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UNEXPLORED BIOLOGICAL PROPERTIES AND PHYTOCHEMICAL CHARACTERIZATION OF METHANOLIC EXTRACTS OF *ACHILLEA AGERATIFOLIA* SUBSP. *SERBICA* (NYMAN) HEIMERL

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ABSTRACT

Phytochemical characterization and biological activities of many plants from *Achillea* genus have been described in scientific reports, while there are no published results regarding *Achillea ageratifolia* subsp. *serbica* (Nyman) Heimerl. Therefore, the aim of this paper was to investigate the different biological activities of methanolic extracts of this plant: antioxidant, antimicrobial, antibiofilm, genotoxic and antimutagenic, and also its phytochemical characterization. Results have demonstrated that extracts of different parts of the plant show significant antimicrobial activity against bacteria that cause food spoilage such as *Staphylococcus aureus* and *Bacillus subtilis*. This extracts also showed the highest antioxidant activity and significantly inhibits *Pseudomonas aeruginosa* ATCC 27853 to form the biofilm. HPLC analysis showed that flavonoid epicatechin was present in the root extract only, whereas rutin and quercetin were found in the extract of aerial part, while myricetin was only present in the inflorescence extract. The extracts of different parts of the plant were not genotoxic in all tested concentrations except the aerial part extract applied in the highest concentration, and also had a significant antimutagenic effect against known mutagens. Based on the results, methanolic extracts of the plant showed significant potential and these results could be used in the further investigation for the possible use in food protection and pharmacy.

Keywords: *Achillea ageratifolia,* antimicrobial, antibiofilm, antimutagenic, genotoxic, phytochemical.

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INTRODUCTION

The *Achillea* genus is common in many regions around the world and has many uses in traditional medicine (Mohammadhosseini *et al.,* 2017). Different extracts and components isolated from this genus have various biological activities (Mohammadhosseini *et al.,* 2017; Si *et al*. 2006; Tuberoso *et al.,* 2009; Saeidnia *et al.,* 2011; Anvari *et al.,* 2016). The most common compounds are flavonoids (flavalones, flavans, and derivatives), the most of which are apigenin, luteolin, and quercetin (Tuberoso *et al.,* 2009).

The oldest known and most familiar plant of this genus, which is widely used in traditional medicine is *A. millefolium* L. (yarrow). Literature data describe antimicrobial, antioxidative, and antiinflamatory activities of this plant (Georgieva *et al.,* 2015). Phenolic acids, flavonoids, tannins, sesquiterpene lactones isolated from this plant are considered responsible for its antiinflamatory activity (Shah *et al.,* 2017). Extracts of this and other plants in the *Achillea* genus, eg. *A. clavennae, A. holosericea*, and *A. lingulata*, have a wide spectrum of antimicrobial activities (Si *et al.,* 2006). It is considered that the antimicrobial activity of plants belonging to

Achillea genus comes from secondary metabolites such as flavonoids, phenolic acids, coumarins, terpenoids and sterols (Saeidnia *et al.,* 2011).

Due to the wide use of *A. millefolium*, other taxons belonging to this genus were, until recently, neglected in phytomedical research, although they possess a significant potential for medical use. One of those plants is *A. ageratifolia* subsp. *serbica* (Nyman) Heimerl, which is an endemic species from *Achillea* genus from Serbia. Past investigations of this taxon were mostly focusing on the phytochemical characterization of extracts as well as an essential oil (Greger *et al.,* 1983; Greger *et al*., 1987a; Greger *et al.,* 1987b; Franzén, 1988; Valant-Vetschera and Wollenweber, 2001; Mladenović and Radulović, 2017).

Ether, hexane, and methanolic extract were investigated of *A. clavennae* L by Stojanović *et al*. (2005) while water, acetone, and ethyl acetate extract of *A. ageratifolia* subsp. *serbica* were investigated by Radojevic *et al*. (2021). To the best of our knowledge, this is the first report about the activities of methanolic extracts of *A. ageratifolia* subsp. *serbica*. Because there is no literature data on methanolic extract of the plant we decided to examine methanolic extracts from different parts of this plant (inflorescence, root, and aerial part). In order to define the phytochemical profile, total phenolic content and concentration of flavonoids were determined and chromatographic analysis of phenolic compounds was performed. Our goal was also to explore biological activities: antioxidant, antimicrobial, antibiofilm, antimutagenic, and genotoxic.

MATERIALS AND METHODS

Plant material and preparation of extracts: In May 2017, the whole plants of *A. ageratifolia* subsp. *serbica* (Nyman) Heimerl were collected, during the flowering season, from the Kablar mountain, in Central Serbia. The investigated site is at 889 m above sea level and is centered on 43°91’23”-N, 20°19’22”- E. Voucher specimens (17448) are deposited in Herbarium of the Institute of Botany and Botanical Garden “Jevremovac”, University of Belgrade (BEOU).

The collected plant material was air-dried in darkness at ambient temperature. Dried, ground plant material was extracted by maceration with methanolic. Briefly, 25 g of plant material was soaked with 125 ml of solvent. The plant material was macerated three times at room temperature using fresh solvent every 24 h (Azwanida, 2015). The filtrates obtained were combined and then evaporated to dryness using a rotary evaporator at 40°C, water extract on a water bath. The obtained extracts were kept in sterile sample tubes and stored at -20°C.

Total phenols and flavonoids content: The total phenol content was determined using Folin-Ciocalteu’s method (Wootton-Beard *et al.,* 2011) and expressed as milligram of gallic acid equivalent per gram of the extract (mg GAE/g of extract). The concentrations of flavonoids were determined using the aluminum chloride method (Quettier-Deleu *et al.*, 2000) and were expressed as milligrams of rutin equivalent per gram of extract. The methods were described in detail in the previous paper (Vasić *et al.,* 2012).

