

RUMEX L. SPECIES INDUCE APOPTOSIS IN 1301, EOL-1 AND H-9 CELL LINES

MAGDALENA WEGIERA, HELENA D. SMOLARZ* and ANNA BOGUCKA-KOCKA

Chair and Department of Pharmaceutical Botany, Medical University, Chodźki 1, 20-093 Lublin, Poland

Abstract: The *Rumex* L. (dock) species for many centuries have been used in medical treatment, through their adstringent, spasmolytic or cholagogic action. In the present study, the *in vitro* screening of cytotoxic activities of ethanol extract from roots, leaves and fruits of six *Rumex* species: *R. acetosa* L., *R. acetosella* L., *R. confertus* Willd., *R. crispus* L., *R. hydrolapathum* Huds. and *R. obtusifolius* L. were performed. We found remarkable cytotoxic activities on leukemic 1301 and EOL-1 cell lines and T cell line at concentration dependent manners. Cytotoxic activity was determined in two ways: trypan blue test and annexin-V FITC and propidium iodide assay. Received IC₅₀ values of investigated extracts on 1301, EOL-1 and H-9 cell lines ranged from 0.22, 0.17 and 0.04 to 2.56, 1.91 and 1.83 mg/mL, respectively. Analysis of morphological changes demonstrated that the extract exerted cell-death *via* apoptosis. The overall activities of *Rumex* species support the traditional use of the extract from the fruits of *R. confertus*, *R. crispus*, *R. hydrolapathum* and *R. obtusifolius* in the treatment of cancer.

Keywords: *Rumex*, ethanol extracts, roots, leaves, fruits, cancer cell lines, apoptosis

The interest of natural drugs as adjunctive therapy for acute and chronic diseases has grown significantly in the recent years. Herbal remedies play an important role in modern medicine and it appears feasible that the compounds from herbs can be helpful in prevention or treatment of different diseases.

There is growing evidence that some forms of complementary and alternative medicine (CAM) are helpful in supportive and palliative cancer care. On the other hand, complementary therapies are used as an adjunct in decreasing the adverse effects of anti-cancer treatments or through alleviating the symptoms of cancer through improving the general well-being of the patient (1). Two most widely and commercially available herbal tonics used as a CAM by some cancer patients, are Essiac® (17.3% of use of the breast cancer survivors in Ontario) and Flor-Essence® (2, 3). Both formulations contain the same four basic herbs: sheep sorrel (*Rumex acetosella* L.), burdock root (*Arctium lappa* L.), slippery elm bark (*Ulmus rubra* Muhl.) and Turkish rhubarb (*Rheum palmatum* L.). In addition, Flor-Essence® contains red clover (*Trifolium pretense* L.), blessed thistle (*Cnicus benedictus* L.), kelp (*Laminaria digitata* L.) Lamour.) and watercress (*Nasturtium officinale*

R. Br.) (2). Essiac® has been used in Canada for more than 80 years – it has been promoted as an Ojibwa Indian cancer cure in Canada since 1922 (4, 5). Although the effects of the tonics have not been thoroughly studied, the effects of the individual herbs used in the Essiac® and Flor-Essence® have been investigated because of their important dietary and medicinal roles. Characteristics of these herbs include estrogenic, cytotoxic, anti-estrogenic, anti-tumor, anti-mutagenic, anti-oxidant, and anti-inflammatory properties, among others. The lay literature suggests that the components act synergistically to convey beneficial properties (2, 6, 7). Evidence for the efficacy of Essiac® and Flor-Essence® against cancer is mostly anecdotal (5, 6, 8) – they are advertised as substances improving health and boosting the immune system (2, 9). Use of Essiac® as an adjunctive therapy will result in improving the quality of life of patients, and potentially in increasing their survival (3). Particular popularity of Essiac® as complementary therapy among breast cancer survivors and patients may be related to the first described use of Essiac® tonic as a cure for a breast lump (2). It has been also used in unconventional therapy among pediatric patients with cancer in Saskatchewan (10). Several unpublished

* Corresponding author: e-mail: h.smolarz@am.lublin.pl

reports were mentioned, and there is some record of *in vitro* anticancer activity in the Essiac® and in some constituent herbs (8). Essiac® has been claimed to inhibit cell proliferation and induce differentiation in human prostate cancer cell lines (11, 12). It may be able to inhibit tumor cell growth while enhancing immune response to antigenic stimulation (11). Essiac® tea also possesses potent antioxidant and DNA-protective activity, properties that are common natural anti-cancer agents (4).

Anthracene derivatives (chrysophanol, physcion, emodin, aloe-emodin, rhein), which are naturally occurring in *Rumex* species (13) seem to be the main biologically active compounds responsible for anti-cancer (14, 15), cytotoxic (16–24), genotoxic and mutagenicity (25, 26) properties.

The main objective of the study was to examine *in vitro* cytotoxic properties of ethanol extracts from the fruits, leaves and roots of six *Rumex* L. species: *R. acetosa* L., *R. acetosella* L., *R. confertus* Willd., *R. crispus* L., *R. hydrolapathum* Huds. and *R. obtusifolius* L. against 1301 – human T cell leukemia lymphoblast, EOL-1 – human eosinophilic leukemia and H9 – clonal derivative of the T cell lines.

EXPERIMENTAL

Plant material

Plant material for the investigation included fruits, leaves and roots of six *Rumex* L. species: *R. acetosa* L., *R. acetosella* L., *R. confertus* Willd., *R. crispus* L., *R. hydrolapathum* Huds. and *R. obtusifolius* L. collected from natural habitat at the end of June (leaves) and in September (fruits and roots) of 2007, near Lublin in Poland.

The voucher specimens are deposited in Department of Pharmaceutical Botany, Medical University of Lublin, Poland (voucher specimens no. RF.01.07 – RF.06.07, RL.01.07 – RL.06.07 and RR.01.07 – RR.06.07).

Extraction

The air-dried and powdered plant materials (2 g) from fruits, leaves and roots of each of the taxons were extracted with 25 mL 80% aq. ethanol at room temperature. After 24 h, samples were filtered and extracted twice with 20 mL 80% aq. ethanol (15 min) in supersonic water bath at room temperature. The combined extracts were evaporated from the solvent to dry residue under reduced pressure at 40°C. For further investigation 0.1–50 mg/mL DMSO solutions of these samples were used.

