Original Article

Ethyl Acetate Extracts of Two *Artemisia* Species: Analyses of Phenolic Profile and Anticancer Activities Against SW-480 Colon Cancer Cells

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Abstract

Because *Artemisia vulgaris* L. and *Artemisia alba* Turra are traditional medicinal plants used for the treatment of different diseases, we evaluated the cytotoxic/apoptotic activity of ethyl acetate extracts from these natural products against human colon cancer cells SW-480. The extracts contained a large amount of the total polyphenols and flavonoids. The phenolic profile showed the presence of phenolic acids (gallic, *p*-coumaric, vanillic, and ferulic acids) and flavonoids (rutin, myricetin, luteolin, quercetin, and apigenin). 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay indicated that antiproliferative activities of both *A. vulgaris* and *A. alba* extracts increased with the extension of time exposure, with decreasing IC_{50} values. Mitomycin C (MMC) alone had antiproliferative activity, but in combination with plant extracts caused stronger effect with lower IC_{50} values. Flow cytometry analyses showed that *A. alba* extract induced higher percentage of SW-480 cells in the early stage of apoptosis (33.5 ± 1.6 vs 0.7 ± 0.1, $P < 0.05$), whereas the *A. vulgaris* extract significantly increased the percentage of cells in necrosis (82.4 ± 5.0 vs 53.9 ± 2.3, $P < 0.05$). In conclusion, *A. alba* extract can be considered a potential source of bioactive components with anticancer activity or be used as a dietary food supplement or supplement to chemotherapy due to its synergistic effect with the MMC.

Keywords

Artemisia vulgaris, *Artemisia alba*, polyphenols, flavonoids, cytotoxicity, apoptosis

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The genus *Artemisia* is a diverse genus of plants of about 500 species[1] which due to their healing properties have a long history in the treatment of human ailments in various parts of the world. The flora of Europe is represented by 60 species of this genus, whereas in the flora of Serbia there are already 9 species.[2] They are characterized by significant quantitative and qualitative composition of secondary metabolites that exhibit a wide range of biological effects and contribute to their practical application, which are known in folk medicine, as well as in the pharmaceutical and food industries.[3]

Artemisia species are used for the treatment of different diseases such as hepatitis,[4] malaria,[5] and different cancers.[6] Previous pharmacological investigations of *Artemisia* species reported that these plants contain biologically active compounds such as flavonoids, coumarins, monoterpenes, lactones, sterols, and sesquiterpene lactones.[7,8] The aim of this study was the comparative analysis of anticancer and apoptotic activities of ethyl acetate extracts obtained from *Artemisia vulgaris* L. and *Artemisia alba* Turra plants against

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Table 1. Total Phenolic (Milligram of Gallic Acid Equivalent Per Gram of Extract) and Total Flavonoid Content (Milligram of Rutin Equivalent Per Gram of Extract) in Ethyl Acetate Extracts of Plants.

Plant material	Total phenolic content	Total flavonoid content
A. vulgaris	57.5 ± 0.6	131.7 ± 0.9
A. alba	39.2 ± 0.3	50.9 ± 0.4

Table 3. IC_{50} Values of the Investigated Extracts and Mitomycin C (MMC) in Individual Treatments and Co-treatments.

	IC_{50} (μg/mL)			
	Treatment		Co-treatment	
	24 h	72 h	24 h	72 h
A. vulgaris	>500	248.4	>500	66.7
A. alba	169.4	4.4	149.2	24.3
MMC	427.4	71.0	-	-

SW-480 colon cancer cells regarding the quantitative and qualitative content of phenolic compounds of extracts. In order to define a phytochemical profile, total phenolic and flavonoid contents were determined using spectrophotometric method, and chromatographic analysis of phenolic compounds was determined using high-performance liquid chromatography (HPLC) method. The results of the total amount of phenolic and flavonoid contents in ethyl acetate extracts of *A. vulgaris* and *A. alba* are given in Table 1.