HPLC analysis

Chemicals and standards: All chemicals used for HPLC analysis were of analytical grade purity and purchased from Sigma-Aldrich (Deisenhofen, Germany). HPLC-grade acetonitrile, water, and formic acid were purchased from (Fisher scientific UK, Leics, UK). Standard solutions of phenolic acids (chlorogenic and caffeic) and flavonoids (epicatechin, rutin, myricetin, quercetin, apigenin and luteolin) were used as reference standards for peak identification. The concentration of standard solutions of phenolic acids was 25 µg/ml and the concentration of flavonoids standard solutions was 40 µg/ml. Additional dilutions were made for calibration curves. Absolute methanolic was used as solvent and diluent.

Instrumentation: The separation, identification and quantification of flavonoids and phenolic acids was performed on an HPLC system (Shimadzu Prominence, Kyoto, Japan) consisted of CBM-20A system controller, DGU-20A3 degasser, analytical pump LC-20AT, manual injector 7125, and SPD-M20A photodiode array detector that scans the wavelength interval by 190–800 nm. Separation was carried out using a Luna (Phenomenex, Torrance, CA, US) C18 column (250×4.6 mm, 5 µm particle size). The temperature of the column was set at 30°C. The following gradient method with water (solvent A) and acetonitrile (solvent B), which both contain 0.1% formic acid, was used as the mobile phase: 0-1 min 5% B, 1-5 min 5-10% B, 5-25 min 10-80% B, 25-27 min 100% B and 27-30 min 5% B. The flow rate of the mobile phase was set at 1 ml/min, and the injection volume of all samples was 20 µl. The HPLC profiles were monitored at 280, 325, and 360 nm. The responses of the detector were recorded using LC Solution software version 1.2 (Shimadzu, Kyoto, Japan).

Phenolic compounds in the methanolic extracts of *A. ageratifolia* aerial part, inflorescence, and the root were identified by comparing retention times and UV-Vis’s absorption spectra of unknown peaks with co-injected reference standards. The compounds identified were confirmed by spiking the sample with the standard compound and monitoring the changes in the peak shape and spectral characteristics. Each standard solution was made by diluting 1 mg of each compound in 10 mg of methanol, and then two-fold serial dilutions were prepared (8 different mass concentrations). The quantification was performed using a standard curve of standard mixtures

containing chlorogenic acid, epicatechin, rutin, myricetin, quercetin, apigenin, and luteolin in the range of 0.78–100 µg/mL. Each plant extract was analyzed in triplicate and data are reported as the mean ± SD.

Determination of antioxidant activity (DPPH assay): The ability of the plant extract to scavenge DPPH free radicals was assessed using the method described by Takao *et al*. (1994). Ascorbic acid was used as a positive control. The experiment was performed in triplicate. Based on the obtained results, % inhibition of DPPH radicals and the IC_{50} value were determined. The percentage of inhibition was calculated using the following equation:

$$\% \text{ inhibition} = (A_{control} - A_{sample}) / A_{control}) \times 100 \quad (1)$$

where $A_{control}$ was the absorbance of the control sample and A_{sample} is the absorbance of the extract. The IC_{50} value is the effective concentration at which 50% of DPPH radicals were scavenged. It was obtained from the graph of scavenging activity (%) versus the concentration of samples. The method was described in detail in the previous paper (Vasić *et al.,* 2012).

Antimicrobial activity

Test microorganisms and suspension preparation: Antimicrobial activity of methanolic extracts was tested against 10 strains of bacteria. Strains of Gram-positive bacteria: *Bacillus pumilus* NCTC 8241, *B. subtilis* ATCC 6633, *B. subtilis*, *Staphylococcus aureus*, *S. aureus* ATCC 25923. Gram-negative bacteria: *Pseudomonas aeruginosa* ATCC 27853, *Proteus mirabilis* ATCC12453, *Escherichia coli*, *E. coli* ATCC 25922, *Salmonella enterica*, and 2 strains of yeast: *Rhodotorula mucilaginosa* and *Candida albicans* ATCC 10231). All clinical isolates were a generous gift from the Institute of Public Health, Kragujevac. The other microorganisms were provided from a collection held by the Microbiology Laboratory, Faculty of Science, University of Kragujevac. The suspensions were prepared by the direct colony method. The turbidity of the initial suspension was adjusted using 0.5 McFarland densitometer (DEN-1, BioSan, Latvia). The initial bacterial and yeast suspensions were additionally diluted in 1:100 ratio in sterile 0.85% saline.

Microdilution method: The antimicrobial activity was tested by determining the minimum inhibitory concentration (MIC) and the minimum microbicidal concentration (MMC) using the microdilution method with resazurin (Sarker *et al.,* 2007). The stock concentration of tested plant extracts was 10000 µg/ml. Twofold serial dilutions of plant extracts were made in sterile 96-well plates containing Mueller-Hinton broth for bacteria and Sabouraud dextrose broth for fungi. The tested concentration range was from 5000 µg/ml to 39 µg/ml. A detailed description of this method is presented in a previous paper (Vasić *et al.,* 2012). Doxycycline and fluconazole, dissolved in a nutrient liquid medium, were used as positive controls. Stock solutions of crude extracts were obtained by dissolving in 10% DMSO which was used as a negative control. Each test included growth control and sterility control. All tests were performed in duplicate, and MICs were constant.

