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The phytochemical composition and biological activities of different types of extracts of *Achillea ageratifolia* subsp. *serbica*

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ABSTRACT:
Achillea ageratifolia subsp. *serbica* is a poorly investigated endemic of the Balkan area and there are almost no data about the potential application of this plant. The purpose of this study was the investigation of the phytochemical composition, antioxidant, antimicrobial, and antibiofilm activity of its water, acetone, and ethyl acetate extracts. The phenolic composition and flavonoids were established using high-performance liquid chromatography with photodiode array detection (HPLC-PDA) analysis, while the chemical composition of the ethyl acetate extracts of the aerial parts of the plant was investigated by gas chromatography–mass spectrometry (GC and GC-MS). Antioxidant and antimicrobial activity, as well as the inhibition ability of biofilm formation on *Staphylococcus aureus* ATCC 25923 and *Pseudomonas aeruginosa* ATCC 27853 biofilms, were also investigated. The total phenolic content ranged from 18.61 mgGAE/g to 43.78 mgGAE/g of extract. The total flavonoid content ranged from 10.79 mgRUE/g to 34.02 mgRUE/g. The acetone and ethyl acetate extracts of the aerial parts contained chlorogenic acid, myricetin, apigenin, and luteolin. Analysis of the volatile specialized metabolites in the ethyl acetate extract of the aerial parts allowed for the identification of thirty-two constituents. The highest antioxidant activity was detected in the water extract of the aerial parts of the plant (IC_{50} = 641.06 μg/ml) and in the ethyl acetate extract of the root (IC_{50} = 675.33 μg/ml). *Bacillus subtilis* and *S. aureus* showed higher sensitivity to the tested extracts, while the antifungal activity of the marked extracts was significant. All extracts showed a moderate inhibitory effect on the ability of biofilm formation of *P. aeruginosa* ATCC 27853, while only the ethyl acetate inflorescence extract showed an effect on the biofilm formation of *P. aeruginosa* ATCC 27853. The tested extracts showed the potential for further investigation and possible application as biofungicides.

Keywords:
antimicrobial activity, antibiofilm activity, antioxidant activity, phenols, flavonoids, Asteraceae

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INTRODUCTION

An endemic of the Balkan Peninsula, *Achillea ageratifolia* subsp. *serbica* (Nyman) Heimerl belongs to the Asteraceae family. The genus *Achillea* is widespread through Europe, temperate areas of Asia, and North America (Simonetti 1990). Many studies indicate the wide application of bioactive components isolated from the representatives of the genus *Achillea* (Tuberoso *et al*. 2009; Saeidnia *et al*. 2011; Mohammadhosseini *et al*. 2017). The medicinal properties of the plant are related to the presence of specialized metabolites, such as flavonoids, phenolic acids, coumarins, terpenoids, and sterols (Saeidnia *et al*. 2011). Apigenin, luteolin, and quercetin are the most frequently identified flavonoids (Tuberoso *et al*. 2009), while *Achillea millefolium* L. is the most studied plant species of the genus *Achillea*, used in folk medicine (Fierascu *et al*. 2015; Georgieva *et al*. 2015; Shah & Peethambaran 2017; Abou Baker 2020). Phenolic acids, flavonoids, tannins, sesquiterpenes, and lactones are just some of the compounds isolated from *A. millefolium*, which are related to anti-inflammatory activity (Shah & Peethambaran 2017). *Achillea millefolium* is well-known for its usage in pharmacy and medicine, but other species that belong to this genus have been poorly investigated. Si *et al*. (2006) indicated that plant extracts of *Achillea clavennae* L., *A. holosericea* Sm., *A. lingulata* Waldst. & Kit., and *A. millefolium* have a wide spectrum of antibacterial activity against *Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa*, and *Salmonella enteritidis* and antifungal activity against *Aspergillus niger* and *Candida albicans*.

Achillea ageratifolia subsp. *serbica* is a poorly investigated species. According to the rulebook on the declaration and protection of protected and strictly protected wild species of plants, animals, and fungi (SGRS 2010-2016), this plant species is protected in Serbia. So far, there are scarce data on the medicinal properties of the plant. Mladenović & Radulović (2017) identified over 300 compounds accounting for 95.5-97.2% of the essential oils obtained from the dry parts of *A. ageratifolia* subsp. *serbica*. In terms of bioactive metabolites, so far, it has been shown that *A. ageratifolia* subsp. *serbica* is rich in flavonoids, especially 6-hydroxykaempferol 3,6-dimethyl ether and hispidulin (Valant-Vetschera & Wollenweber 2001).

