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Cytotoxic and antioxidant activity of *Achillea alexandri-regis*

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The cytotoxicity and antioxidant properties of herb extracts of *Achillea alexandri-regis* were studied. Combined chloroform and ethylacetate extracts exhibited a pronounced cytotoxic effect against HeLa cancer cells ($IC_{50} = 25.92 \pm 4.96$ μg/ml), and lower cytotoxicity against K562 leukemia cells ($IC_{50} = 48.59 \pm 18.31$ μg/ml). The methanol extract was found to be a moderately cytotoxic *in vitro* agent against HeLa and K562 cells. No suppressive activity was detected on non-malignant peripheral blood mononuclear cells (PBMC). The antioxidant activity of the methanol extract was assessed by DPPH radical scavenging. The methanol extract of *A. alexandri-regis* showed concentration dependent DPPH radical scavenging activity with $IC_{50} = 36.14 \pm 0.05$ μg/ml.

Achillea alexandri-regis Bornm. & Rudski is a stenoendemic species of Serbian flora, found only in its *locus classicus*, the Ošljak mountains which form the northern part of the Šara mountains (southern Serbia) (Gajić 1976). Previous pharmacological studies showed that a methanol extract of the aerial parts has a significant dose dependent anti-inflammatory effect in the carrageenan-induced rat paw edema test. Also, pre-treatment with the methanol extract prevented experimental ulceration induced by ethanol (Kundaković et al. 2000). The herb extract inhibited lipid peroxidation and showed hydroxyl and superoxide radical scavenging activity in various biological systems (Kundaković et al. 1998), as well as weak antimicrobial activity (Mirković et al. 1999).
The composition of the essential oil was determined by Ristić et al. (1996). Our latest chemical analysis of the aerial parts showed the presence of triterpenoids, flavonoids, phenolic acids and lignans (Kundaković et al. 2003).
The aim of the present study is a further evaluation of the pharmacological properties of the endemic plant *A. alexandri-regis*. Thus, its cytotoxicity on human cervix adenocarcinoma cells (HeLa), and leukemia K562 cells, as well on non-malignant peripheral blood mononuclear cells (PBMC) was studied. Its radical scavenging activity was further evaluated using the DPPH test. The results are presented in the Table 1.
The MTT-microculture tetrazolium assay was used to evaluate the cytotoxicity against the K562 leukemia cells and PBMC, while the KBR spectrophotometric assay was used to test the cytotoxicity of the combined chloroform and ethylacetate extracts, as well the methanol extract against HeLa cells.
Our results show that the combined chloroform and ethylacetate extracts exhibited a pronounced cytotoxic effect against HeLa cancer cells with an $IC_{50} = 25.92 \pm 4.96$ μg/ml. Lower cytotoxicity was observed against K562 leukemia cells ($IC_{50} = 48.59 \pm 18.31$ μg/ml). Triterpene diol 3-O-fatty acid esters have recently been identified in combined chloroform and ethylacetate extracts (Kundaković et al. 2003). The triterpene diols present, although as 3-O-fatty acid esters' could be considered to be active principles. Their anti-tumor promoting and cytotoxic effects have been reported by Ukiya et al. (2002).
The methanol extract was found to be a moderate *in vitro* cytotoxic agent against HeLa cells with an ($IC_{50} = 158.50 \pm 15.50$ μg/ml), and K562 cells ($IC_{50} > 122.2$ μg/ml).
There is a certain selectivity in toxicity against malignant and healthy cell lines. Both the combined chloroform and ethylacetate extracts, and the methanol extract showed no suppressive activity against normal PBMC cells before or after activation with phytohaemaglutinin (PHA) (AC: $IC_{50} > 200$ μg/ml; AM: $IC_{50} > 316$ μg/ml).
The antioxidant activity of the methanol extract was further assessed by DPPH radical scavenging. The methanol extract of *A. alexandri-regis* showed concentration dependent DPPH radical scavenging activity. The highest DPPH radical scavenging activity (90%) was observed at a concentration of 0.1 mg/ml. Fifty percent of DPPH scavenging activity was obtained with an $IC_{50} = 36.14 \pm 0.05$ μg/ml. This is in agreement with a recent study of Candan et al. (2003) that evaluated the water-soluble part of the methanol extract of *Achillea millefolium* subsp. *millefolium*. For this extract, DPPH scavenging activity was also obtained at high concentrations with an $IC_{50} = 45.60 \pm 1.30$ μg/ml. Compared to the reference compounds, ascorbic acid ($IC_{50} = 4.09 \pm 0.08$ μg/ml) and rutin ($IC_{50} = 2.5 \pm 0.1$ μg/ml), the methanol extract has lower antioxidative capacity.
The pharmacological effects demonstrated, namely anti-inflammatory, antiulcer and antioxidant activity, as well as its low toxicity, attributed to complex chemical composition make *A. alexandri-regis* an interesting subject for further studies.

Table 1: Cytotoxicity and DPPH radical scavenging activity of *Achillea alexandri-regis* herb extracts (IC_{50} μg/ml ± S. D.).

