

NATURAL DRUGS

CYTOTOXIC EFFECT OF SOME MEDICINAL PLANTS FROM *ASTERACEAE*
FAMILY ON J-45.01 LEUKEMIC CELL LINE – PILOT STUDYMAGDALENA WEGIERA*, HELENA D. SMOLARZ, MARCIN JĘDRUCH, MAGDALENA KOR-
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Abstract: In this study the *in vitro* cytotoxic properties of ethanol extracts from the herbs, inflorescences and roots of selected *Asteraceae* species: *Arctium lappa*, *Artemisia absinthium*, *Calendula officinalis*, *Centaurea cyanus*, *Tanacetum vulgare* and *Tragopogon pratensis* on J-45.01 human acute T leukemia cell line was examined. All tested samples possess antileukemic properties and induce cells death *via* apoptosis. The correlation between antileukemic activity and total polyphenol content was determined.

Keywords: *Asteraceae*, J-45.01, leukemic cell line, cytotoxic properties, polyphenol content

The *Asteraceae* family occurs commonly in the world (about 25.000 species) and in Poland is represented by about 333 species. The species are a source of many biologically active compounds like essential oils (1–4), polyphenolic compounds (5, 6), flavonoids (5–11), terpenoids (8–10, 12–17), phenolic acids (7, 10, 18), alkaloids (19), lignans (7), saponins (8, 15, 20), stilbenes, sterols (10), polysaccharides (12) and many others.

Due to their bio-active properties plants from *Asteraceae* family are commonly used in treatment of various diseases. *Artemisia absinthium* L. (known as wormwood) is a well known in many countries for her healing properties. It acts insecticidally, antihelminthically (1, 13), antimalarially (14, 21), antiseptically (22) and shows anti-inflammatory and antioxidant properties (22). Wormwood has been also used for a long time in traditional medicines for cardiac stimulation (22), the treatment of gastric pain, to stimulate appetite, digestion (2, 21, 22) and to improve memory and mental function (22). *Arctium lappa* L. (burdock) is especially known in traditional Chinese medicine (7). This plant is a rich source of flavonoids and lignans and it is helpful in the treatment of inflammatory disorders. Moreover, it has been used as an antiaging, antioxidant, antimutagenicity, anticarcinogenicity and antitumoral remedy (7, 23). *Calendula officinalis* L. has found many medicinal applica-

tions both in Europe and in America (20). Many studies have shown that it possesses anti-inflammatory, antiseptic, antimicrobial, diaphoretic, antispasmodic and tonic properties and also has the ability to heal eruptive skin diseases and abrasions (15, 16, 20). Saponin-rich extract from *Calendula officinalis* was found to have cytotoxic and antitumoral activity (18). Anti-HIV properties of extracts from *Calendula officinalis* flowers were observed as well (9). Another species from the wide range of *Asteraceae* family, which has been found many applications both in folk and modern medicine, is *Tanacetum vulgare* L. Extracts from this species have been used for treating many health problems such as digestive disorders, rheumatism, fever and ulcers (12). It acts anti-inflammatorily (11, 12, 24) and also has been reported to have antimicrobial, antitumor and antioxidant properties (24). *Tragopogon pratensis* L. shows diaphoretic and antitussive properties (25).

As shown above, some species of the *Asteraceae* family are used as remedies for many health problems, including tumor diseases, which are the second most common cause of death.

The objective of this study was to examine the *in vitro* cytotoxic properties of ethanol extracts from the herbs, inflorescences and roots of the selected *Asteraceae* species: *Arctium lappa*, *Artemisia absinthium*, *Calendula officinalis*, *Centaurea*

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cyanus, *Tanacetum vulgare* and *Tragopogon pratensis* against J-45.01 human T cell leukemia cell line.