Antibiofilm activity – the effect on forming and formed biofilm: In 96-well microtiter plates 100 µl of TSB (tryptone soy broth) broth was added. One hundred µl of the tested extract was added into the first row of the plates, with an initial concentration of 10000 µg/ml, and serial twofold dilutions were made down to 80 µg/ml. Subsequently, 10 µl of bacterial suspension (1.0 McFarland for Gram positive and 0.5 McFarland for gram negative bacteria) was added into each well. Microtiter plates were then incubated for 48 h at 37ºC. After incubation, the well content was washed out with 200 µl of 0.85% saline. Formed biofilm was fixed with 100 µl of methanolic, and after evaporating, 100 µl 0.1% of crystal violet dye was added to each well (room temperature, 20 min). Wells have then washed out again and the biofilm was decolored by adding 200 µl of ethanol. Optical densities (OD) of stained adherent bacteria were determined with a micro-ELISA plate reader at a wavelength of 630 nm (OD630 nm). Sterile broth or broth with extracts served as a control to check sterility and non-specific binding of media. To compensate for background absorbance, OD readings from sterile medium, extracts, fixative, and dye were averaged and subtracted from all test values (O'Toole *et al.,* 1998).

The difference in the procedure of measuring the effect on the formed biofilm is that varying concentrations of the extract were added into the microtiter plates with the formed biofilm of tested bacteria. The antibiofilm activity was tested on *S. aureus* ATCC 6538 and *P. aeruginosa* ATCC 27853. Results were expressed as biofilm inhibitory concentration, BIC_{50}. All tests were performed in duplicate, and BIC was constant.

Genotoxic and antimutagenic activity

The single-cell gel electrophoresis (Comet assay): Comet assay was performed as described by Singh *et al*. (1988) with a few modifications. Peripheral venous blood was obtained from two healthy donors aged 26 and 27 years which gave written informed consent according to the Helsinki Declaration. Lymphocytes were isolated using Histopague-1077 and treated with methanolic extracts from different parts of the plant (inflorescence, root and whole aerial part) in four different concentrations (125, 250, 500, and 1000 µg/ml) for half an hour at 37ºC. After the incubation period, cells viability was determined using a Trypan blue assay. Treated cells were suspended in 100 µl of 1% low melting point agarose (LMPA), and then 90 µl of suspension was layered onto microscopic slides in a quantity and kept at 4ºC for 5 min. Afterwards the slides were transferred into ice-cold prepared lysis

solution at pH 10 for 2 h (2.5 M NaCl, 100 mM EDTA, 10 mM tris, 1% Triton X-100, 10% DMSO). The slides were then kept in an electrophoresis buffer (10 M NaOH, 200 mM EDTA) at 4ºC for 30 min. Electrophoresis was run at 25 V and 300 mA for 30 min at 4°C in an ice bath, after which the slides were submerged in neutralization buffer (0.4 M Tris-HCl, pH 7.5) three times for 5 min and stained with 50 µl ethidium bromide for 10 min. Negative control (without treatment) and positive controls only with hydrogen peroxide (H_2O_2, final concentration of 10 µg/ml) or only with mitomycin C (MMC, final concentration of 0.5 µg/ml) were included parallel.

DNA damage quantification: The slides were examined using a fluorescent microscope (Nikon E50i) at 400x magnification. A total of 100 cells per donor were visually analyzed (50 from each of two replicate slides). DNA damage was quantified by tail length. Cells were divided into five categories based on DNA damage: 0 - no damage; 1 - low level damage; 2 - medium level damage; 3 - high level damage; 4 - total damage. The genetic damage index (GDI) calculated based on the size and intensity of the "comet" tails, using the formula (Collins, 2004):

GDI = Class1 + 2 x Class2 + 3 x Class3 + 4 x Class4 / Class0 + Class1 + Class2 + Class3 + Class4.

Statistical analysis: Data are presented as means ± standard deviations where appropriate. All statistical analyses were performed using the SPSS package. (SPSS for Windows, ver. 17, 2008) (Chicago, IL, USA). One-way analysis of variance (ANOVA) with Turkey's post hoc test was used to compare differences among groups in the comet assay. The relationship between GDI values and tested extracts concentrations were determined by the Pearson correlation coefficient. A difference at $p < 0.05$ was considered significant.

RESULTS

Phytochemical analysis: Yield, the concentration of total phenols and flavonoids in methanolic extracts of different parts of *A. ageratifolia* subsp. *serbica* are shown in Table 1.

Table 1. Yield, concentration of total phenolic, flavonoid and antioxidant activity in methanolic extracts of different parts of *A. ageratifolia* subsp. *Serbica*.

Part of plant	Yield %	Total phenolic content (mg GA/g of extract) *	Flavonoid content (mg RU/g of extract) *
Aerial part	6.52	39.83 ± 4.67	20.33 ± 1.07
Inflorescence	8.90	53.61 ± 2.69	26.71 ± 0.96
Root	3.75	70.78 ± 1.39	7.79 ± 0.77

* Each value shown is the mean value ± standard deviation

The highest concentration of total phenols was measured in the root extract, and the highest concentration of flavonoids was measured in the inflorescence extract (Table 1).

RP-HPLC analysis of *A. ageratifolia* subsp. *serbica* plant extracts: HPLC chromatograms of *A. ageratifolia* subsp. *serbica* extracts are presented in Figures 1, 2, and 3, showing peaks of identified compounds in these extracts. HPLC analysis demonstrated that all three extracts, aerial part, inflorescence, and root contained chlorogenic acid, apigenin, and luteolin. Flavonoid epicatechin was found only in the root extract; rutin and quercetin in the extract of aerial part, and myricetin in the inflorescence extract. Concentrations of identified phenolic compounds in extracts, expressed as mg per g of dry extract, are presented in Table 2. Chlorogenic acid is the dominant component in all three extracts, with the highest content in the root. The aerial part extract also contained high amounts of rutin, while inflorescence extract contained an inconsiderable amount of apigenin.