Four phenolic acids (gallic, vanillic, *p*-coumaric, and ferulic) and 5 flavonoids (rutin, myricetin, luteolin, quercetin, and apigenin) were identified and quantified by HPLC method and their concentrations in *A. vulgaris* and *A. alba* extracts are shown in Table 2. Some other authors also analyzed the chemical constituents of these extracts.[9,10]

Plant extracts exert multifunctional effects on cancer cells, among which it is very important whether those extracts possess cytotoxic character or not. The cytotoxic activities of ethyl acetate extracts obtained from *A. vulgaris* and *A. alba* were determined by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay and expressed by IC_{50} values. The results are presented in Table 3 and Figure 1. Considering individual treatments, we found that *A. alba* extract exerted a more significant cytotoxic activity than *A. vulgaris*. To evaluate the potential cytotoxic activity, mitomycin C (MMC) and *A. vulgaris* or *A. alba* extracts were concomitantly added to the cell culture. The data show that the exposure of the SW-480 cell line to the extracts and MMC significantly reduced the IC_{50} values, to 24.3 μg/mL with *A. alba* +MMC and 66.7 μg/mL with *A. vulgaris* + MMC. Both *A. vulgaris* and *A. alba* extracts lowered cell viability in a dose- and time-dependent manner.

Plants and their bioactive substances are mainly used in combination with cytostatic, because of its positive effects on health and potential role in the prevention and/or treatment of cancer.[11] These combined treatments may increase the efficacy of treatment, simultaneously decreasing the side effects of cytostatic. One of the frequently used antitumor cytostatic for the treatment of different cancers (breast, cervical, colorectal, pancreas, lung) is MMC.[12] For these reasons, *Artemisia vulgaris* and *A. alba* extracts and low dose of MMC were investigated for possible synergistic effects. Combinational treatment results, expressed via combination index (CI) value, showed better synergistic effect for *A. vulgaris* compared to *A. alba* extract. After 24 hours, *A. alba* and *A. vulgaris* extracts exerted synergistic effects in the concentration range of 50 to 500 μg/mL, whereas after 72 hours the synergistic effects were detected only in the highest concentrations (250 and 500 μg/mL). CI values indicate

Table 2. Chemical Composition of Ethyl Acetate Extracts of *A. vulgaris* and *A. alba* ($\times 10^{-1}$ μg/L extracts).

Compounds	Time (min)	*A. vulgaris*	*A. alba*	*t* test (t_{crit} 4.3026)
Gallic acid	5.11	6.6 ± 0.4	5.5 ± 0.3	−27 .47
Vanillic acid	14.53	5.6 ± 0.3	3.2 ± 0.6	−125.57
p-Coumaric acid	17.60	5.6 ± 0.3	9.2 ± 0.3	474.83*
Ferulic acid	18.75	2.2 ± 0.4	4.3 ± 0.5	8.378*
Rutin	16.69	2.8 ± 0.4	12.8 ± 0.2	723*
Myricetin	20.95	1.5 ± 0.6	4.3 ± 0.5	82.75*
Luteolin	24.04	2.0 ± 0.5	1.6 ± 0.6	−62.54
Quercetin	24.37	2.3 ± 0.3	1.9 ± 0.3	−16.98
Apigenin	26.92	1.5 ± 0.3	/	/
Artemisinin	24.21	0.5 ± 0.3	0.3 ± 0.1	−601.0

*Statistically significant difference in content of *p*-coumaric acid, ferulic acid, rutin, and myricetin between *A. vulgaris* and *A. alba* (*t* test: paired two sample for means).