MATERIALS AND METHODS

Plant material

The different plant organs from selected species of the *Asteraceae* family were used in this study: roots and herbs of *Arctium lappa* (RAc, HAc), roots and herbs of *Artemisia absinthium* (RAt, HAAt), herbs and inflorescences of *Centaurea cyanus* (HCe and ICe) and roots, herbs and inflorescences of *Calendula officinalis* (RCa, HCa and ICa), *Tanacetum vulgare* (RTa, HTa and ITa) and *Tragopogon pratensis* (RTr, HTr and ITr). These abbreviations are consistently used in the text. The plant samples were collected from natural habitat at the end of June (herbs), July (inflorescences) and in the September (roots) of 2009, near Lublin (Poland). Voucher specimens were deposited at the Chair and Department of Pharmaceutical Botany, of the Medical University of Lublin (Poland).

Plant extraction

One gram of air-dried and powdered plant material was extracted with 35 mL of 70% aqueous methanol solution. The extraction was carried out for 1 h in boiling water bath using a cooler. The received extract was cooled down to room temperature and filtered to 100 mL volumetric flask. Then, the material remaining was extracted again with 70% aq. methanol solution in the same conditions as before. After filtering, the obtained mixtures were combined in volumetric flask and filled to 100 mL with distilled water (extract A). Extract A was used for phytochemical analysis to determine the total polyphenol content. A part of the extract A (50 mL) was evaporated and condensed in liquid nitrogen to dry residue (extract B, subject to biological assay).

Cell lines and culture medium

The J-45.01 cell line (Jurkat; human acute T leukemia cell line from ECACC, cat. no. 88042803) was used in this work. It was cultured by ECACC protocol at the concentration of 5×10^5 cells/mL. All cultures were grown in an incubator (Biotech) in humidified atmosphere of 5% CO₂, for 24 h at 37°C. 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 (100 U/mL), streptomycin (100 µg/mL) and amphotericin B (2.5 µg/mL) (Gibco, Carlsbad, USA).

Trypan blue assay

The J-45.01 cells in concentration 5×10^5 cells/mL were stimulated *in vitro* with various concentrations of ethanol extracts (0.04–1.0 mg/mL). The cells were incubated for 24 h at 37°C in humidified atmosphere of 5% CO₂. At the end of this period, the medium from each plate was removed by aspiration. Then, 10 µL suspension of the cells were incubated for 5 min with 10 µL of 0.4% trypan blue solution (Sigma). Thereafter, the presence of nonviable cells, which were dark blue and viable cells, which excluded the dye, was analyzed in Olympus BX41 microscope. The study was an average of 10 randomly selected fields in the preparation, so that the sum of all cells was not less than 200. The percentage of viable cells in controls was higher than 95%. The IC₅₀ doses (concentrations at which cell viability was 50%) were set by means of MS Excel spreadsheet, using data received from tree independent test.

Annexin V assay

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 and the culture medium was removed. Then, they 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 (12 µg/mL). Thereafter, samples were analyzed by an Olympus BX41 light and fluorescence microscope for the presence of viable cells (annexin V⁻/PI⁻); early apoptotic (annexin V⁺/PI⁻) and late apoptotic/necrotic cells (annexin V⁺/PI⁺). Fluorescein isothiocyanate-labeled annexin V could bind the outer membranes of apoptotic cells and discriminate apoptotic cells in the early stage from necrotic cells when used with propidium iodide. The extracts mediated apoptosis was expressed as the percentage of apoptotic cells/total cells. Cell morphology using a BX41 Olympus light and fluorescence microscope was also examined. Data were processed according to the MultiScan software. All the samples were analyzed at the concentrations close to IC₅₀ values or at the concentration with 50% of living cells in breeding.

Determination of total polyphenols

The determination of a total polyphenol compounds in the examined species was carried out

according to the spectrophotometric method (26). One mL of sample solution was added to 4 mL of distilled water and 1 mL of Folin-Ciocalteu reagent. After 3 min, 4 mL of Na_2CO_3 (70 g/L) was added. The absorption of the blue color was measured at 660 nm. The concentration of phenols in the sample was estimated from standard curve of gallic acid in the concentration range of 10–70 mg/L. Results were expressed as gallic acid equivalents (mg gallic acid equivalents/g dried extract). All samples were analyzed in six replications.