Table 2. *A. ageratifolia* subsp. *serbica* phenolic composition.

Compound	t_r (min)	Concentration (mg/g dry extract ± SD)*		
		Aerial part (MeOH)	Inflorescence (MeOH)	Root (MeOH)
Chlorogenic acid	13.08	11.635 ± 0.15	17.830 ± 0.18	39.260 ± 0.26
Epicatechin	13.69	-	-	1.375 ± 0.05
Rutin	14.12	5.463 ± 0.07	-	-
Myricetin	18.96	-	1.719 ± 0.05	-
Quercetin	18.85	0.411 ± 0.007	-	-
Apigenin	20.49	0.411 ± 0.012	3.626 ± 0.05	0.080 ± 0.003
Luteolin	20.82	2.661 ± 0.04	1.407 ± 0.03	0.354 ± 0.009

* Each value shown is the mean value ± standard deviation

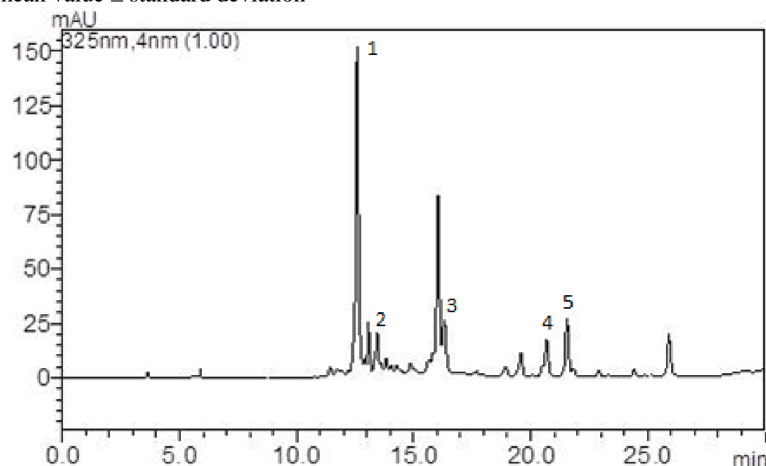

Figure 1. HPLC chromatogram of *A. ageratifolia* aerial part extract (1-chlorogenic acid, 2-rutin, 3-quercetin, 4-apigenin, 5-luteolin).

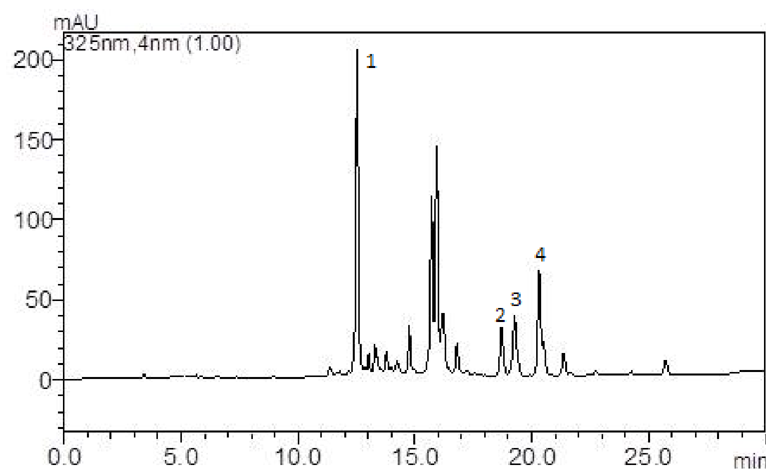

Figure 2. HPLC chromatogram of *A. ageratifolia* inflorescence extract (1-chlorogenic acid, 2-myricetin, 3-apigenin, 4-luteolin).

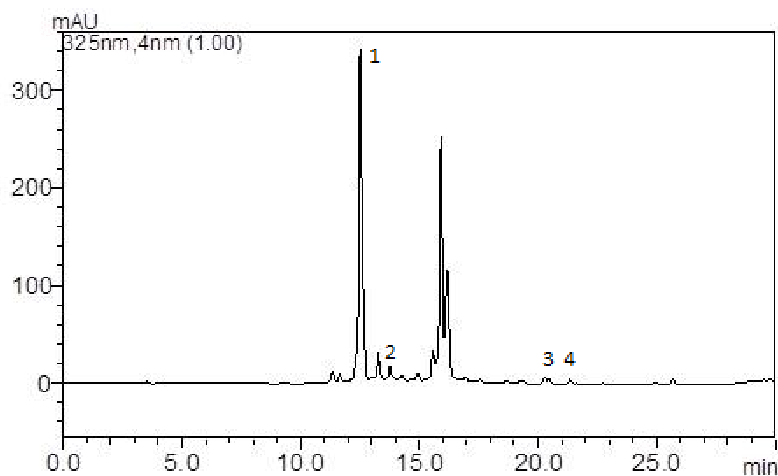

Figure 3. HPLC chromatogram of *A. ageratifolia* root extract (1-chlorogenic acid, 2-epicatechin, 3-apigenin, 4-luteolin).

Antioxidant activity: The antioxidant activity of methanolic extracts of *A. ageratifolia* subsp. *serbica* was evaluated for the first time in this research. The highest antioxidant activity was demonstrated for the root extract (Table 3).

Table 3. Antioxidant activity of different parts of *A. ageratifolia* subsp. *serbica* methanolic extracts.

Part of plant	Antioxidant activity (IC_{50} value µg/ml)[*]
aerial part	206.99 ± 0.70
inflorescence	223.42 ± 0.19
root	106.36 ± 0.39

[*] Each value shown is the mean value ± standard deviation.