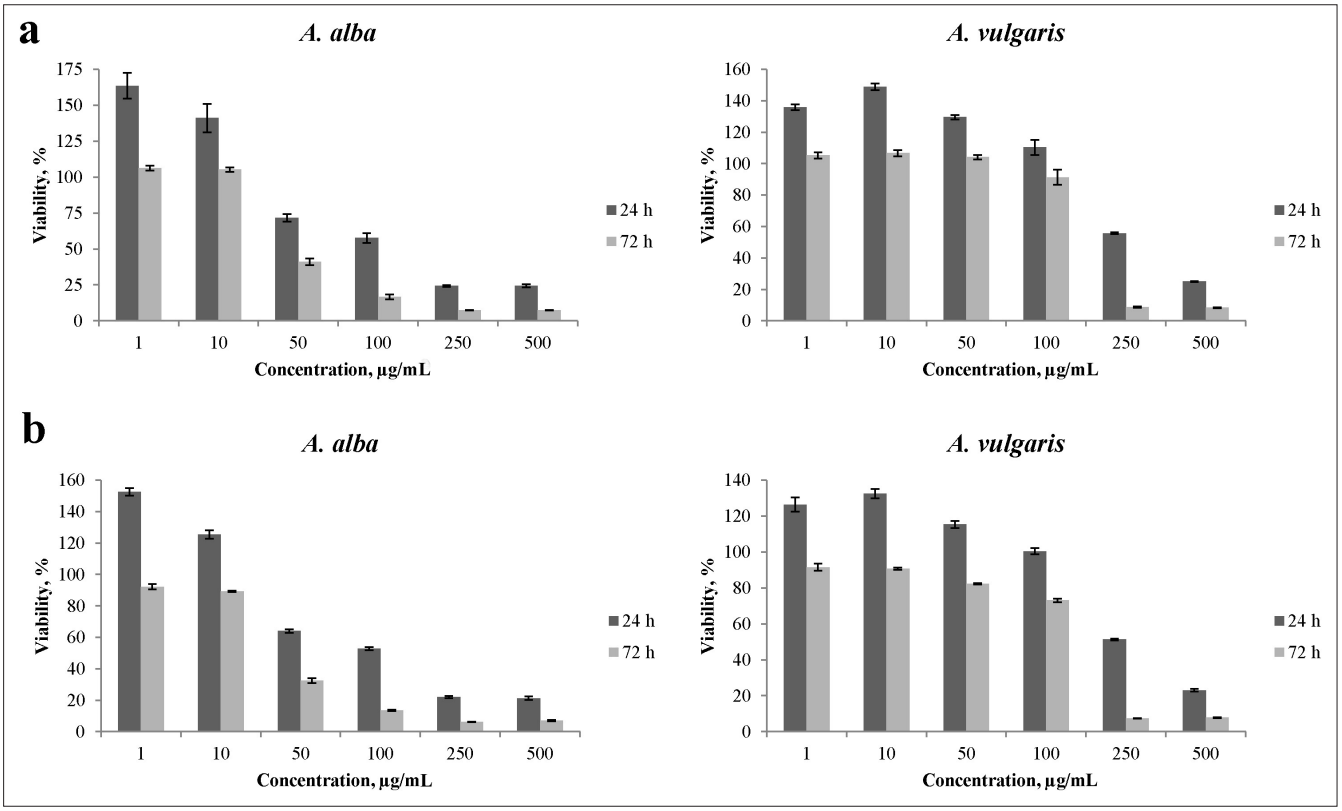

Figure 1. The dose–response curves of the effects of *A. vulgaris* and *A. alba* on SW-480 cell growth after 24 and 72 hours of exposures. All values are mean ± standard error, $n = 3$. (a) Individual extract treatment; (b) co-treatment of extracts with 0.5 µg/mL MMC. MMC, mitomycin C.