Statistical analysis

All results are expressed as the mean of three to six experiments. The relationship between antileukemic properties and the total polyphenol content was evaluated in two ways: graphically with MS Excel and statistically with GraphPad In Stat 3 software, where the Spearman rank correlation coefficient (r) was determined. Values of $p < 0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

The effect of ethanol extracts from different part of six species of *Asteraceae* family on J-45.01 human acute T leukemia cell line was examined. The cytotoxicity with trypan blue exclusion test was conducted in a range of concentrations (0.04–1.0

mg/mL) to determine the IC_{50} value (after 24 h stimulation). The received IC_{50} values are reported in Table 1.

The strongest antileukemic activity was found for the inflorescences of *Tanacetum vulgare*, for which the IC_{50} dose of 0.20 mg/mL was determined. Large cytotoxic activity was also recorded for the roots of *Calendula officinalis* ($\text{IC}_{50} = 0.23$ mg/mL) and for the herb of *Centaurea cyanus* ($\text{IC}_{50} = 0.25$ mg/mL). Similar antileukemic properties ($\text{IC}_{50} = 0.33$ – 0.38 mg/mL) were shown by the herbs and roots of *Arctium lappa*, *Tanacetum vulgare*, the herbs of *Artemisia absinthium*, *Tragopogon pratensis* and the inflorescences of *Calendula officinalis*. The largest IC_{50} value characterized the inflorescences of *Centaurea cyanus* (0.77 mg/mL).

Apoptotic effect of plant extracts was estimated by annexin V assay. Annexin V and propidium iodide stained the cells with impaired integrity of the plasma and nucleus membrane. The J-45.01 cells were stimulated by the extracts at a concentration close to the IC_{50} value. On the basis of the characteristic morphological changes in the stimulated leukemic cells intensive apoptotic response was observed.

In this experiment, after 24 h extract stimulation, we found similar number of living cells in all analyzed cell cultures (46.33–50.97%). The number of cells in the early stages of apoptosis ranged from

Table 1. IC_{50} values and total polyphenol content of ethanol extracts from *Asteraceae* family.

Plant sample	IC_{50} value [mg/mL]*	Total polyphenol content [mg/g gallic acid]**
RAc	0.35 ± 0.032	23.50 ± 1.766
RAt	0.60 ± 0.054	7.30 ± 0.904
RCa	0.23 ± 0.018	4.38 ± 0.787
RTa	0.30 ± 0.019	24.00 ± 2.472
RTr	0.64 ± 0.045	4.18 ± 0.854
HAc	0.34 ± 0.021	22.93 ± 1.271
HAt	0.35 ± 0.022	22.36 ± 1.393
HCa	0.41 ± 0.013	11.12 ± 1.255
HCe	0.25 ± 0.017	20.43 ± 3.106
HTa	0.36 ± 0.021	30.42 ± 2.051
HTr	0.38 ± 0.018	16.23 ± 1.608
ICa	0.33 ± 0.013	16.69 ± 0.929
ICe	0.77 ± 0.040	12.49 ± 1.335
ITa	0.20 ± 0.008	39.39 ± 3.790
ITr	0.66 ± 0.033	35.41 ± 2.432

* (n = 3), ** (n = 6)

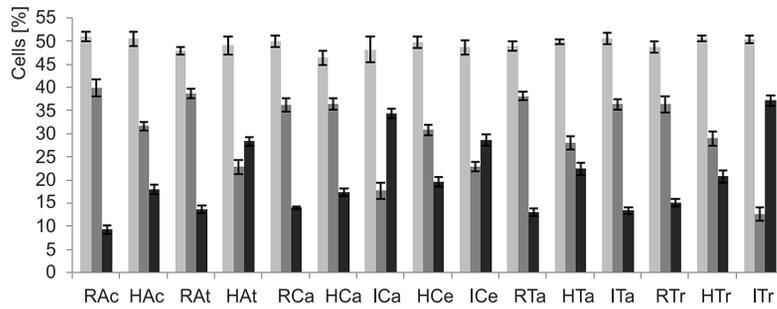


Figure 1. Amount of living (A), early apoptotic (E), late apoptotic and necrotic (L/N) J-45.01 cells after 24 h stimulation at IC_{50} values. The number of cells was determined by the annexin V-FITC labelled and propidium iodide test. Data are the means of 3 different experiments and are expressed as the percentage of the control

