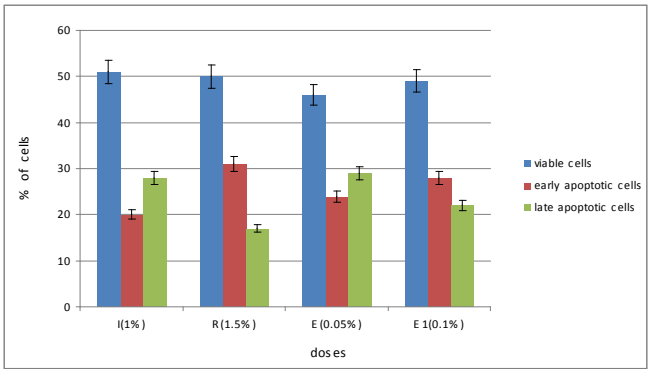
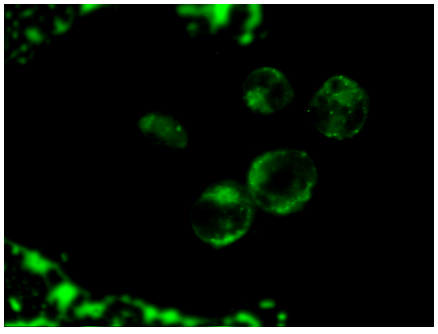
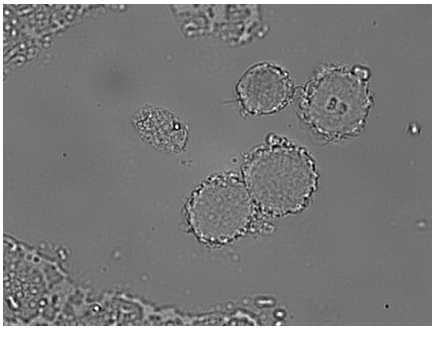


Fig. 2. The valuation of the Jurkat human T-lymphoblastic cell lines (ECACC) after 24 h incubation IC_{50} doses examined extracts (Annexin V assay). Explanations: I(1%) – 1% fraction LA; R(1.5%) – 1.5% fraction RA; E(0.05%) – ethanolic extract from fresh leaves (Fr. LF); E1(0.1%) – ethanolic extract from dry leaves (Fr. LD)

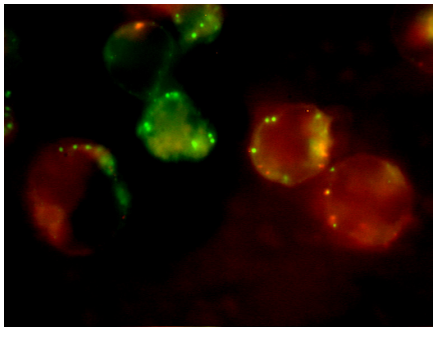


A

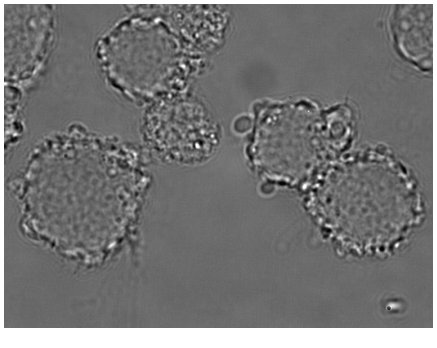


B

Fig. 3. The cells of Jurkat line during the early stage of apoptosis, after stimulated with 0.05% ethanolic extract from fresh leaves of *J. sobolifera* – characteristic shrinkage of the cytoplasm, plasma membrane blebbing and presence of apoptotic bodies are observed (B). A – green fluorescence of annexin V conjugated with FITC, absence of red fluorescence of PI. Magnification 600 x



A



B

Fig. 4. The cells of Jurkat line during the late stage of apoptosis, after stimulated with 0.05% ethanolic extract from fresh leaves of *J. sobolifera* - characteristic shrinkage of the cytoplasm and presence of apoptotic bodies are observed (B). A - green fluorescence of annexin V conjugated with FITC, red fluorescence of PI. Magnification 1000 x