Antimicrobial with antibiofilm activity: The antimicrobial with the antibiofilm activity of methanolic extract of the plant has been investigated in this research. Results are presented in Tables 4 and 5. Gram positive bacteria were more sensitive than Gram negative bacteria. The effective concentration of the root extract is MIC 2500 µg/ml for *P. aeruginosa* ATCC 27853. Most sensitive Gram positive bacteria were food isolates *B. subtilis* and *S. aureus* (Table 4). Yeasts had a moderate sensitivity, where the most significant effect was with the aerial extract on *C. albicans* ATCC 10231 (Table 4).

Table 4. Antimicrobial activity methanolic extracts of different parts of *A. ageratifolia* subsp. *Serbica.*

Plant parts	Aerial part		Inflorescence		Root		Doxycycline/Fluconazole	
Tested specie	**MIC[1]**	**MMC[2]**	**MIC**	**MMC**	**MIC**	**MMC**	**MIC**	**MMC**
B. pumilus NCTC 8241	5000	5000	5000	5000	5000	5000	0.98	7.81
B. subtilis	78	156	625	1250	78	78	1.95	31.25
B. subtilis ATCC 6633	>5000	>5000	>5000	>5000	>5000	>5000	0.11	1.95
S. aureus H	78	313	625	625	625	625	0.22	3.75
S. aureus ATCC 25923	625	625	625	1250	625	625	0.45	7.81
P. aeruginosa ATCC 27853	>5000	>5000	5000	>5000	2500	>5000	62.5	125
P. mirabilis ATCC12453	5000	5000	5000	5000	5000	5000	15.63	62.5
E. coli	>5000	>5000	>5000	>5000	>5000	>5000	15.63	31.25
E. coli ATCC 25922	>5000	>5000	>5000	>5000	>5000	>5000	15.63	31.25
S. enterica	>5000	>5000	>5000	>5000	>5000	>5000	15.63	31.25
R. mucilaginosa	2500	5000	1250	1250	2500	5000	62.5	1000
C. albicans ATCC 10231	156	5000	1250	5000	2500	5000	62.5	1000

[1] MIC values for plant extracts and antibiotic are given as µg/ml – means inhibitory activity.
[2] MMC values for plant extracts and antibiotic are given as µg/ml – means microbicidal activity.

Table 5. The influence of methanolic extracts on the formation of biofilm.

Plant parts	Aerial part	Inflorescence	Root	Tetracycline
Tested species		BIC_{50} [1]		
P. aeruginosa ATCC 27853	>10 000	3 243.7	2 312	156

[1] Minimum biofilm inhibition concentration for plant extracts and antibiotic are given as µg/ml

Genotoxic and antimutagenic activity: The results of the comet assay for separate and combined treatment of extracts with known mutagens are shown in Table 6 and Figure 4. In separate treatments, all tested concentrations of extracts from the plant increased the GDI values (from 0.46 ± 0.07 to 2.38 ± 0.20 for inflorescence; from 0.45 ± 0.04 to 1.96 ± 0.08 for root; from 0.25 ± 0.04 to 2.08 ± 0.05 for the aerial part) in comparison to negative controls (0.19 ± 0.01), but significantly only in the highest tested concentration (1000 µg/ml) of the extract from whole aerial part. Pearson correlation coefficient demonstrated that all plant extracts increased GDI values in a dose dependent manner ($r = 0.912$, $p < 0.05$ for inflorescence; $r = 0.852$, $p < 0.05$ for root; $r = 0.856$, $p < 0.05$ for whole aerial part) (Figure 4).

In the combined treatments, there was a significant decrease in H_2O_2-induced GDI values in the treatments with high tested concentrations of extracts (500 and 1000 µg/ml) from both inflorescence and whole aerial part, and with all tested concentrations except the lowest (125 µg/ml) from the root, in comparison to positive control. Pearson correlation coefficient between the concentrations of extracts and GDI values were significantly negative ($r = -0.723$, $p < 0.05$ for inflorescence; $r = -0.879$, $p < 0.05$ for root; $r = -0.877$, $p < 0.05$ for whole aerial part). In the treatment against MMC, it is noticed significant decrease induced GDI values in all tested concentrations of extracts from all plant parts except the lowest (125 µg/ml) from both inflorescence and root. Pearson correlation coefficient between the extract's concentrations and

MMC-induced GDI values were significantly negative ($r = -0.751$, $p < 0.05$ for inflorescence; $r = -0.784$, $p < 0.05$ for root; $r = -0.812$, $p < 0.05$ for whole aerial part) (Figure 4).

The analyses of comets distribution in separate treatment showed positive correlation between increasing number of damaged cells and tested concentrations of extracts (Pearson, $r = 0.843$, $p < 0.05$ for inflorescence; $r = 0.810$, $p < 0.05$ for root; $r = 0.825$, $p < 0.05$ for whole aerial part), while in the treatment of extracts against mutagen–induced damage correlation was negative (Pearson for H_2O_2, $r = -0.702$, $p > 0.05$ for inflorescence; $r = -0.898$, $p < 0.05$ for root; $r = -0.866$, $p < 0.05$ for whole aerial part) and (Pearson for MMC, $r = -0.159$, $p > 0.05$ for inflorescence; $r = -0.816$, $p < 0.05$ for root; $r = -0.790$, $p < 0.05$ for whole aerial part).