Based on the fact that there is almost no scientific data related to the biological activity of this plant, the aim of the study was the evaluation of the chemical composition of the different types of extracts made from the aerial parts of the plant, as well as from the inflorescence and the root separately. The goal of the research was also to determine the antioxidant, antimicrobial and antibiofilm activity of the mentioned plant extracts in order to investigate their potential usage.

MATERIAL AND METHODS

Plant material and preparation of extracts. All of the plant samples of *A. ageratifolia* subsp. *serbica* were collected in May 2017, during the flowering season. The plant material was collected from the Kablar Mountain in Central Serbia, at 889 m above sea level. Determination was based on Gajić (1974) and Richardson (1976). The voucher specimens are deposited in the Herbarium of the Institute of Botany and Botanical Garden "Jevremovac", University of Belgrade - Faculty of Biology (BEOU) under accession number 17448.

The collected plant material was air-dried in darkness at an ambient temperature. The dried, aerial plant material was extracted by maceration with water, acetone, and ethyl acetate. The inflorescence and the root were extracted by maceration with ethyl acetate. Briefly, 25 g of plant material was soaked in 125 ml of solvent. Maceration of the aerial plant material, as well as the inflorescence and the root, was carried out three times, at room temperature, using fresh solvent every 24 hours. The obtained filtrates were evaporated to dryness using a rotary evaporator at 40°C. The obtained extracts were kept in sterile sample tubes and stored at -20°C until use.

Determination of total phenolic and flavonoid content. The total phenolic content was determined using the Folin-Ciocalteu method (Wootton-Beard *et al*. 2011) and expressed as milligram of gallic acid equivalents per gram of extract (mgGAE/g of extract). The aluminum chloride method (Quettier-Deleu *et al*. 2000) was used to determine the total flavonoid content, which was expressed as milligram of rutin equivalents per gram of extract (mgRUE/g of extract).

HPLC equipment and chromatographic analysis of phenolic compounds: RP-HPLC analysis. Phenolic acids and flavonoids in the acetone and ethyl acetate extracts from the aerial parts of *A. ageratifolia* subsp. *serbica* were identified by comparing the retention times and UV-Vis absorption spectra of unknown peaks with co-injection reference standards. The identified compounds were confirmed by spiking the sample with the standard compound and monitoring the changes in the peak shape and spectral characteristics. Both extracts were analyzed in triplicate and the data are reported as the mean ± SD. The method was described in detail in Mihailović *et al*. (2019).

GC-FID and GC-MS analyses. The chemical composition of the ethyl acetate extracts isolated from the aerial parts of *A. ageratifolia* subsp. *serbica* was investigated by GC and GC-MS analysis. The GC-MS analyses were performed in triplicate on a Hewlett-Packard 6890N gas chromatograph equipped with a DB-5MS phenyl methyl siloxane capillary column (30 m × 0.25 mm, film thick-

ness 0.25 µm, Agilent Technologies, USA) and coupled with a 5975C mass selective detector from the same company. The injector and interface were operated at 250 and 320°C, respectively. The oven temperature was raised from 70 to 315°C at a heating rate of 5°C/min and then isothermally held for 10 min. Helium was used as the carrier gas at 1.0 ml/min. The samples (5 µl of the extract solution in diethyl ether) were injected in a pulsed split mode (split ratio 40:1). The MS conditions were as follows: ionization energy 70 eV, acquisition mass range 35–700 amu, and scan time 0.34 s. GC-FID analysis was carried out under the same experimental conditions using the same column as described for the GC-MS analysis.

The extract constituents were identified by comparing their linear retention indices determined relative to the retention times of C8–C34 n-alkanes on the DB-5MS column (van den Dool & Kratz 1963) with those reported in the literature (Adams 2007), and through a comparison of their mass spectra with those from a number of commercial and in-house MS libraries (NIST11, Wiley06, MassFinder and Adams) by the application of AMDIS software (DTRA/NIST 2002). Also, a homemade MS library with the spectra corresponding to pure substances was used, and finally, wherever possible, the identification was achieved by co-injection with an authentic sample. The percentage composition was computed from the GC-FID peak areas without the use of correction factors.