Cell lines and culture medium

The three human cell lines: 1301 (human T cell leukemia lymphoblast), EOL-1 (human eosinophilic leukemia) and H-9 (human T cell) were used in this work. They were cultured by ECACC protocol at the concentration of 5×10^5 cells/mL. All cultures were incubated in the air atmosphere humidified 5% CO₂, for 24 h at 37°C in incubator (A-Biotech, Wrocław, Poland). The growing medium consisted of: RPMI 1640 medium (Sigma, St. Louis, USA), 10% heat inactivated fetal bovine serum (Sigma, St. Louis, USA), 2 mM L-glutamine and antibiotics: penicillin at concentration 100 U/mL, streptomycin at concentration 100 µg/mL and amphotericin B at concentration 2.5 µg/mL (Gibco, Carlsbad, USA). At the time of seeding, the cells were exposed to ethanol extracts, at the following final concentrations: 0.1–50 mg/mL. This range of concentration of ethanol did not caused cell death at all. Cells were observed using a BX41 Olympus light/fluorescence microscope. The data were processed according to the MultiScan Software. All tests were performed in triplicate.

Trypan blue assay

Two lines of human leukemic cells and one normal cell line were stimulated *in vitro* with various concentrations of EtOH extracts from fruits, leaves and roots of six species of *Rumex* genus. The viable cells were counted using trypan blue dye exclusion assay. The percentage of cell viability was determined by dividing the number of viable cells by the number of all cells. The seeding cultures at concentration 5×10^5 cells/mL were treated with different concentrations of testing extracts and incubated for 24 h. At the end of this period, the medium from each plate was removed by aspiration. Then, 10 µL suspension of cells was incubated for 5 min with the 10 µL 0.4% trypan blue solution (Sigma). Thereafter, an Olympus BX41 microscope was used to analyze the presence of nonviable cells which were dark blue, and viable cells which excluded the dye. About 10 randomly selected fields were analyzed in the preparation, so that the sum of all cells was not less than 200. In parallel, cells viability was examined in the control test – the share of dead cells in each control sample does not exceed 5% of all cells. The control cultures with the negative DMSO concentration were also performed. The IC₅₀ (inhibitory concentration – that is such a concentration of stimulant, which in 24 h cell culture gave 50% of surviving cells) was set by means of MS Excel spreadsheet and standard deviation.

Annexin V assay

The measurement of phosphatidylserine redistribution in plasma membrane was conducted according to the protocol outlined by the manufacturer in the Annexin V-FITC Apoptosis Detection kit (Sigma Chemical Co, USA), as previously described (27). In brief, 1×10^6 cells were washed and re-suspended in HEPES buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 5 mM CaCl₂) containing annexin V-FITC (1:50) and 1 µg/mL of propidium iodide for 15 min, before being analyzed with a fluorescence microscope.

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-h cell cultures were centrifuged at 800 rpm for 10 min at room temperature (RT) and the culture medium was removed. Then, the cultures were incubated for 10 min in the buffer comprising: 10 mM Hepes [N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) hemisodium salt]/NaOH, pH 7.4; 140 mM NaCl; 2.5 mM CaCl₂; annexin V labelled with 0.65 µg/mL of FITC and propidium iodide at the concentration of 12 µg/mL (protocols by manufacturer).

Thereafter, samples were analyzed by an Olympus BX41 light and fluorescence microscope for the presence of viable cells – annexin V-/PI-; early apoptotic cells – annexin V+/PI-; late apoptotic/secondary necrotic – annexin V+/PI+ (by manufacturer procedure). Fluorescein isothiocyanate-labeled annexin V could bind the outer membranes of apoptotic cells and discriminate apoptotic cells in the early stage against necrotic cells when used with propidium iodide. The extract 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 and laser scanning confocal microscopy (Carl Zeiss, Jena, Germany). The data were processed according to the MultiScan software. Apoptotic cells (compaction and margination of nuclear chromatin, cytoplasmic condensation and membrane blebbing and cell shrinkage) were observed. All the samples were analyzed at the concentrations close to IC₅₀ values or at the concentration with 50% of living cells in breeding.

Table 1. IC₅₀ values of roots, leaves and fruits of selected *Rumex* L. species (mg/mL ± S.E.M.; n = 3).

Ethanol extract [mg/mL]		Cell line		
		1301	EOL-1	H-9
<i>R. acetosa</i>	roots	0.41 ± 0.006	0.41 ± 0.023	0.32 ± 0.003
	leaves	2.21 ± 0.309	1.91 ± 0.049	1.66 ± 0.051
	fruits	1.79 ± 0.112	0.40 ± 0.008	0.55 ± 0.008
<i>R. acetosella</i>	roots	1.56 ± 0.018	1.23 ± 0.147	0.99 ± 0.006
	leaves	1.99 ± 0.016	1.91 ± 0.022	1.83 ± 0.021
	fruits	2.56 ± 0.198	1.05 ± 0.013	1.00 ± 0.087
<i>R. confertus</i>	roots	0.22 ± 0.002	0.23 ± 0.004	0.10 ± 0.011
	leaves	0.77 ± 0.019	0.59 ± 0.019	0.54 ± 0.019
	fruits	0.75 ± 0.004	0.18 ± 0.021	0.33 ± 0.062
<i>R. crispus</i>	roots	0.44 ± 0.023	0.43 ± 0.008	0.04 ± 0.002
	leaves	1.24 ± 0.024	0.68 ± 0.017	0.75 ± 0.005
	fruits	0.62 ± 0.006	0.20 ± 0.011	1.42 ± 0.107
<i>R. hydrolapathum</i>	roots	0.31 ± 0.003	0.18 ± 0.002	0.15 ± 0.004
	leaves	0.67 ± 0.078	0.60 ± 0.012	0.36 ± 0.027
	fruits	0.42 ± 0.005	0.17 ± 0.002	0.05 ± 0.003
<i>R. obtusifolius</i>	roots	0.52 ± 0.028	0.40 ± 0.039	0.20 ± 0.019
	leaves	0.47 ± 0.004	0.44 ± 0.003	0.32 ± 0.010
	fruits	0.80 ± 0.017	0.95 ± 0.025	0.36 ± 0.022

Statistical analysis

All results are expressed as the mean of three different experiments. The GraphPad InStat 3 software was used for the statistical calculations.

We evaluated relationship between cytotoxic properties (IC₅₀ values) and content of anthracene derivatives (13). Values of $p < 0.05$ were considered to be statistically significant and $p < 0.01$ very significant.