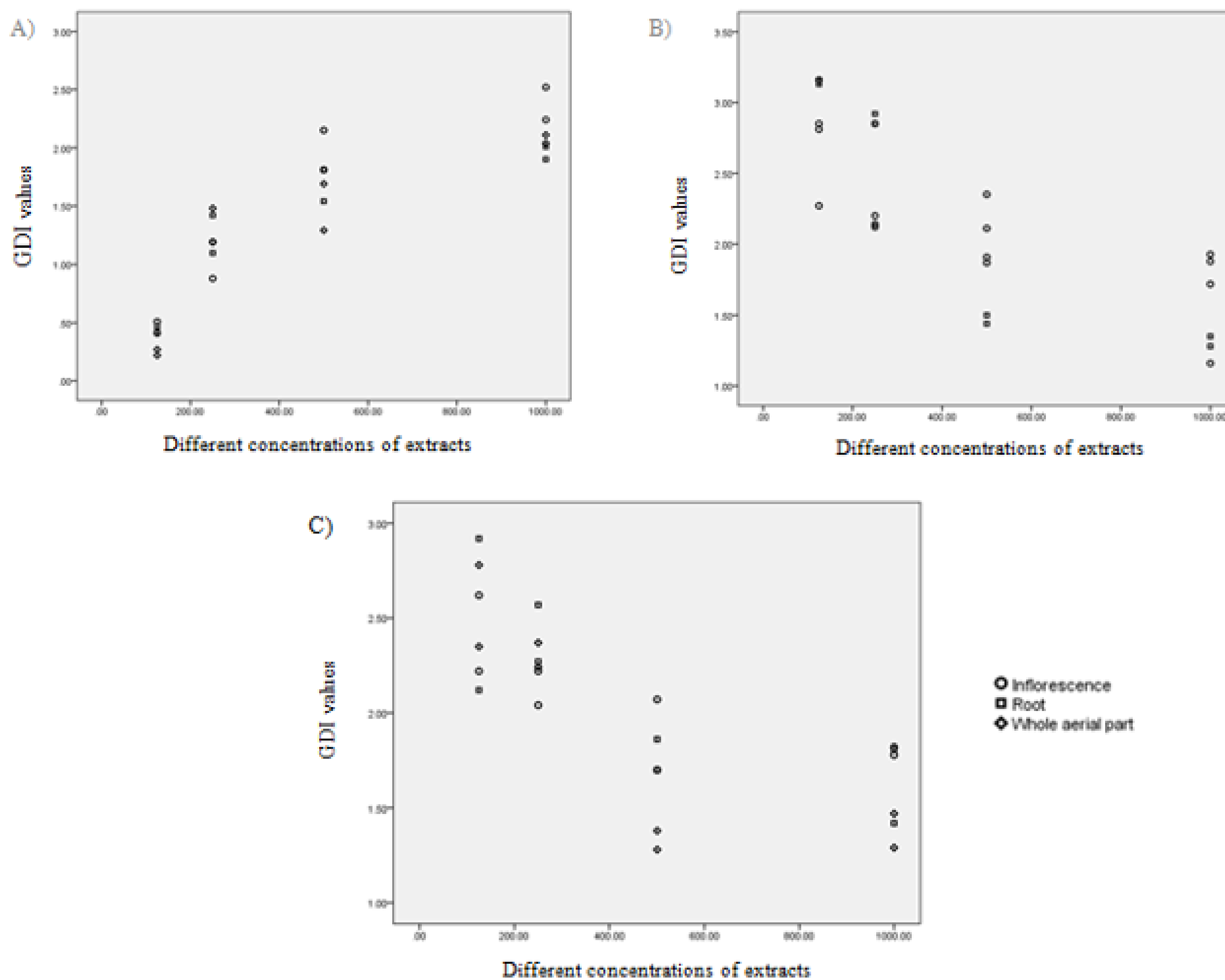

Figure 4. Correlation graph between the GDI values and concentrations of extracts from different parts of *A. ageratifolia* subsp. *serbica* in separate (A), and combined treatments with H_2O_2 (B) and MMC (C)

Table 6. Genotoxic and antimutagenic effect of methanolic extracts from different parts of *A. ageratifolia* subsp. *serbica* in cultured human lymphocytes using comet assay.

Controls/ parts of plant	**Treatment (□g/mL)**	**No. of analyzed** Cells	**0**	**1**	**2**	**3**	**4**	**GDI (mean □SD)**
	Negative control	200	86.50	8.50	4.50	0.50	/	0.19 ± 0.01
Controls	Positive control H_2O_2 (10)	200	/	/	8.00	41.50	50.50	3.43 ± 0.13
	Positive control MMC (0.5)	200	/	2.00	10.00	36.00	52.00	3.38 ± 0.03
	125	200	70.50	19.50	3.50	6.50	/	0.46 ± 0.07
	250	200	33.00	38.00	20.00	8.50	0.50	1.04 ± 0.22