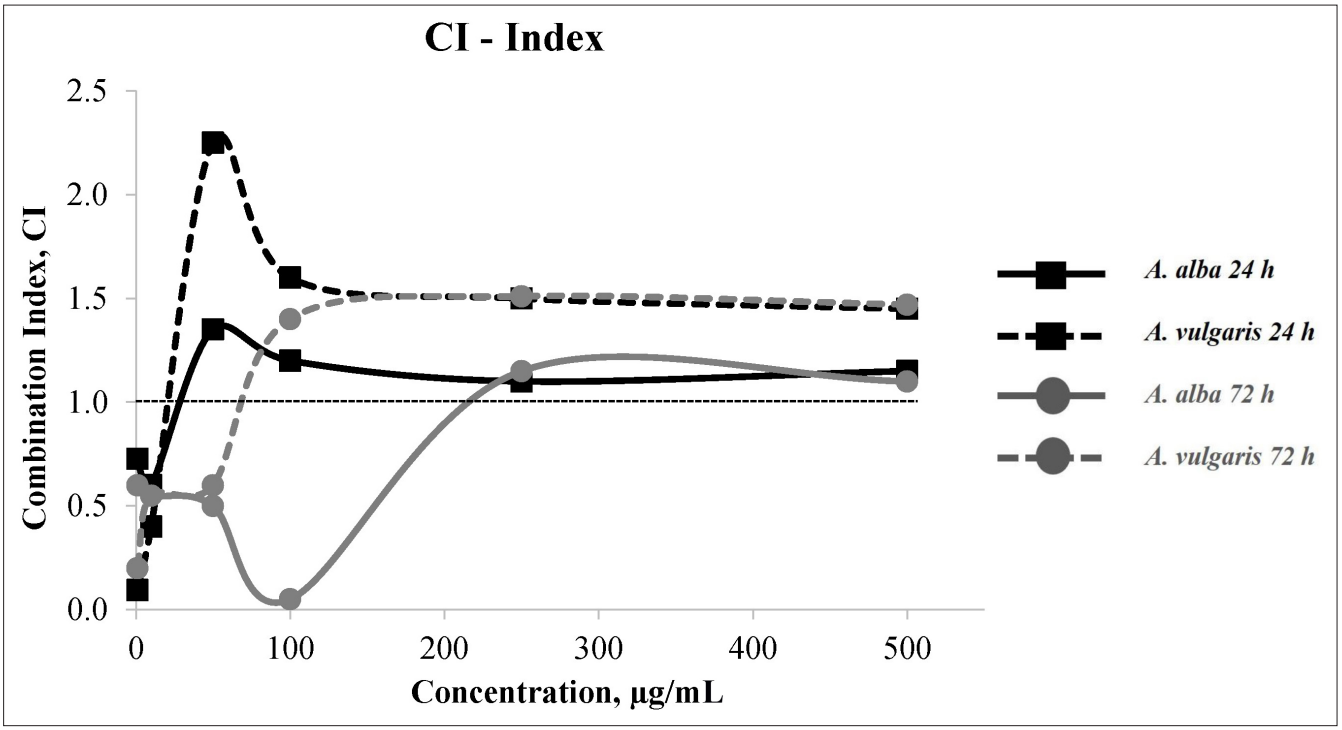

Figure 2. Combination index (CI) plots of *A. vulgaris* and *A. alba* in co-treatment with MMC on SW-480 cells.

Table 4. Apoptosis and Necrosis of SW-480 Cells Induced by 24 hours Exposure to the Ethyl Acetate Extracts of *A. vulgaris* and *A. alba*.

	Control	*A. vulgaris*	*A. alba*
Viable cells (%)	99.2 ± 0.1	6.9 ± 1.9*	4.5 ± 0.4*
Early apoptotic cells (%)	0.4 ± 0.2	0.7 ± 0.1*	33.5 ± 1.6**
Early necrotic or late apoptotic cells (%)	0.2 ± 0.0	6.4 ± 1.3*	8.1 ± 1.4*
Necrotic cells (%)	0.2 ± 0.1	82.4 ± 5.0*	53.9 ± 2.3**

*Statistically significant difference between untreated control and early apoptotic and early necrotic or late apoptotic SW-480 cells treated with IC_{50} concentrations of *A. vulgaris* and *A. alba* extracts.
**Statistically significant difference between untreated control and appropriate plant and between *A. vulgaris* and A. *alba* extracts.

better synergistic effect after 24 hours from treatment when compared to 72 hours (Figure 2).

Secondary metabolites have strong cytotoxic and apoptotic activities on different human cancer cell lines.[13,14] Sak[15] reported that the combination of plants and their bioactive components with cytostatic can induce apoptosis and thereby increase the destruction of cancer cells. The effects of ethyl acetate extracts obtained from *A. vulgaris* and *A. alba* on the type of SW-480 cell death were evaluated by flow cytometry using annexin V-FITC staining. Our results show that *A. alba* extract caused higher percentage of apoptotic cells (33.5% ± 1.6%), whereas the dominant type of cell death in *A. vulgaris* extract was necrosis (82.4% ± 5.0%) in comparison to control cells (Table 4).