RESULTS

Cytotoxic activity of 6 species of the *Rumex* L. genus was analyzed using two human leukemic lines: 1301 (human T lymphoblastic cell) and EOL-1 (human eosinophilic leukemia) and normal H-9 (normal T lymphocytes) cell line. The study was made for ethanol extracts from roots, leaves and fruits of selected *Rumex* species: *R. acetosa*, *R. acetosella*, *R. confertus*, *R. crispus*, *R. hydrolapathum* and *R. obtusifolius*.

Determination of cytotoxicity by trypan blue test

At the first stage of the cytotoxic assays, the IC₅₀ doses were set (Table 1).

All examined extracts (with concentrations ranging from 0.04 to 2.56 mg/mL) indicate cytotoxic activity upon leukocytes: both on leukemia cell lines 1301 and EOL-1 and a normal H-9 cell line. Comparing the level of the cytotoxicity of plant extracts against 1301 cell line, it was found that the most active species is *R. hydrolapathum*. The IC₅₀ values for roots, leaves and fruit are respectively 0.31, 0.67 and 0.42 mg/mL. The other extracts from the roots of *R. confertus* L., *R. acetosa*, *R. crispus* and *R. obtusifolius* (with IC₅₀ values: 0.22, 0.41, 0.44 and 0.52 mg/mL, respectively) also deserve distinction. The extracts from leaves and fruits of *R. obtusifolius* (0.47 and 0.80 mg/mL), from fruits *R. crispus* (0.62 mg/mL) and from fruits and leaves of *R. confertus* (0.75 and 0.77 mg/mL) have similar cytotoxic activity. The weakest stimulants for this cell line proved to be extracts from *R. acetosella* (1.56–2.56 mg/mL), and fruits and leaves of *R. acetosa* (1.79 and 2.21 mg/mL).

Comparing IC₅₀ values for each of the tested species one can observe the following chronology of cytotoxic activity for roots: *R. confertus* > *R. hydrolapathum* > *R. acetosa* > *R. crispus* > *R. obtusifolius* > *R. acetosella*; for leaves: *R. obtusifolius* > *R. hydrolapathum* > *R. confertus* > *R. crispus* > *R. acetosella* > *R. acetosa* and for fruits: *R. hydrolapathum* > *R. crispus* > *R. confertus* > *R. obtusifolius* > *R. acetosa* > *R. acetosella*.

The extracts from fruits of docks show the strongest cytotoxic activity on human leukemic cell line EOL-1. The lowest IC₅₀ value among the analyzed extracts was obtained from fruits of *R. hydrolapathum* – 0.17 mg/mL, high cytotoxicity was exerted by root extracts of *R. hydrolapathum* (0.18 mg/mL).

Decreasing cytotoxic activity defined by IC₅₀ values of individual extracts for EOL-1 cell line is as follows: for roots: *R. hydrolapathum* > *R. confertus* > *R. obtusifolius* > *R. acetosa* > *R. crispus* > *R. acetosella*; for leaves: *R. obtusifolius* > *R. confertus* > *R. hydrolapathum* > *R. crispus* > *R. acetosella* > *R. acetosa* and for fruits: *R. hydrolapathum* > *R. confertus* > *R. crispus* > *R. acetosa* > *R. obtusifolius* > *R. acetosella*.

Comparing the IC₅₀ values between the two leukemic cell lines 1301 and EOL-1, we can conclude that in all cases the line EOL-1 seems to be more sensitive to the extracts used in the experiment. Only extracts of the fruits of *R. obtusifolius* are characterized by greater activity on the cell line 1301. It was also noted that within a given species (for two leukemia lines) there were small differences in IC₅₀ values for: the roots and leaves of *R. acetosa*, the roots of *R. confertus* and *R. crispus* and *R. acetosella* leaves, *R. hydrolapathum* and *R. obtusifolius*. The lowest IC₅₀ value of all the extracts was obtained from the species *R. hydrolapathum* and *R. confertus* for EOL-1 cell line. For fruits and roots, the IC₅₀ values were 0.17–0.18 (*R. hydrolapathum*) and 0.18–0.23 mg/mL (*R. confertus*) and 0.59 and 0.60 mg/mL for extracts from the leaves (*R. confertus* and *R. hydrolapathum*, respectively). The least active of all extracts were the extracts from *R. acetosella*, both in EOL-1 and 1301 cell lines. From the obtained data it follows that the extracts from roots of *Rumex* L. species have the strongest cytotoxic activity on leukemic 1301 cell line, while the fruits are the most active parts of plants against the cell line EOL-1. Within all dock species the leaves are characterized by the weakest cytotoxicity, however, in the case of *R. acetosella* (line 1301) and *R. obtusifolius* (both lines) fruits proved to be the least active parts of the plants. It was found that the strongest cytotoxic activity, and thus the lowest IC₅₀ value (0.17 mg/mL), was shown for the extract from the fruits of *R. hydrolapathum* on leukemic EOL-1 cell line. In turn, the highest IC₅₀ value, was established for the extract from the fruits of *R. acetosella* against 1301 cell line (IC₅₀ = 2.56 mg/mL).

At the next stage of work, the cytotoxic properties of the extracts affecting the viability of normal human T lymphocytes (H-9 cell line) were exam-

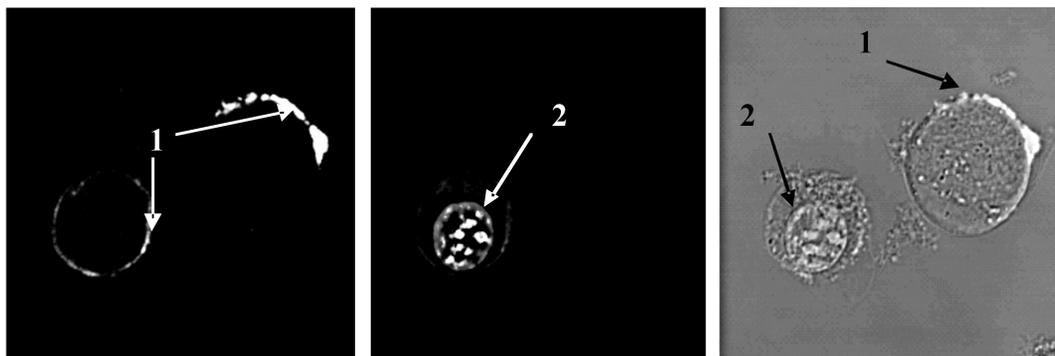


Figure 1. Detection of apoptotic cells with FITC-labeled annexin V and propidium iodide. Images shows an early (annexin V⁺/PI⁻) and late (annexin V⁺/PI⁺) stage of apoptosis in EOL-1 cells, stimulated by ethanol extract of *R. confertus* fruits (0.10 mg/mL, 24-h culture). Cells were fixed and then immunostained with anti-annexin V antibody followed by FITC-conjugated secondary antibody to show annexin V protein. They were also stained with PI to visualize the nuclei. The images were scanned using laser scanning confocal microscopy (Carl Zeiss, Jena, Germany). Pointer 1 indicates fluorescence representing annexin V signals, pointer 2 indicates fluorescence representing nuclei signals.