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 (vitamin C) was used as a positive control. The experiment was performed in triplicate. Based on the obtained results, the percentage inhibition of DPPH radicals and the IC_{50} value were determined. The percentage of inhibition was calculated using the following equation:

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

where $A_{control}$ was the absorbance of the control sample and A_{sample} 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 concentration of samples, described in detail in Vasić *et al.* (2012).

Antimicrobial activity. The antimicrobial activity of the tested extracts was tested against 10 strains of bacteria (5 strains of Gram-positive bacteria: *Bacillus pumilus* NCTC 8241, *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 25923 and clinical isolates of *Bacillus subtilis* and *Staphylococcus aureus*; 5 strains of Gram-negative bacteria: *Pseudomonas aeruginosa* ATCC 27853, *Proteus mirabilis* ATCC 12453, *Escherichia coli* ATCC 25922, and clinical isolates of *Salmonella enterica* and *Escherichia coli*; and 2 strains of yeast: *Candida albicans* ATCC 10231 and clinical isolate *Rhodotorula mucilaginosa*). 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 of microorganisms were prepared by the direct colony method. The turbidity of the initial suspension was adjusted using a 0.5 McFarland densitometer (DEN-1, BioSan, Latvia). The initial bacterial and yeast suspensions were additionally diluted in 1:100 and 1:1000 ratio in sterile 0.85% saline.

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 the 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 yeast. The tested concentration range was from 5000 to 39 µg/ml. Doxycycline and fluconazole, dissolved in a nutrient liquid medium, were used as positive controls. Stock solutions of crude extracts obtained by dissolving in 10% DMSO were used as negative controls. Each test included growth control and sterility control. All tests were performed in duplicate.

Antibiofilm activity. The antibiofilm activity on *S. aureus* ATCC 6538 and *P. aeruginosa* ATCC 27853 was tested with the method described in O'Toole *et al.* (2000). In 96-well microtiter plates 100 µl of TBS (tryptone soy broth) broth was added. 100 µl of the tested extract was added into the first row of the plates, with an initial concentration of 10000 µg/ml, and serial two-fold dilutions were made down to 80 µg/ml. Subsequently, 10 µl of bacterial suspension (0.5 McFarland) was added into each well. The microtiter plates were then incubated for 48 hours at 37°C. The rest of the experiment was done as described in Muruzović *et al.* (2016).

The difference in the procedure of measuring the effect on the formed biofilm is that varying concentrations of the extract were added to the microtiter plates with the biofilm formation of the tested bacteria. The results were expressed as biofilm inhibitory concentration, (BIC_{50}), defined as the lowest concentration of extract that showed 50% inhibition on the biofilm formation (Chaieb *et al.* 2011). All tests were performed in duplicate and BIC_{50} was calculated. Tetracycline was used as a positive control.

Data analysis. All data were presented as means ± standard deviations where appropriate, using Microsoft Excel (Redmond, Washington, DC, USA). The paired–samples t-test was used to compare the content of phenols and flavonoids in different types of extracts. The data were analyzed using SPSS version 20 software (SPSS Inc., Chicago, IL, USA).

RESULTS

The highest percentage of extraction yield was recorded from the water extract of the aerial parts of *A. ageratifolia* subsp. *serbica* (16.16%), while the lowest was obtained from the ethyl acetate extract of the root (0.95%). It could be concluded that the extraction yield was solvent-dependent.

The total phenol and flavonoid content in the tested extracts. Specialized plant metabolites are soluble in different solvents, and this was the criteria for selecting the water, acetone and ethyl acetate for the extraction of phenols and flavonoids.

The highest content of total phenolic compounds was obtained in the acetone extract from the aerial parts (43.78 mgGAE/g), while the lowest content was measured in the water extract from the aerial parts (18.61 mg GAE/g) (Table 1).

The highest flavonoid content was obtained in the acetone extract from the aerial parts (34.02 mgRUE/g), while the lowest content was obtained in the ethyl acetate extract of the root (10.79 mgRUE/g) (Table 1). Generally, the highest phenol and flavonoid content was measured in the acetone extract from the aerial parts of the plant (43.78 mgGAR/g and 34.02 mgRUE/g).

