Some immunotropic effects of *Rhaponticum carthamoides*

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Abstract

Rhaponticum (leuzea) carthamoides (Compositae) has been used for many years in traditional Chinese, Tibetan, Mongol and Russian medicine as an adaptogenic and anabolic remedy. Some other biological effects of this plant were also described. This paper presents an influence of hydroalcoholic extract of this plant on some aspects of cellular and humoral immunity in mice. Additionally, the effect of this extract on the neovascular reaction induced in mice skin after grafting of cells isolated from human kidney cancer was evaluated. Oral administration of the extract for 5 days, in daily doses of 0.25 mg per mouse, resulted in an increase of chemokinetic activity of splenic lymphocytes and anti-SRBC antibody production. No influence of the extract on metabolic activity of mice blood granulocytes nor on the angiogenic activity of human kidney cancer cells were observed.

Key words: *Rhaponticum carthamoides, Leuzea, mice, immunity, human cancer, angiogenesis.*

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Introduction

Rhaponticum (*leuzea*) *carthamoides* (*Compositae*) has been used for over 5000 years in traditional Chinese, Tibetan, and Mongol medicine, mainly as an adaptogenic and anabolic remedy. This plant, which grows in southern Siberia, has also been used for a long time in Russia. Extract of this plant was included in the Russian pharmacopeia in 1961 as a natural remedy for improving physical and mental performance, as well as for improving recovery following illness. Various types of extracts and individual compounds, isolated from this plant have been found to possess pharmacological and biological effects [1-7]. However, information about its immunotropic activity is scarce [8-11].

This paper presents an influence of hydroalcoholic extract of this plant on some aspects of cellular and humoral immunity in mice. Additionally, the effect of this extract on the neovascular reaction induced in mice skin after grafting of cells isolated from human kidney cancer was evaluated.

Material and methods

The study was performed on 4-8 weeks old female and male inbred Balb/c mice, 20-22 g of body mass, delivered from the Polish Academy of Sciences' breeding colony.

For all experiments the animals were handled according to the Polish animal protection law and NIH (National Institutes of Health) standards. All experiments were accepted by the local Ethical Committee.

Preparation and administration of the extract

Rhaponticum carthamoides plants were cultivated, their rhizomes collected and identified in the Warsaw University of Life Sciences-SGGW. Preparation of 70% alcoholic extract and its chemical analysis was done as previously described [12].

Mice were fed the extract (0.25 mg daily) in 10% ethyl alcohol or 10% alcohol only (controls), in 40 µl volume, with the aid of Eppendorff pipette. This dose corresponds to 125 mg given to a person weighting 70 kg (applying the

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coefficient of 7 for adjusting differences between mice and human in relation of the surface to body mass). In immunological experiments, mice were fed the extract for 5 days, then some mice were sacrificed and their blood and spleen were collected for further preparation. The others were injected intraperitoneally with 0.2 ml of 10% sheep red blood cells (SRBC) suspension. In experiments with human cancer cells, mice were fed extract (or 10% alcohol) for 3 days after intradermal cells grafting.

Spleen cells chemokinesis (spontaneous migration) assay *in vitro* was performed according to the Sandberg method [13] with a modification [14, 15]. Briefly, splenocytes were isolated from spleens under sterile conditions by straining through stainless sieve and cotton gauze and centrifugation on Lymphoprep in order to remove erythrocytes. Isolated splenocytes were resuspended in Parker culture medium with 5% inactivated FCS, at the final concentration of 30×10^6 cells/ml. Afterwards, siliconized capillary tubes were filled with cell suspension, sealed with plasticine, centrifuged (5 min 450 g) and fixed on the glass plates. Cells levels were marked. After 24 h incubation (37˚C, 5% CO_2 humidified atmosphere) the distances of migration were measured in milimeters (mm) at a magnification of 6.5 × and presented as migration units (1 MU = 0.18 mm).