ined. It was found out that among all the tested docks species, extract from *R. hydrolapathum* showed the highest cytotoxic activity and *R. acetosella* was the weakest. IC₅₀ for the roots of *R. crispus* and the fruits of *R. hydro;apathum* were established 0.04 mg/mL and 0.05 mg/mL respectively. The leaves of the investigated dock species had the weakest cytotoxic properties, with the exception of *R. crispus* and *R. obtusifolius*.

The results indicate that the tested extracts have cytotoxic activity both on leukemic 1301 and EOL-1 cell lines and normal T lymphocytes. In most cases, the IC₅₀ values for H-9 cell line were lower than for the 1301 and EOL-1 cell lines. In many cases these differences are negligible. Cytotoxicity difference (the difference in IC₅₀ values) of certain plant extracts against the H-9 cell line, is as follows: 0.03 (roots of *R. hydrolapathum*), 0.05 (fruits of *R. acetosella*), 0.12 (fruits of *R. hydrolapathum*) and 0.20 (roots of *R. obtusifolius*). It is interesting that the extracts from the fruits of *R. confertus* and *R. acetosa* are characterized by a smaller cytotoxic effect against normal cell line than the leukemic cell lines. It is worthwhile to point out that the IC₅₀ value of the extract from the fruits of *R. crispus* is a 7-fold higher for the H-9 than for the EOL-1, and 2-fold higher than for the 1301 cell line. To a lesser extent, this beneficial difference can be observed in the case of extracts from fruits of *R. acetosa* and leaves of *R. confertus* with respect to the EOL-1 cell line.

Morphological changes in cultured cells

The next stage of our investigations concerned the observation of the influence of extracts upon morphological changes of cells and the assessment

of the number of living, early apoptotic, late apoptotic and necrotic cells with annexin V-FITC and propidium iodide test. Apoptotic changes in cells were observed by annexin V assay. The percentage of viable cells in control cultures was higher than 95%.

A membrane-impermeant red fluorescent nucleic acid dye PI was used to distinguish apoptotic cells from necrotic cells (Fig. 1). Early apoptotic cells still have intact cytoplasmic membranes and therefore exclude PI which is widely used for cell viability assays. Necrotic cells are not able to exclude PI because of their damaged membranes. Incubation of cells with the FITC-labeled annexin (green fluorescence) and PI (red fluorescence) allows the discrimination between intact cells (FITC⁻/PI⁻), early apoptotic cells (FITC⁺/PI⁻) and necrotic cells (FITC⁺/PI⁺).

On the basis of the observation of morphological changes it was proved that the examined ethanol extracts cause the death of cells of the examined lines of the leukocytes apoptosis. Redistribution of membrane phosphatidylserine from the inner leaflet of the plasma membrane to the outer surfaces is common in many apoptotic cells (28). Treatment with investigated extracts resulted in an increase of cell population that was positive for annexin V staining. The data showed that they induced the exposure of phosphatidylserine on the surface of cells in a concentration-dependent manner. The apoptotic morphological changes such as nuclear, chromatin condensation, DNA fragmentation, blebbing, changes to the cell membrane such as a loss of membrane asymmetry and attachment, cell shrinkage, were observed in cells.

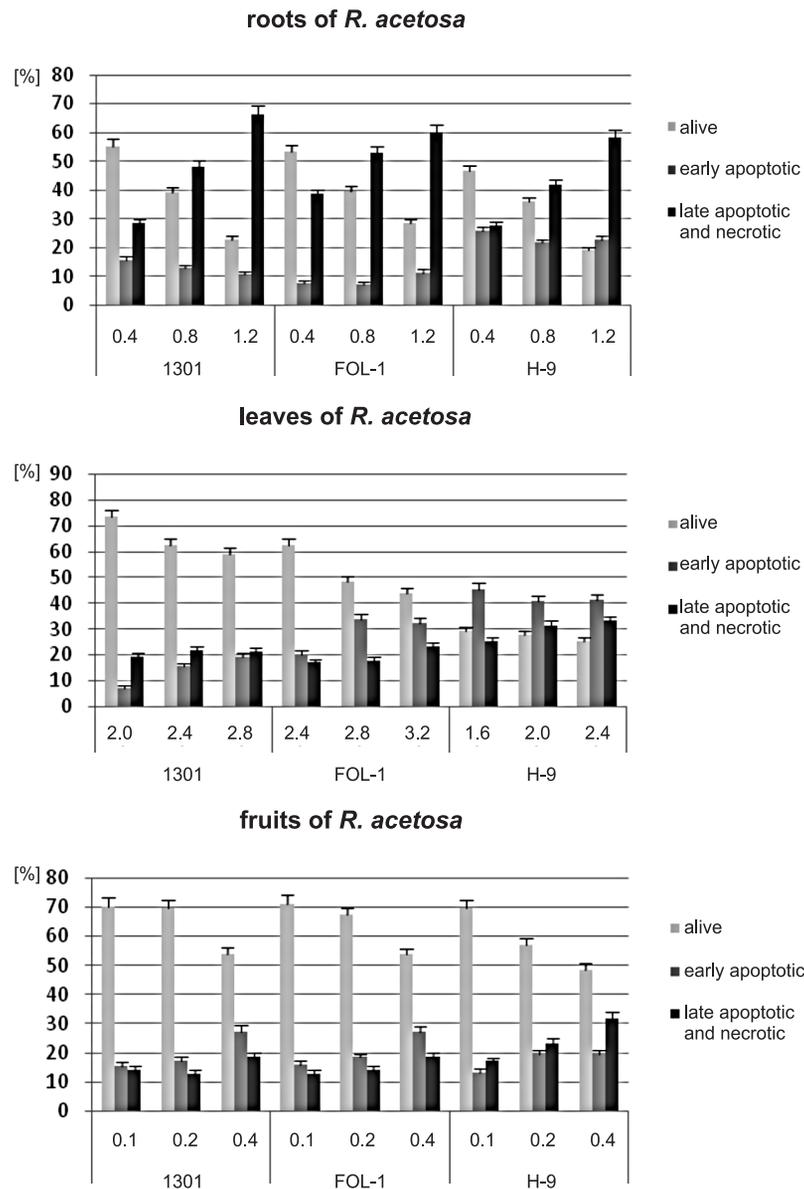


Figure 2. Comparison of the number of viable, early apoptotic and late apoptotic and necrotic cells in 1301, EOL-1 and H-9 cell line stimulated by different concentration (mg/mL) of ethanol extracts from roots, leaves and fruits of *R. acetosa*

Analysis of apoptosis in dose depended manner

The results of the influence of the ethanol extracts from the roots, leaves and fruits of six *Rumex* species on the apoptotic process in 1301, EOL-1 and H-9 cell lines by annexin V method are presented in Figures 2–7.