Extract or compound	Cytotoxicity			DPPH test
	HeLa cells	K562	PBMC(+/−PHA)	
AC	25.92 ± 4.96	48.59 ± 18.31	> 200	–
AM	158.50 ± 15.50	122.2	> 316	36.14 ± 0.05
Ascorbic acid	–	–	–	4.09 ± 0.08
Rutin	–			2.5 ± 0.1

Experimental

Whole plant *A. alexandri-regis* was collected in July 1995 on Mount Ošljak (Serbia). A voucher specimen is preserved in the Institute of Botany Herbarium, Botanical Garden, University of Belgrade (BEOU, No. 8402). The dried, aerial parts of *Achillea alexandri-regis* (280 g) were rinsed with acetone to remove resin from the plant surface. The plant material was then dried and ground into powder. Extraction was performed on a Soxhlet apparatus with petroleum ether, toluene, chloroform, ethylacetate, methanol and 50% methanol (V/V). The solvents were evaporated under reduced pressure at 40 °C. All extracts were analyzed by TLC. For cytotoxicity and antioxidant activity evaluation, chloroform and ethylacetate extracts (AC) and methanol and 50% methanol extracts (AM) were combined.

1. *In vitro* cytotoxicity assays

Stock solutions of AC and AM extracts were prepared in dimethyl sulfoxide (DMSO) at concentrations of 6.5 mg/ml and 25 mg/ml, respectively. The solutions were filtered through a 0.22 μm Millipore filter before use, and diluted with nutrient medium to various working concentrations. The nutrient medium was RPMI 1640 medium, without phenol red, supplemented with L-glutamine (3 mM), streptomycin (100 μg/ml), and penicillin (100 IU/ml), 10% heat inactivated (56 °C) fetal bovine serum, FBS and 25 mM HEPES. The pH of the nutrient medium was adjusted to 7.2 with bicarbonate solution. All reagents were Sigma products.

Human cervix adenocarcinoma HeLa cells were cultured as monolayers in the nutrient medium, while human myelogenous leukemia K562 cells were maintained as a suspension culture. The cells were grown at 37 °C in a 5% CO_2 and humidified air atmosphere. HeLa cells were seeded (2,000 cells per well) into 96-well microtiter plates and 20 h later, after the cell adherence, five different concentrations of the test extracts were added to the wells. The extracts under investigation were added to suspensions of leukemia K562 cells (3,000 cells per well) two hours after cell seeding. The final concentrations of extracts were the same in both HeLa and K562 cell cultures.

Peripheral blood mononuclear cells (PBMC) were separated from whole heparinized blood from a healthy volunteer by Lymphoprep^{TM} gradient centrifugation. Interface cells, washed three times with Haemaccel (aqueous solution supplemented with 145 mM Na^+, 5.1 mM K^+, 6.2 mM. Ca^{2+}, 145 mM Cl^- and 35 g/L gelatin polymers, pH = 7.4) were counted and resuspended in nutrient medium. PBMC were seeded (150,000 cells per well) into nutrient medium or in nutrient medium enriched with (5 μg/ml) phytohaemaglutinin, PHA (Wellcome) in 96-well microtiter plates. Two hours after seeding, the extracts were added to the wells, in triplicate, to five different final concentrations.

All experiments were done in triplicate, in two independent experiments. Nutrient medium was added to the control wells. Nutrient medium with corresponding concentrations of extracts, but without cells was used as a blank.

HeLa cell survival was determined using the Kenacid BlueR (KBR) dye binding method (Clothier 1995). After 72 h of continuous extract exposure, the target cells were washed twice with PBS and then fixed with a mixture of methanol and acetic acid (3 : 1). The fixed samples were stained with 0.04% KBR and washed. The dye bound to the cellular proteins was dissolved in 1 M sodium acetate in 70% ethanol. Absorbance of the dissolved dye was measured at 570 nm 2 h later.

Survival of K562 cells and PBMC was determined by the MTT test (Ohno and Abe 1991), 72 h after addition of agents. MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, was dissolved (5 mg/ml) in phosphate buffer saline (pH 7.2) and filtered through a 0.22 μm Millipore filter, before use. Briefly, in the MTT test, 20 μl of MTT solution (5 mg/ml PBS) was added to each well. Samples were incubated for four hours at 37 °C in a 5% CO_2 and humidified air atmosphere. Then, 100 μl of 10% SDS (sodium dodecylsulfate) was added. Absorbance at 570 nm was measured the next day.

To calculate the percentage of cell survival in both tests, the absorbance of a sample with cells grown in the presence of various concentrations of the investigated agent was divided by the control absorbance, and multiplied by 100. The absorbance of the blank control was always subtracted from the absorbance of the corresponding sample. IC_{50} concentration was defined as the concentration of an agent inhibiting cell survival by 50%, compared to the control.

2. *DPPH test*

DPPH radical scavenging activity of methanol extract was measured according to the method described by Cuendet et al. (1997). Dry methanol extract was dissolved in methanol to concentrations of 0.05–10%. Sample solutions (4 ml) containing the methanol extract at different concentrations were mixed with 1 ml of 0.5 mmol/l DPPH (Sigma) solution in methanol, and allowed to react in the dark at room temperature. The absorbance was measured at 517 nm after 30 min and the percent age activity was calculated. Dose-response curves were constructed and IC_{50} values were calculated. All measurements were performed in triplicate. Ascorbic acid (La Chema) and rutin (Fluka) were used as reference compounds.

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