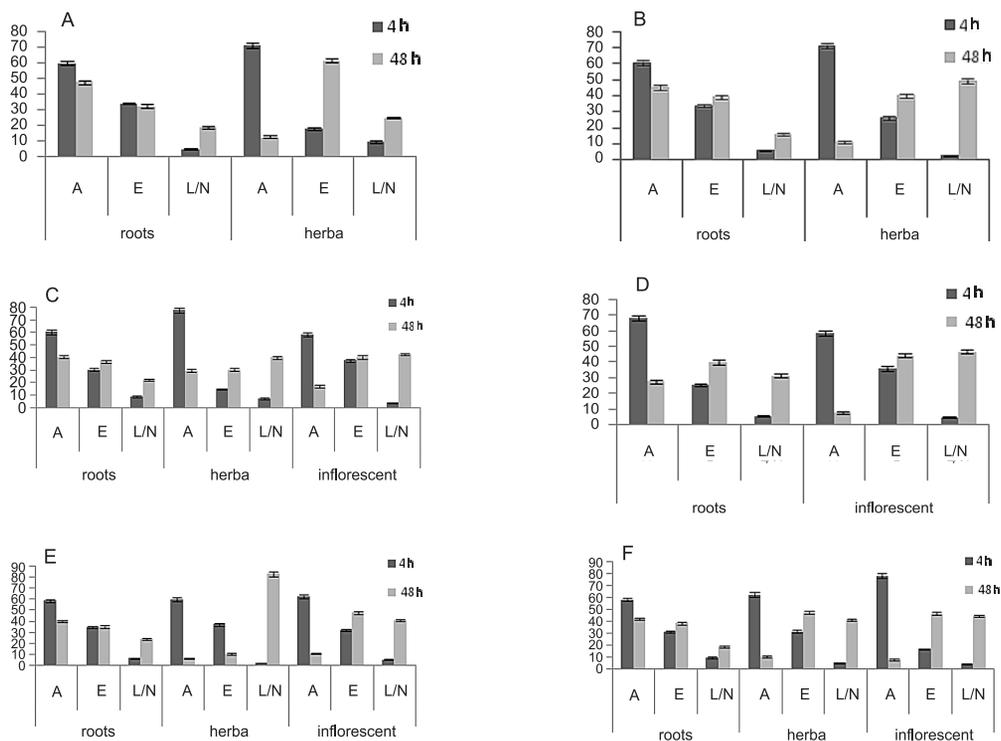


Figure 2. Amount of living (A), early apoptotic (E), late apoptotic and necrotic (L/N) J-45.01 cells after 4 and 48 h stimulation at IC_{50} values. Description: A) *A. lappa*, B) *A. absinthium*, C) *C. officinalis*, D) *C. cyanus*, E) *T. vulgare* and F) *T. pratensis*. The number of cells was determined by the annexin V-FITC labelled and propidium iodide test. Data are the means of 3 different experiments and are expressed as the percentage of the control

36.20 to 39.90%, while the number of cells in the late stage apoptotic and necrotic cells were differentiated (average value for all samples was 20.27%). The percentage of early and late apoptotic J-45.01 cells observed for the investigated extracts are demonstrated in Figure 1.

The proportional time-dependent decrease in the number of living cells and the proportional increase in the number of the early apoptotic, late

apoptotic and necrotic cells, in time-dependent manner, better visible after 4 and 48 h stimulation, is shown in Figure 2.