In all investigated extracts of *R. acetosa* the highest level of the apoptotic (early apoptotic, late apoptotic and necrotic) cells showed the normal H-9 cell line (Fig. 2).

At the concentration 0.8 mg/mL of ethanol extract from the roots the percentage of apoptotic cells for 1301, EOL-1 and H-9 cell lines were as follows: 61.0, 60.5 and 63.7%, respectively. For ethanol extract from leaves (2.4 mg/mL) and fruits (0.2 mg/mL) the levels of apoptotic cells were: 37.3 (1301), 37.3 (EOL-1) and 74.7% (H-9) and 30.5 (1301), 32.8 (EOL-1) and 43.1% (H-9), respectively.

The ethanol extracts of *Rumex acetosella* from

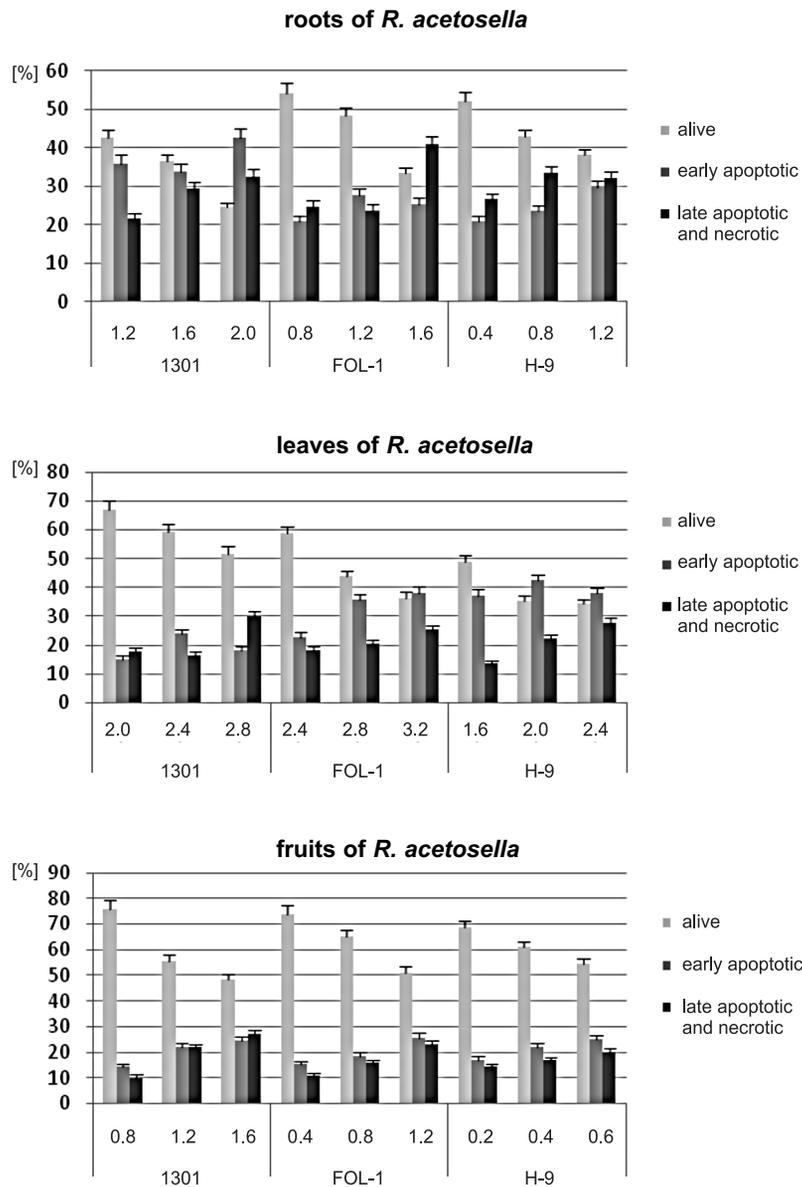


Figure 3. Comparison of the number of viable, early apoptotic and late apoptotic and necrotic cells in 1301, EOL-1 and H-9 cell line stimulated by different concentration (mg/mL) of ethanol extracts from roots, leaves and fruits of *R. acetosella*

the leaves and fruits have the strongest influence on apoptotic process in the normal H-9 cell line: 65.7% (2.4 mg/mL ethanol extract from the leaves) and 45.4% (0.8 mg/mL ethanol extract from the fruits) (Fig. 3).

The highest level of apoptotic cells was observed for the normal T lymphocytes stimulated by 0.8 mg/mL of 69.8% in ethanol extract from roots and 1.2 mg/mL of 60.0% in ethanol extract from leaves of *R. confertus* (Fig. 4). Similar levels of

apoptotic cells were observed in two leukemic cell lines: 1301 (42.5%) and EOL-1 (43.1%), which were stimulated by ethanol extracts from fruits (at concentration 0.4 mg/mL). Levels of early apoptotic and late apoptotic and necrotic cells were also similar – 32.6 and 9.9% in 1301 cell line and 31.5 and 11.6% in EOL-1 cell line, respectively.