Chemiluminescence test (CL)

Mice were bled in anaesthesia from retro-orbital plexus and sacrificed with Morbital. CL was measured using the method of Easmon and Cole with some modifications [16] at room temperature, in scintillation counter (RackBeta 1218, LKB, Sweden). Briefly: samples of 0.05 ml heparinised blood were diluted 1 : 4 with PBS (Biomed Lublin, Poland) supplemented with 0.1% BSA (Sigma-Aldrich, USA) and 0.1% glucose (Polfa, Poland). Next, 0.05 ml of this diluted blood was mixed with 0.2 ml of luminol (Sigma-Aldrich, USA) solution (10^{-5} M) in PBS and placed in a scintillation counter in the "out of coincidence" mode for background chemiluminescence measurement. Then, the cells were activated by addition of 0.02 ml solution of opsonised zymosan (10 mg/ml) and chemiluminescence activity was measured for the next 15 min. Counting of leukocytes and blood smears examination was performed by routine methods and the results were shown as the maximum value of chemiluminescence (cpm) obtained for 10^3 granulocytes.

Study of antibody production

Mice were bled in anaesthesia (3.6% chloral hydrate), from retroorbital plexus, 7 days after immunization. The antibody level was evaluated with haemagglutination assay in inactivated (56˚C, 30 min) sera. After performing a series of sera dilutions, 0.5% SRBC were added and the mixture was incubated for 60 min at room temperature, then centrifuged (10', 150 g) and shaken (but not stirred). The hemagglutination titer was evaluated in a light microscope – as the last dilution in which at least 3 cell conglomerates were present in at least 3 consecutive fields at objective magnification 20× and the results were presented as log titers.

Tumor-induced angiogenesis test (TIA)

Material was obtained surgically from the patient with kidney tumor (Ca.clarocellulare). About 5 g of tumor tissue was dispersed mechanically in a sterile ice-cold PBS and subjected to enzymatic digestion by use of collagenase 0.1 mg/ml (Sigma) and DNA-se 0.001 mg/ml (Serva) dissolved in PBS for 45 min on magnetic shaker in room temperature. Then, obtained suspension was filtered through a sieve, washed twice in PBS and suspended in Parker culture medium in concentration of 5×10^6 cells per ml. Viability of tumor cell suspension as assessed by trypan blue exclusion test was about 80%.

Cutaneous angiogenesis assay was performed according to [17] with own modifications [18, 19]. Briefly, multiple 0.05 ml samples of cell suspension were injected intradermally into partly shaved, narcotised Balb/c mice (3-4 mice per group, 4-6 injections per mouse). In order to facilitate the localisation of injection sites later on, the suspension was coloured with 0.1% of trypan blue. On the day of grafting and on the following two days mice were fed tested substance, or 10% ethyl alcohol as a control. After 72 hours mice were sacrificed with lethal dose of Morbital. All newly formed blood vessels were identified and counted in dissection microscope, on the inner skin surface, at magnification of 6×, in 1/3 central area of microscopic field. Identification was based on the fact that new blood vessels, directed to the point of cells injection, differ from the background vasculature in their tortuosity and divarications. All experiments were performed in anaesthesia (3.6% chloral hydrate, 0.1 ml per 10 g of body mass).

Statistics

Statistical analysis was performed by unpaired *t* test with Welch correction (GraphPad InStat3).

Results

The results of the influence of *Rhaponticum* on antibody production are presented on the Figure 1. Treatment of mice for 5 days before antigen (SRBC) injection significantly increased the antibody titer of their sera, in comparison with the controls.

Figure 2 presents the effect of feeding mice *Rhaponticum* extract on chemokinetic activity of lymphocytes isolated from their spleens. Migratory activity of these cells *in vitro* significantly increased in comparison with the controls.

We have not observed differences in the number and chemiluminescent activity of blood leukocytes between the

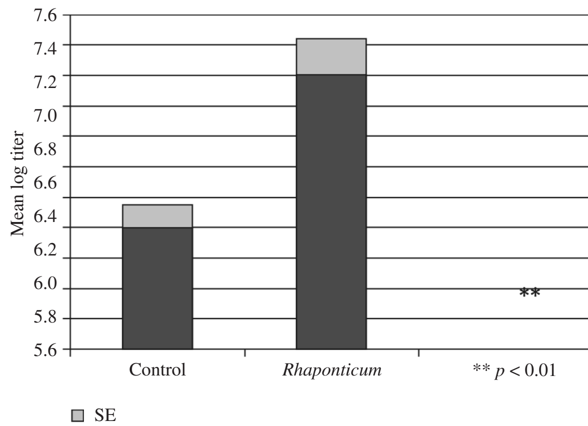

Fig. 1. The effect of *Rhaponticum carthamoides* extract on the ability of mice to produce anti-SRBC antibodies (11 control sera and 11 sera collected from *Rhaponticum*-treated mice)