Comparing the differences between the number of cells at different time intervals it was determined that programmed cell death in many cases does not proceed rapidly, but in a mild way. In most cases the number of late apoptotic and necrotic cells after 48 h of stimulation was about 2–2.5 times high-

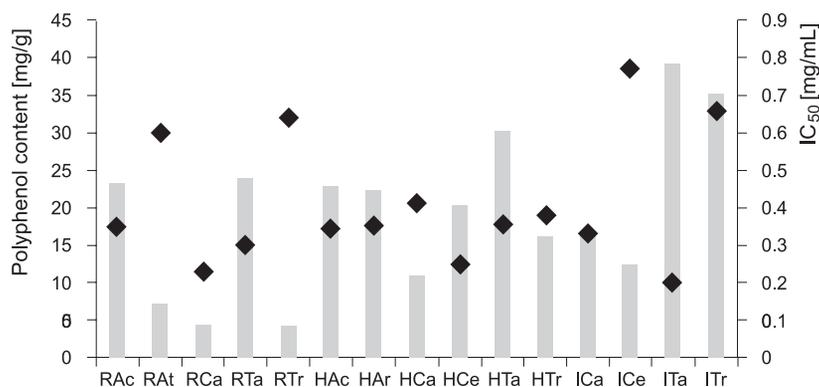


Figure 3. Correlation between total polyphenol content [mg gallic acid equivalents/g dried extract – columns] and antileukemic properties – IC₅₀ value [mg/mL – points].

er than after 4 h. Approximately 10-fold increase was observed for the inflorescences of *Calendula officinalis*, *Tragopogon pratensis* and *Centaurea cyanus*. A very large jump (more than 30 times) of the amount of late apoptotic and necrotic cells in culture is distinguished for the extract from the herb *Tanacetum vulgare* (2.7% after 4 h and 82.5% after 48 h) (Fig. 2E). Over 20-fold increase was observed for the herb of *Artemisia absinthium* (Fig. 2B). It is noted, that all extracts from the roots are characterized by the similar number of cells in the early stages of apoptosis (30.45–38.85%), weakly depending on time. In J-45.01 culture, after 48 h of stimulation, extracts from inflorescences of *Tragopogon pratensis* caused a very large drop in the number of viable cells (89%) compared to the reading at 4 h (Fig. 2F). For the roots of *Artemisia absinthium*, *Calendula officinalis*, *Tanacetum vulgare* and *Tragopogon pratensis*, after 48 h, a similar number of live cells and cells in the early stages of apoptosis (about 40%) was found in all test. Indeed, the apoptotic cells (compaction and margination of nuclear chromatin, cytoplasmic condensation and membrane blebbing and cell shrinkage) were observed.

In the next stage of investigations, the total content of polyphenol compounds was detected according to the Singleton and Rossi method. The highest content of polyphenol compounds among all tested extracts (inflorescence, herbs and roots) was found for *Tanacetum vulgare* (39.39, 30.42 and 24 mg/g gallic acid, respectively) (Table 1). High level of polyphenols was recorded also in the inflorescences of *Tragopogon pratensis* (35.41 mg/g gallic acid) and it is similar to the total phenolic content

examined for the aerial parts (leaves and flowers) for the other *Tragopogon* species: *T. budjnurdense*, *T. hololeucum*, *T. chiliophyllum*, *T. sonboli*, *T. tabrisianum* and *T. kotschy* (32.15, 47.11, 37.24, 32.60, 28.90 and 44.40 mg/g gallic acid, respectively) (27). Quite high amount of polyphenol compounds was also detected in roots and herbs of *Arctium lappa* (23.50 and 22.93 mg/g gallic acid) and herbs of *Artemisia absinthium* (22.36 mg/g gallic acid).

For evaluation of relationship between antileukemic activity and the total content of polyphenol compounds in the studied extracts, the MS Excel spreadsheet and statistic program GraphPad In Stat3 were used. Graphical comparison of the results showed a clear positive relationship between the highest polyphenol content and the strongest cytotoxicity (low IC₅₀ value) only for the inflorescences of *Tanacetum vulgare* (Fig. 3). The opposite correlation (small polyphenol content and weak antileukemic properties) was observed for the roots of *Artemisia absinthium* and *Tragopogon pratensis*. According to GraphPad In Stat3, p-values of less than 0.05 were considered significant.

In conclusion, the screening of plant extracts for their cytotoxic and apoptotic properties has shown that medicinal herbs from Asteraceae family might have also antileukemic importance.

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