The ethanol extracts from the roots of *R. crispus* at 0.8 mg/mL concentration showed similar, highest mortality of leukemic EOL-1 cell line

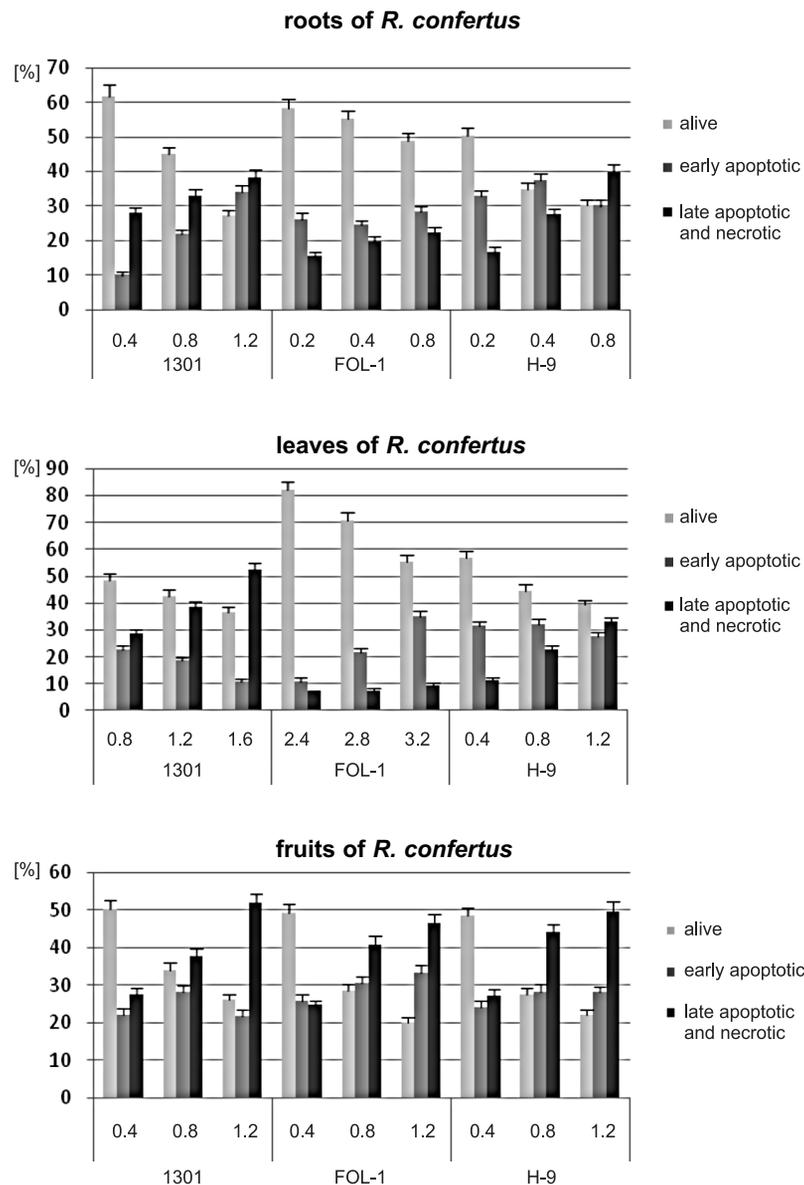


Figure 4. Comparison of the number of viable, early apoptotic and late apoptotic and necrotic cells in 1301, EOL-1 and H-9 cell line stimulated by different concentration (mg/mL) of ethanol extracts from roots, leaves and fruits of *R. confertus*

(71.5% of apoptotic cells) and normal H-9 cell line (72.4% of apoptotic cells) (Fig. 5). The percentage values of early apoptotic and late apoptotic cells were 30.6 and 40.9% and necrotic cells – 28.4 and 44.0%, respectively. At concentration of 1.2 mg/mL of extracts from leaves (58.1%) and at concentration of 0.2 mg/mL of ethanol extracts from fruits (38.3%), the highest level of apoptotic cells were observed in H-9 cell line. However, the extracts from fruits showed almost identical level of apop-

totic cells in 1301 and EOL-1 cell lines: 70.7 and 70.8%, respectively.

The highest levels of apoptotic cells were observed in the leukemic EOL-1 cell line after stimulation with the extracts from both roots and fruits of *R. hydrolapathum* (Fig. 6). At the concentration of 0.4 mg/mL of ethanol extract from the roots and at the concentration 0.2 mg/mL of ethanol extract from the fruits the percentage of apoptotic cells for 1301, EOL-1 and H-9 cell lines

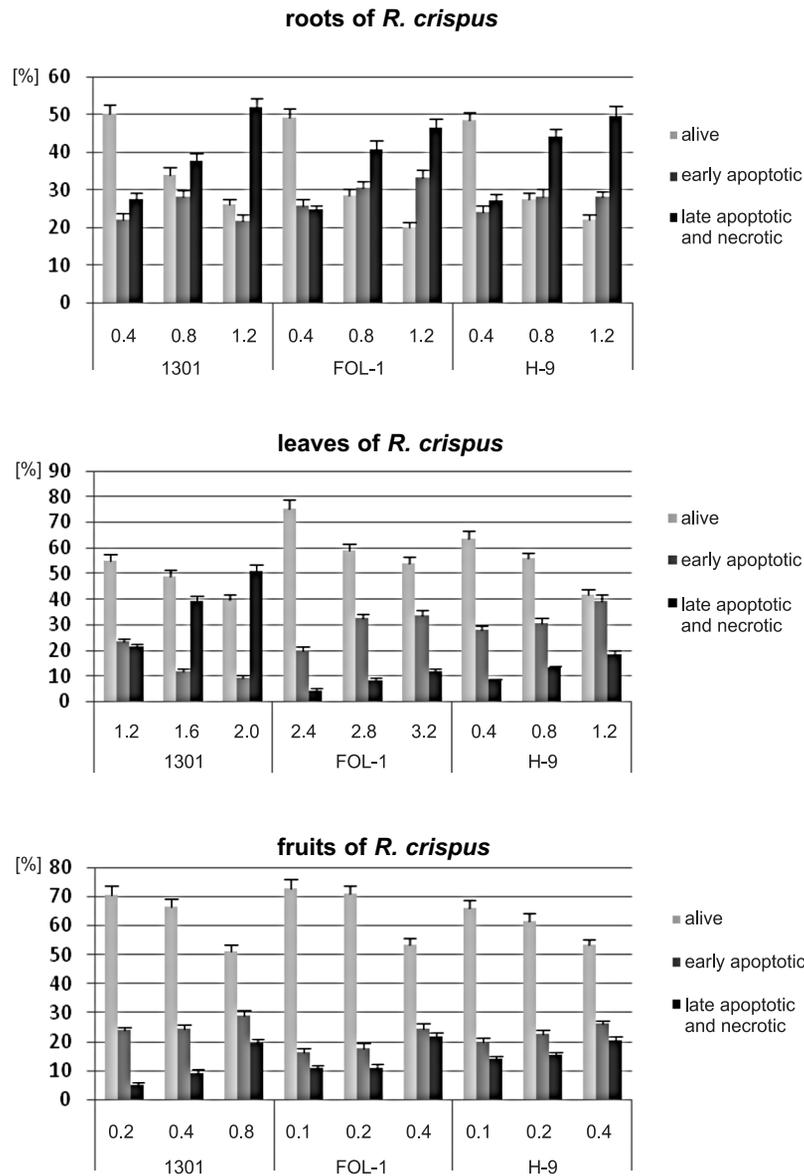


Figure 5. Comparison of the number of viable, early apoptotic and late apoptotic and necrotic cells in 1301, EOL-1 and H-9 cell line stimulated by different concentration (mg/mL) of ethanol extracts from roots, leaves and fruits of *R. crispus*

was: 43.6, 58.6 and 54.4% and 26.3, 58.3 and 48.1%, respectively. However, the normal T lymphocytes stimulated by ethanol extracts of leaves showed the highest level of apoptotic cells – 52.1% at 0.8 mg/mL.