	500	200	9.00	25.50	31.50	26.50	7.50	1.98 ± 0.24
	1000	200	1.50	18.00	25.50	39.50	15.50	2.38 ± 0.20
Inflorescence	125 + H_2O_2	200	3.50	9.50	21.50	43.00	22.50	2.72 ± 0.63
(separate and	250 + H_2O_2	200	4.50	15.00	29.00	26.50	25.00	2.53 ± 0.46
combined	500 + H_2O_2	200	4.50	20.50	30.50	36.50	8.00	2.23 ± 0.17**
treatments)	1000 + H_2O_2	200	17.00	25.00	22.50	21.50	14.00	1.91 ± 0.04**
	125 + MMC	200	5.50	17.00	23.50	38.00	16.00	2.42 ± 0.28
	250 + MMC	200	6.50	23.50	30.50	29.50	10.00	2.13 ± 0.13**
	500 + MMC	200	10.00	27.50	32.00	25.00	5.50	1.89 ± 0.26**
	1000 + MMC	200	7.00	31.00	39.00	21.00	2.00	1.80 ± 0.03**
	125	200	71.00	17.00	8.50	3.50	/	0.45 ± 0.04
	250	200	35.50	22.00	25.00	16.00	1.50	1.26 ± 0.23
	500	200	16.00	27.50	32.00	22.00	2.50	1.72 ± 0.16
	1000	200	11.50	20.50	31.00	35.00	2.00	1.96 ± 0.08
Root	125 + H_2O_2	200	0.50	8.00	27.00	37.00	27.50	2.83 ± 0.03
(separate and	250 + H_2O_2	200	12.50	20.50	21.50	32.50	13.00	2.13 ± 0.01**
combined	500 + H_2O_2	200	15.00	23.50	29.00	20.50	12.00	1.89 ± 0.03**
treatments)	1000 + H_2O_2	200	29.00	27.00	20.00	19.00	5.00	1.44 ± 0.39**
	125 + MMC	200	8.00	25.00	22.00	18.00	27.00	2.52 ± 0.57
	250 + MMC	200	2.50	18.50	30.50	31.50	17.00	2.42 ± 0.21**
	500 + MMC	200	14.00	30.00	26.50	23.00	6.50	1.78 ± 0.11**
	1000 + MMC	200	24.50	26.50	19.00	23.00	7.00	1.62 ± 0.27**

Controls/ parts of plant	**Treatment (□g/mL)**	**No. of analyzed cells**	**0**	**1**	**2**	**3**	**4**	**GDI (mean □SD)**
	Negative control	200	86.50	8.50	4.50	0.50	/	0.19 ± 0.01
Controls	Positive control H_2O_2 (10)	200	/	/	8.00	41.50	50.50	3.43 ± 0.13
	Positive control MMC (0.5)	200	/	2.00	10.00	36.00	52.00	3.38 ± 0.03
	125	200	80.00	15.50	4.50	/	/	0.25 ± 0.04
	250	200	34.50	14.00	35.00	16.50	/	1.34 ± 0.21
	500	200	27.00	17.50	35.50	19.50	0.50	1.50 ± 0.30
	1000	200	11.00	17.00	34.50	28.50	9.00	2.08 ± 0.05*
Whole aerial part	125 + H_2O_2	200	/	1.50	15.50	50.50	32.50	3.14 ± 0.01
(separate and	250 + H_2O_2	200	1.50	5.50	26.50	36.00	30.50	2.89± 0.05
Combined	500 + H_2O_2	200	32.00	20.00	24.50	16.00	7.50	1.47 ± 0.04**
treatments)	1000 + H_2O_2	200	35.50	23.50	19.50	17.00	4.50	1.32 ± 0.05**
	125 + MMC	200	6.00	11.00	23.50	39.50	20.00	2.57 ± 0.30**
	250 + MMC	200	11.00	9.50	35.00	27.00	17.50	2.31 ± 0.09**
	500 + MMC	200	37.00	19.00	25.00	12.00	7.00	1.33 ± 0.07**
	1000 + MMC	200	34.50	19.00	24.00	19.00	3.50	1.38 ± 0.13**

*significant increase GDI in comparison to negative control cells (ANOVA, $p < 0.05$).
**significant decrease GDI in comparison to positive control cells (ANOVA, $p < 0.05$).

DISCUSSION

Phytochemical analysis: The number of total phenols and flavonoids in the methanolic extract of the aerial section of *A. wilhemsii* K. Koch (Fathi *et al.,* 2011) is significantly lower than those in *A. ageratifolia* subsp. *serbica*. The content of total phenolic compounds in the methanolic extracts of aerial sections of *A. coarctata* Poir., *A. kotschyi* Boiss. and *A. lycaonica* Boiss. and Heldr. (Agar *et al.,* 2015) is higher than the content of total phenols in the methanolic extract of the aerial section of *A. ageratifolia* subsp. *serbica*, but similar to the concentrations of methanolic extracts of the root and inflorescence.

RP-HPLC analysis of *A. ageratifolia* subsp. *serbica* plant extracts: To the best of our knowledge, this is the first report about the phenolic composition of methanolic extracts of *A. ageratifolia* subsp. *serbica*. A large number of studies about *Achillea* species are based mostly on *A. milefolium* polyphenol profile, which showed that chlorogenic acid, rutin, luteolin, and apigenin were dominant phenolic compounds in this plant, which is in agreement with our results (Benetis *et al.,* 2008).

Phytochemical studies have revealed that many components of this genus (flavonoids such as flavonols, flavons, and their derivatives; apigenin, luteolin, and

quercetin make the majority of the total) are biologically active and numerous data are confirming their traditional use like an herbal medicine (Tuberoso *et al.,* 2009). Besides the pyrrolidine and dehydropirilidin, from the root extracts of *A. ageratifolia* (Sm.) Benth and Hook. f., new pyrrolidines have been isolated (Greger *et al.,* 1987b). Research on 11 high-mountain species of *Achillea* genus (isolation flavonoid compounds) have demonstrated that *A. ageratifolia*subsp. *serbica* is characterized by the presence of 3,6,7-trimetoxi and 6-hydroxy flavones (Valant-Vetschera and Wollenweber, 2001). Previous intensive studies of *A. ageratifolia* subsp. *serbica* have succeeded in isolating new isobutylamides (Greger *et al.,* 1983). Other phytochemical investigations mostly relate to polyacetylenes and terpenoids. Therefore, phytochemical and biological studies of this genus and species should be intensified and these results may have a role in the possible pharmaceutical use of this plant.

Antioxidant Activity: When compared to the antioxidant activity of methanolic extracts of aerial sections of *A. Wilhemsii* (Fathi *et al.,* 2011), as well as *A. coarctata*, *A. kotschyi* and *A. lycaonica* (Agar *et al.,* 2015), the antioxidant activity of any part of *A. ageratifolia* subsp. *serbica* is lower.