Table 2. Results of cytotoxic test of extracts from *J. sobolifera* leaves

Fraction (doses,%)	% of necrotic cells	% of living cells
I (0.5%)	30	70
I (1%)	56	44
I (1.5%)	65	35
R (0.5%)	35	65
R (1%)	40	60
R (1.5%)	50	50
E (0.05%)	45	55
E (0.1%)	60	40
E (0.5%)	91	9
E1 (0.1%)	46	54
E1 (0.2%)	59	41
E1 (1%)	83	17

Table 3. Viability and apoptotic effect of the *Jovibarba sobolifera* extracts.

Fraction (doses,%)	% of living cells	early apoptotic cells	late apoptotic cells
I (1%)	51	20	28
R (1.5%)	50	31	17
E (0.05%)	46	24	29
E1(0.1%)	49	28	22

Studies of biological activity of aqueous fractions (polysaccharide) made by various methods: the Levan's test (mitotic index), the test with the trypan blue (cytotoxicity) and the test with Annexin V and propidium iodide (apoptosis), indicate a high biological activity of these fractions.

Results of biological research are received enter to farthest research, attempt of explanation of influence of analyzed fractions from leaves of *J. sobolifera* on mechanism of operation in processes of survivals and deaths of tumor cells.

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SUMMARY

The fractions of polysaccharides from rosette leaves and roots, and ethanolic extracts from fresh and dried leaves of *Jovibarba sobolifera* (Sims.) Opiz were investigated. All isolated polysaccharide fractions were mainly composed of glucose, rhamnose and galactose. Biological activity testing using the Allium test showed mitostatic activity for all fractions. It was discovered that mucilage contained in *Jovibarba sobolifera* impedes the linear growth of onion roots and causes mitostatic activities disorder. It appeared that mucilage extracted from herbs is more active. Additionally, having stimulated the Jurkat line cells by examined fractions, the evaluation of cytostatic activity and the evaluation of the number of cells in the early stage of apoptosis, was carried out. Water solutions of mucilage of herbs and roots, and ethanol extracts made of dried and fresh herbs were subjected to examination. The conducted tests revealed that ethanol extracts of *Jovibarba sobolifera* had the strongest effect. Cytostatic activity of examined fractions (polysaccharides and ethanolic extracts) were stated using the test with trypan blue (0.4% aqueous solution of blue) also.

Keywords: cytotoxic activity, apoptosis, Allium test, *Jovibarba sobolifera* (Sims.) Opiz, *Crassulaceae*

STRESZCZENIE

Badaniom poddano frakcje polisacharydowe z liści i korzeni, a także ekstrakty etanolowe ze świeżych i wysuszonych liści *Jovibarba sobolifera* (Sims.) Opiz. Wszystkie wyizolowane frakcje polisacharydowe składały się głównie z glukozy, ramnozy i galaktozy. Zastosowany test Allium ujawnił aktywność mitotyczną wszystkich frakcji. Stwierdzono, że śluz zawarty w *Jovibarba sobolifera* zakłóca wzrost liniowy korzeni cebul i powoduje zaburzenia aktywności mitotycznej. Śluz wyekstrahowany z ziela okazał się bardziej aktywny. Dodatkowo, stymulując linie komórkowe Jurkat badanymi frakcjami, przeprowadzono ocenę aktywności cytostatycznej oraz ocenę liczby komórek we wczesnej fazie apoptozy. Badaniom poddano wodne roztwory śluzu z ziela i korzeni, a także wyciągi etanolowe z suchego i świeżego ziela. Przeprowadzone testy wykazały, że najsilniejszą aktywnością odznaczają się wyciągi etanolowe z *Jovibarba sobolifera*. Aktywność cytostatyczną badanych frakcji (polisacharydy i wyciągi etanolowe) przeprowadzono także za pomocą testu z trypanem blue (0.4% roztwór wodny).

Słowa kluczowe: aktywność cytotoksyczna, apoptoza, Allium test, *Jovibarba sobolifera* (Sims.) Opiz, *Crassulaceae*