Our results clearly showed that *A. alba* extract which had higher antiproliferative potential and IC_{50} of 169.4 µg/mL caused higher percentage of SW-480 cells in early apoptosis, whereas *A. vulgaris* extract significantly induced necrosis. However, *A. alba* extract with less content of total phenolics and flavonoids contained more significant amounts of phenolic acids and flavonoids in particular, compared to *A. vulgaris* extract. The result indicates that the total quantity of polyphenols and flavonoids was less important than their chemical quantification. A similar conclusion was given by Dai and Mumper.[16]

The higher contents of *p*-coumaric acid, ferulic acid, rutin, and myricetin in *A. alba* extract can be used as potential explanations of apoptotic effect. They may be responsible for the difference in cytotoxic and apoptotic activities between *A. vulgaris* and *A. alba* extracts too. A number of recently published studies by the use of different methods reported that *p*-coumaric acid, ferulic acid, rutin, and myricetin inducing apoptosis have good inhibitory activity against various cancer cell lines. So Jaganathan et al[17] reported that *p*-coumaric acid inhibited the growth of HCT-15 and HT-29 colon cancer cells by inducing apoptosis. Eroğlu et al[18] and Fahrioğlu et al[19] concluded that ferulic acid had inhibitory effect in prostate and pancreatic cancer lines. Similarly, Nasr Bouzaiene et al[20] reported that both coumaric and ferulic acid inhibited proliferation in human lung cancer and colon adenocarcinoma cells. Studies of rutin showed that flavonoid had cytotoxic effects on hepatic (HCT) cells[21] and activated apoptosis against human neuroblastoma cells.[22] Other studies have demonstrated that myricetin had anticancer effect inducing cytotoxic and apoptotic effects in different lines of cancer cells, HTC-15 human colon cancer[23] and SKOV3 ovarian cancer.[24]

Experimental

Plant Material Collection and Preparation of Extracts

Aerial flowering parts of *A. vulgaris* and *A. alba* were collected from natural populations in the region of Goč Mountain in central Serbia. The voucher specimens were confirmed and deposited at the Herbarium of the Department of Biology and Ecology, Faculty of Science, University of Kragujevac. The collected plant material was air-dried in darkness at ambient temperature. The dried plant material was cut up and stored in tightly sealed dark containers until needed. The prepared plant material (10 g) was coarsely crushed and extracted with 200 ml of water and ethyl acetate. After 48 hours, the obtained extracts were filtered through a paper filter (Whatman®, No. 1) and evaporated under reduced pressure at 40°C by the rotary evaporator. The obtained extracts were stored in sterile sample bottles for further processing.

Determination of Total Phenolic Content in the Plant Extracts

The content of phenolics in the plant extracts was measured by using Folin–Ciocalteu's method[25] and expressed as milligram of gallic acid equivalent per gram of extract.

Determination of Flavonoids in the Plant Extracts

The content of flavonoids in the plant extracts was measured by using spectrophotometric method[26] and expressed in terms of milligram of rutin equivalent per gram of extract.

HPLC Analysis of Materials

All chemicals were of analytical grade. Plant extract solutions and complete medium were filtered through Filtropur 0.2 µm (Sarstedt, Germany). All reagents were of HPLC reagent grade purity unless stated otherwise. Acetonitrile was obtained from J.T. Baker (Deventer, The Netherlands) whereas orthophosphoric acid was from Merck (Darmstadt, Germany). HPLC-grade water was produced by using Milli-Q water purification system (Millipore, Milford, MA, USA) and was used for the preparation of all solutions and reagents.

Separation of the Ethyl Acetate Extracts of A. vulgaris and A. alba by HPLC

The HPLC method, modified from the methods of Stefkov et al,[27] was used for the separation of phenolic acid and flavonoids in *A. vulgaris* and *A. alba* extracts. The HPLC system (Shimadzu, Kyoto, Japan) consisted of degasser DGU-20A3, analytical pumps LC-20AT, 7125 injector and SPD-M20A diode array detector, and CBM-20A system controller. The chromatographic separations were performed using reversed-phase Luna C18 Phenomenex (Torrance, CA, USA) (250 mm × 4.6 mm, particle size 5 µm) column. The flow rate was 1.0 mL/min and the injection volume was 20 µL. The analyses of the phenolic compounds were carried out at 30°C using 2 gradients of acetonitrile and water–orthophosphoric acid, pH 3. The detector wavelength was set at 254 and 290 nm. The chromatographic data were processed using LC Solution computer software (Kyoto, Japan, Shimadzu). Gallic acid, vanillic acid, caffeic acid, chlorogenic acid, ferulic acid, *p*-coumaric acid, rutin, quercetin, apigenin, myricetin, luteolin, and artemisinin were used as standards. Identification and quantitative analysis were done by comparison with standards.