Antimicrobial and antibiofilm activity: The aerial extracts of *A. damascene*, *A. clavennae*, *A. holosericea*, *A. lingulate,* and *A. milleofilum* have a wide spectrum of antimicrobial activity (Barbour *et al.,* 2004; Stojanović *et al.,* 2018). Methanolic extract of the *A. wilhelmsii*'s aerial has the inhibitory effect against *S. aureus*, *B. cereus,* and *E. coli*, at concentrations ranging between 6.25 and 25 mg/ml (Mohammadi-Sichani *et al.,* 2011). Methanolic extracts of *A. ageratifolia* subsp. *serbica* have a much stronger antimicrobial activity, especially against bacteria that cause food spoilage such as *S. aureus*, *B. subtilis* and *S. aureus* ATCC 25923 (MIC 78-625 µg/ml). The aerial extract shows slightly higher activity when compared to the inflorescence and the root (Table 4).

The antibiofilm activity of methanolic extract of this plant was investigated for the first time in this research. The effect was evaluated on the ability of biofilm formation and on already formed biofilm of *S. aureus* ATCC 25923 and *P. aeruginosa* ATCC 27853. Antibiofilm activity was determined by measuring the BIC_{50} (Table 5). The methanolic extracts of *A. ageratifolia* subsp. *serbica* did not inhibit the formed biofilms for either bacterium as well as on the ability of *S. aureus* ATCC 25923 to form biofilm. The extracts of the root and the inflorescence have demonstrated a certain inhibitory effect on the ability of biofilm formation of *P. aeruginosa* ATCC 27853 (Table 5). Radojević *et al*. (2021) indicated that water, acetone, and ethyl acetate extracts of *A. ageratifolia* subsp. *serbica* showed no effect on the biofilm formation ability of *S. aureus* ATCC 25923, except for the ethyl acetate extract from the aerial parts of the plant, with BIC_{50} at 1770.7 µg/ml. However, Radojević *et al*. (2021) indicated that water, acetone, and ethyl acetate extracts of *A. ageratifolia* subsp. *serbica* showed an inhibitory effect on the biofilm formation of P. *aeruginosa* ATCC 27853 (BIC_{50} from 2304.75 µg/ml to 3232.8 µg/ml). Only the ethyl acetate extract of the inflorescence exhibited an effect on the formed biofilm of *P. aeruginosa* ATCC 27853 (BIC_{50} at 9170.85 µg/ml). Based on the results, it could be concluded that methanolic extract showed the limited antibiofilm effect to tested bacterial species, compared to water, acetone, and ethyl acetate extracts.

The available literature contains data only regarding the antibiofilm activity of *A. ageratum* L. essential oil on the *C. albicans* biofilm. Vavala *et al*. (2009) published that essential oil had an inhibitory effect (BIC 60% in a concentration of 9 µg/ml) on *C. albicans* forming a biofilm.

Genotoxic and antimutagenic activity: To our knowledge, there are no available literature data on the action of *A. ageratifolia* subsp. *serbica* on the genome of healthy human cells, so we decided to test its genotoxic potential on human peripheral blood lymphocytes *in vitro*. Our results showed that the methanolic extracts obtained from inflorescence, root and aerial parts of the plant were not genotoxic in all tested concentrations except the highest (1000 µg/ml) concentration of the extract obtained from the aerial part. Namely, GDI values increased insignificantly in the treatments with different concentrations of all analyzed extracts, and only the treatment with the highest concentration of the aerial part of the plant caused a significant increase in GDI values, compared to the negative control (untreated cells). These results are in agreement with the results of Teixeira *et al*. (2003) who examined the effect of another species of the *Achillea* genus, *A. millefolium*. They showed no significant chromosome alterations after treatment, as compared to untreated cultured human peripheral blood lymphocytes.

In the treatment against known mutagens (H_2O_2 and MMC) all examined parts of the plant had a significant antimutagenic effect. The frequency of mutagen-induced GDI values decreased dose dependently, i.e. with increased concentration of extracts the induced genomic damage was reduced proportionally. Analysis of the distribution of DNA damage levels showed that the number of H_2O_2 or MMC-induced damaged cells decreased with increasing of extracts concentrations, while the number of undamaged cells increased, in comparison to positive controls (only H_2O_2 or only MMC). The obtained results point to the extremely protective effect of the examined parts of the plant. Düsman *et al*. (2013) came to the same conclusion when they studied the effect of *A. milestones* on cyclophosphamide-induced genome damage in bone marrow cells from Wistar rats.

The antimutagenic activity of extracts can be explained by combining the effects of their components, primarily flavonoids and phenolic compounds, which are known to have pronounced antioxidant effects that can eliminate the effect of free radicals produced by the action of H_2O_2 and MMC (Kunwar and Priyadarsini, 2011; Bouriche *et al.,* 2017).

Conclusion: Based on the results obtained in this research, it may be concluded that the tested extracts of *A. ageratifolia* subsp. *serbica* are important sources of flavonoid epicatechin (root extract) while rutin and quercetin are found in the extract of the aerial part and myricetin in the inflorescence extract. Root extract contains the highest number of phenols and the overall greatest amount of chlorogenic acid and also has the highest antioxidative activity. Extracts of all three different parts of the plant were not genotoxic in all tested concentrations except the highest (1000 µg/ml) from the aerial part. All examined parts of the plant had a significant antimutagenic effect in the treatment against known mutagens (H_2O_2 and MMC). Methanolic extracts of different plant parts show outstanding antimicrobial activity against the bacteria that cause food spoilage. *A. ageratifolia* subsp. *serbica* may have potential and possibility for use pharmacy.

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