Similar levels of apoptotic cells were observed in leukemic cell line EOL-1 (58.5%) and normal H-9 cell line (43.1%), which were stimulated by ethanol extracts from roots of *R. obtusifolius* (at concentration 0.4 mg/mL) (Fig. 7). Levels of early

apoptotic and late apoptotic and necrotic cells were also close – 35.7 and 22.6% in EOL-1 cell line and 24.2 and 23.9% in H-9 cell line, respectively. The extracts from the leaves (at concentration 0.8 mg/mL) showed the similar, highest mortality of leukemic 1301 cell line (30.5% of apoptotic cells) and normal H-9 cell line (36.7% of apoptotic cells). The percentage of early apoptotic and late apoptotic and necrotic cells was: 7.3, 23.2% and 20.2 and 16.7%, respectively. Influenced by extracts from

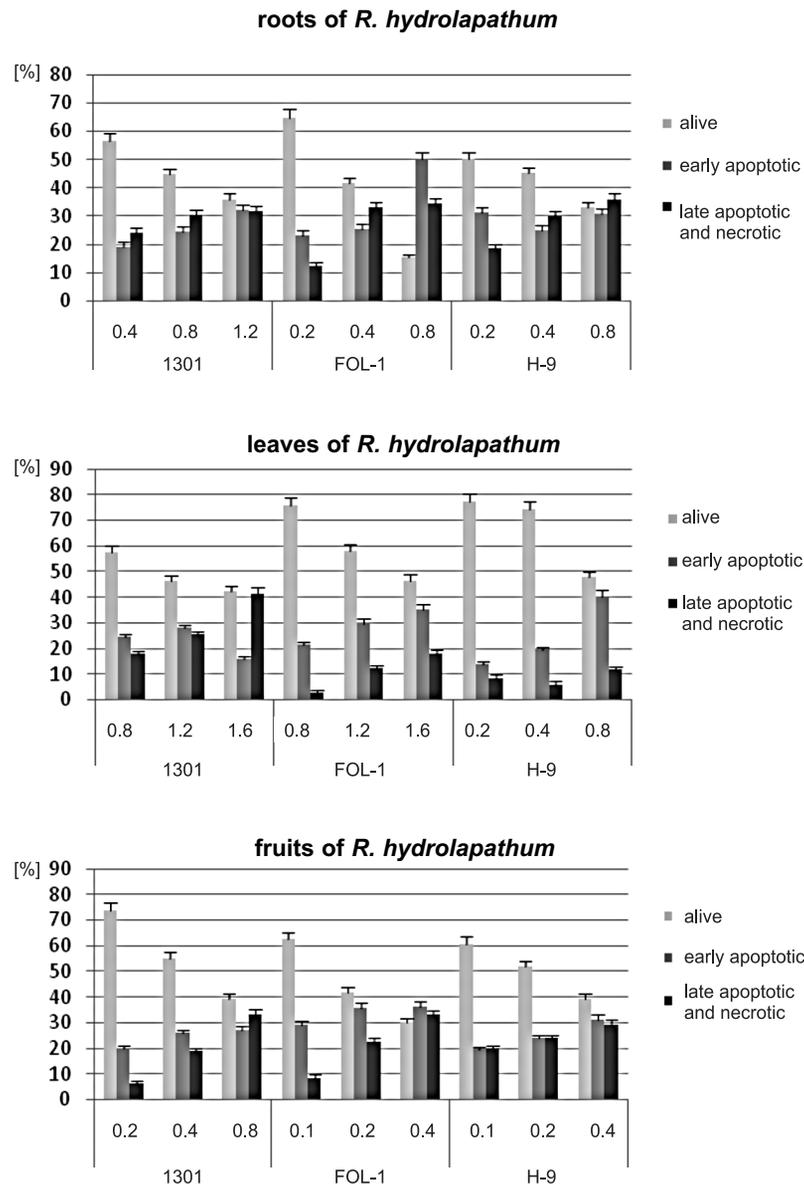


Figure 6. Comparison of the number of viable, early apoptotic and late apoptotic and necrotic cells in 1301, EOL-1 and H-9 cell line stimulated by different concentration (mg/mL) of ethanol extracts from roots, leaves and fruits of *R. hydrolapathum*

fruits of *R. obtusifolius* the normal H-9 cell line showed the highest level of the apoptotic cells – at the concentration of 0.4 mg/mL we observed 79.5% of apoptotic cells, whereas in 1301 and EOL-1 cultures – 25.2 and 51.7%.

Analyzing the data using GraphPad InStat3 software, there was no significant relationship between cytotoxic activity and the total content of anthracene derivatives in the tested extracts ($p > 0.05$).