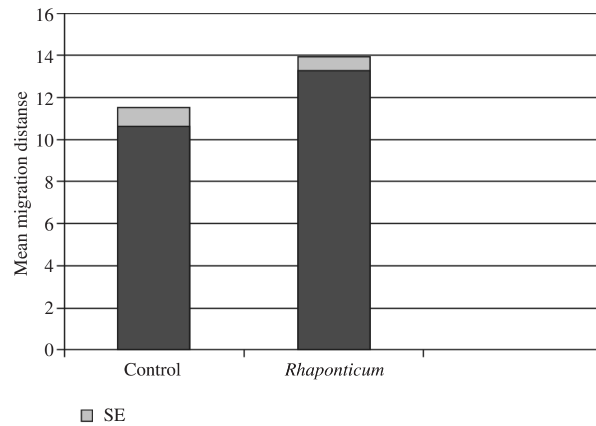

Fig. 2. The effect of feeding mice *Rhaponticum* extract on the chemokinetic activity of their splenic lymphocytes (**$p < 0.01$)

control and *Rhaponticum*-treated mice. The corresponding values were for leukocytes number 4470 ±1083 (controls) and 5430 ±348 (*Rhaponticum*-treated animals); for CL activity: 39335 ±10458 (controls) and 33775 ±11026 (*Rhaponticum*-treated mice). We also have not observed any influence of *Rhaponticum* extract on the neovascular reaction induced in mice skin after grafting of human cancer cells isolated from the tumor of kidney (12.7 ±0.37 versus 12.83 ±0.48).

Discussion

In this paper we present for the first time the ability of *Rhaponticum carthamoides* to increase chemokinetic activity of lymphocytes isolated from the spleens of mice, which were fed extract of *Rhaponticum* rhizomes, as well as its stimulatory effect on antibody production in mice. These results indicate that an extract of *Rhaponticum carthamoides* contains some immunostimulatory substances. The main chemical groups of compounds present in this plant are steroids, particularly ecdysteroids, and phenolics (flavonoids and phenolic acids, responsible for antioxidant action). However, the unique biological activity of crude extracts is probably the result of synergistic action of various compounds [20, 21].

Some experiments performed *in vitro* with human blood lymphocytes indicate that the polysaccharide fraction of *Rhaponticum* leaves markedly enhanced their proliferative response to mitogen PHA. The same authors reported inhibition of the generation of oxygen free radicals by human granulocytes *in vitro*, in the presence of *Rhaponticum* carthamoides extract [9, 10].

Granulocytes provide the first line of defence against microbial pathogens and they have been shown to have the capacity to kill various kinds of them. The most important element of the killing process is generation of reactive oxygen species during the oxidative burst. The production of free oxygen radicals is a critical component of destructive mechanisms of phagocytic cells and is of great importance for protecting against infectious diseases. This process leads to the emission of light proportional to free radical quantity-chemiluminescence (CL). Hence, adequate and safe immunostimulatory remedy administered *in vivo*, should not inhibit production of oxygen free radicals by granulocytes. In our present experiments, *in vivo* administration of *Rhaponticum* extract had no influence on blood leukocytes number and their chemiluminescent activity.

It has been described with regards to healthy people, that *Rhaponticum* preparations increased the threshold of organism defense to various stimuli. Azizov reported an effect of *Leuzea* tincture on humoral immunity of runners for distances of 5000 and 10000 m. In these people, intensive physical activity induced significant decrease of IgG, IgA and C3 component of complement in blood serum. 20-day administration of *Leuzea* contributed to restoration of the lowered immunological parameters [8].

As angiogenesis is very important for tumor growth and metastasis formation, a proper immuno-stimulator should not enhance angiogenic activity of tumor cells. Our experiments performed with kidney cancer cells have revealed no stimulatory influence of *Rhaponticum* extract on the intensity of neovascular reaction induced by these cells in the skin of mice.

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