MTT Assay—Cytotoxic Effect

Materials. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, trypsin-EDTA, phosphate-buffered saline, and penicillin/streptomycin were obtained from Thermo Fisher Scientific, Waltham, MA, USA. Dimethyl sulfoxide and MTT were obtained from Sigma Aldrich, St. Louis, MO, USA.

MTT assay. The cell viability of the colon cancer cells after exposure to the plant extracts was measured by MTT assay.[28] SW-480 cells were seeded in a 96-well microtiter plate (10^4 cells per well). After 24 hours of incubation of cells, the culturing medium was replaced with 100 µL medium containing various doses of ethyl acetate extracts of *Artemisia* at different concentrations (1, 10, 50, 100, 250, and 500 µg/mL) for 24 and 72 hours. To determine the possible synergistic cytotoxic effect, SW-480 cells were treated with plant extracts together with MMC at the concentration of 0.5 µg/mL. MTT assay was performed in individual and in co-treatment with extracts. Cell proliferation was calculated as the ratio of absorbance of the treated group divided by the absorbance of the control group, multiplied by 100 to give a viability percentage. The absorbance of the control cells served as viability of 100%. A plot of the percentage of viability versus sample concentrations was used to calculate the concentration which showed 50% cytotoxicity (IC_{50}). For explaining the possible synergistic, additive, and antagonistic effects in co-treatments of extracts with MMC we compared the effects of cytotoxicity obtained in individual treatments and co-treatments, using the formula explained by Meyer et al.[29]: CI = (control − co-treatment)/(control − treatment) + (control − MMC). Values higher than 1 represent synergistic effect, values of 0.5 to 1 additive effect, and values below 0.5 antagonistic effect.

Flow Cytometric Analysis

Detection of apoptosis was realized using an annexin V-FITC (annexin V-fluorescein isothiocyanate) apoptosis detection kit. For better comparison of the influence of plants, SW-480 cells (15×10^4) were treated with the same concentrations (500 µg/mL) of both *A. vulgaris* and *A. alba* ethyl acetate extracts or DMEM (control) for 24 hours. The untreated cells served as a control.

Annexin V-FITC is a fluorescent dye that binds to phosphatidylserines. The second color, 7-amino-actinomycin D (7-AAD), binds to cellular DNA in the cells where the cell membrane is completely damaged.[30] Annexin V-FITC and 7-AAD were added to each sample, which are subsequently incubated in the dark for 15 minutes. The cells were identified as follows: (a) viable cells if they were negative for annexin V-FITC and 7-AAD; (b) early apoptotic cells if they were positive for marker annexin V-FITC but negative for 7-AAD; (c) early necrotic or late apoptotic if they were positive for annexin V-FITC and 7-AAD; and (d) necrotic cells if they were negative for annexin V-FITC but positive for 7-AAD. The samples were analyzed using a flow cytometer (Beckman Coulter).

Statistical Analysis

The data were expressed as mean ± standard deviation (SD). Statistical analyses for cytotoxic and apoptotic activities were performed using SPSS package (IBM SPSS Statistics 20). Mean differences were established by Student's *t* test. The IC_{50} values were calculated from the dose curves by CalcuSyn software. In all cases $P < 0.05$ was considered statistically significant. For the statistical analysis of difference in polyphenolic compounds between the two extracts, Microsoft Office Excel 2010 software was used (*t* test: paired two sample for means). The percentage of viability, apoptotic cells, and necrotic cells by flow cytometry was determined by means of CXP Software for FC500 Flow Cytometry System.

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Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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