DISCUSSION AND CONCLUSION

Apoptosis is one of the main types of cell death, being the phenomena induced in malignant tumor cells of the blood-vascular system (lymphoma and leukemia), as a result of treatment with radio- and chemotherapy. On that account, the preparations stimulating the course of this process in the cancer cells and/or inhibiting this process in normal cells of the human body are sought after (29). In order to be

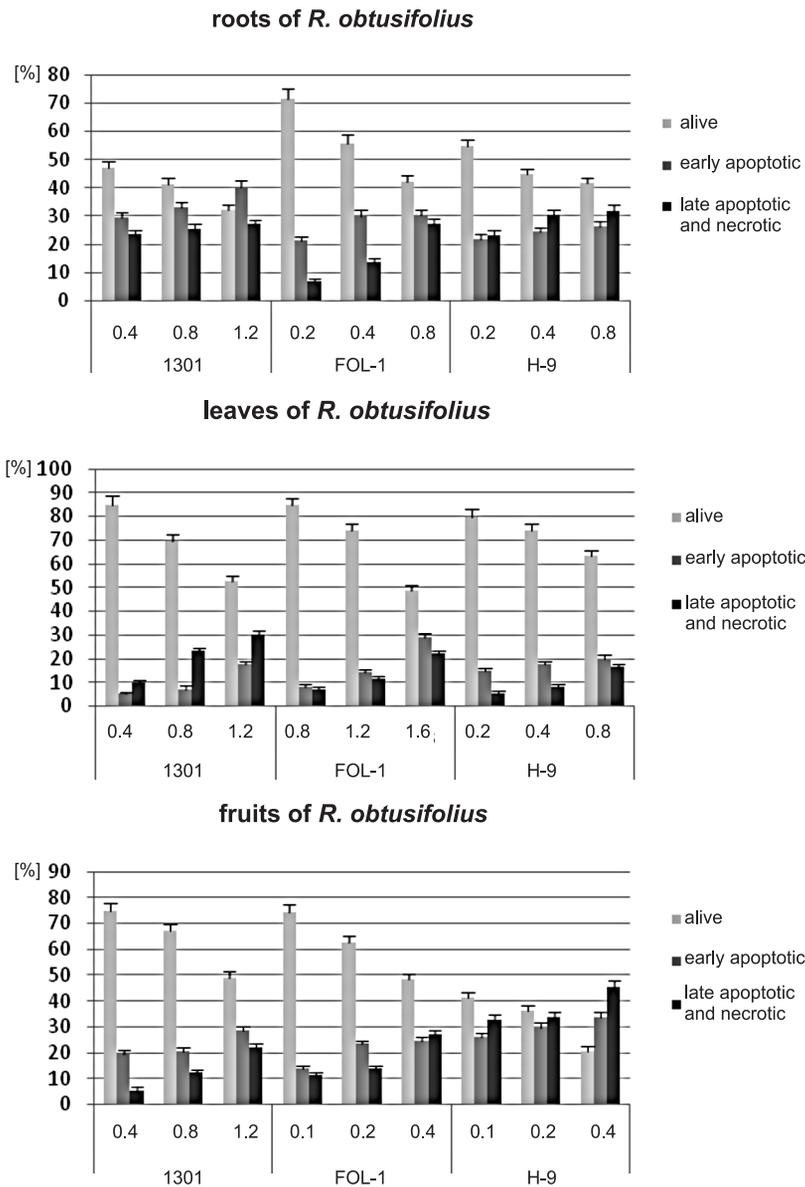


Figure 7. Comparison of the number of viable, early apoptotic and late apoptotic and necrotic cells in 1301, EOL-1 and H-9 cell line stimulated by different concentration (mg/mL) of ethanol extracts from roots, leaves and fruits of *R. obtusifolius*

successful in the fight against cancer, contemporary medicine was forced to seek new preparations, supporting this process, also in the phytotherapy.

The latest news present an assessment of cytotoxicity of various plant extracts, as well as the substances isolated from them. Up till now the effects of ethanol extracts from any species of the *Rumex* genus on human leukemia cell lines 1301 and EOL-1, as well as their impact on the normal T lymphocytes (H-9 cell line) have not been examined yet.

There are, however, sources confirming strong cytotoxic properties of the components that have been isolated from *R. acetosa* against other tumor cell lines. From CH₂Cl₂ fraction obtained from the methanol extract of the herb *R. acetosa* chrysophanol, physcion, emodin and emodin-8-O-β-D-glucopyranoside were isolated. They showed high cytotoxic activity against 5 human tumor cell lines: A549 lung cancer, ovarian SK-OV-3, central nervous system XF498, intestines HCT-15 and

MEL-2 melanoma. Among the tested compounds, emodin showed the best cytotoxicity against all tumor cell lines – the IC₅₀ values were: 3.32, 2.94, 3.64, 2.98 and 3.10 mg/ml for A549, SK-OV-3, XF498 SK-MEL-2 and HCT15 cell lines, respectively. For the other anthraquinones values of IC₅₀ (mg/mL) were as follows: for chrysophanol – 24.76 (A549), 7.28 (SK-OV-3), 5.83 (SK-MEL-2) and > 30.00 (XF498 and HCT15); physcion – 25.16 (A549) 22.41 (SK-OV-3), 26.07 (SK-MEL-2), 26.40 (XF498), 26.88 (HCT15) and emodyno-8-O-β-D-glucopiranoside > 30.00 (all lines), respectively. It has been also proved that the most active emodin exhibits anticancer effect by a nucleotide excision repair and to be effective against the leukemic mouse cell line L1210. High activity of emodin, compared to other isolated compounds may result from its chemical structure necessary for the cytotoxic action is the presence of -OH group at position C-6 of anthracene ring (30). Moreover, in recent studies emodin indicated impact on the process of proliferation and apoptosis in adriamycin-resistant leukemic cell lines HL-60/ADR, setting the value of IC₅₀ = 1.57 mg/mL (31). Other researchers also confirmed high antileukemic activity of the emodin isolated from methanol extract of *Polygonum hypoleucum* Ohwi (*Polygonaceae* family). Emodin is characterized by strong cytotoxic properties of many cell lines with the lowest IC₅₀ values against leukemic line: 1.5 ± 0.2 mg/mL (K562) and 2.8 ± 0.4 mg/mL (Raji). For other compounds IC₅₀ values against all cell lines were higher than 10 mg/mL (32).

When examining cytotoxic properties of dock species, two herbal preparations Essiac® and Flor-Essence®, which include herb of *Rumex acetosella* (5, 12), are worth mentioning. It has been also proved that those herbal tonics induced proliferation of T47D, MDA-MB-231 and MDA-MB-436 breast cell lines at concentrations ranging from 1 to 8%, regardless of the presence of the estrogen receptor (2). Recent studies have confirmed the properties of many cytotoxic compounds from roots of *Rumex crispus*, responsible for killing cells lines DLD-1 (human colon adenocarcinoma cell line) (33).

The present study provides evidence for cytotoxicity and induction of apoptosis by investigated *Rumex* species: *R. acetosa*, *R. acetosella*, *R. confer-tus*, *R. crispus*, *R. hydrolapathum* and *R. obtusifolius*. The detailed molecular mechanism for the anticancer effect of the extracts of fruits, which have the strongest cytotoxic activity, and the isolation and identification of its active components still need to be further investigated.

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