RP-HPLC analysis of the extracts. Based on the results of the HPLC analysis, both the acetone and ethyl acetate extracts of the aerial parts of *A. ageratifolia* subsp. *serbica* shared a similar phenolic composition containing chlorogenic acid, myricetin, apigenin, and luteolin (Figs. 1, 2). The main difference in their phenolic composition was that caffeic acid was identified in the ethyl acetate extract only.

All the identified phenolic compounds were found in higher amounts in the acetone extract than in the ethyl acetate extract, with the exception of caffeic acid, which was

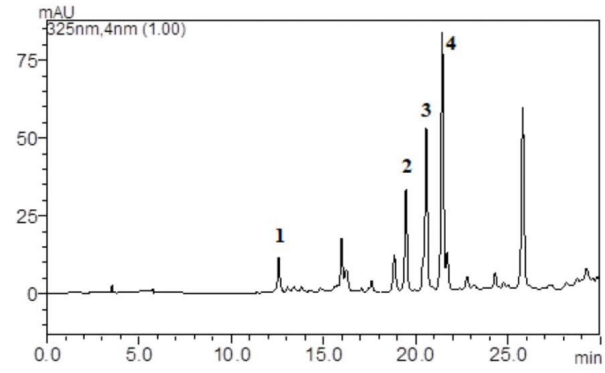

Fig. 1. HPLC chromatogram of the acetone extract from the aerial parts of *Achillea ageratifolia* subsp. *serbica* (1-chlorogenic acid, 2-myricetin, 3-apigenin, 4-luteolin).

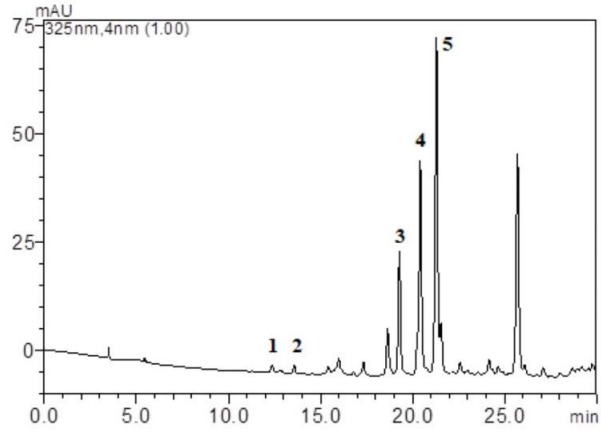

Fig. 2. HPLC chromatogram of the ethyl acetate extract from the aerial parts of *Achillea ageratifolia* subsp. *serbica* (1-chlorogenic acid, 2-caffeic acid, 3-myricetin, 4-apigenin, 5-luteolin).

Table 1. The total phenol and flavonoid content.

Ordinal number of extracts	Type of extracts	Phenols* (mgGAE/g of extract)	Flavonoids* (mgRUE/g of extracts)	IC_{50}* (μg/ml)
1	Aerial parts – water	18.61 ± 1.99[a]	10.90 ± 0.74[b]	641.06 ± 2.40
2	Aerial parts – acetone	43.78 ± 4.88[c]	34.02 ± 6.52[d]	732.07 ± 6.47
3	Aerial parts – ethyl acetate	28.67 ± 1.59[e]	27.86 ± 0.47[e]	988.78 ± 2.80
4	Inflorescence – ethyl acetate	30.11 ± 2.94[f]	29.31 ± 1.29[f]	825.60 ± 2.60
5	Root – ethyl acetate	33.00 ± 4.69[g]	10.79 ± 1.07[h]	675.33 ± 2.21
6	Ascorbic acid (vitamin C)	/	/	5 ± 2.64

*mean value ± standard deviation; different letters indicate significant differences between the mean values of phenols and flavonoids for different extract types

found in the ethyl acetate extract only (Table 2). The dominant component in both extracts was luteolin, with a higher content (9.663 mg/g DW) in the acetone extract than in the ethyl acetate extract (9.109 mg/g DW). Flavonoid apigenin was found in the acetone extract at a concentration of 1.500 mg/g DW, while in the ethyl acetate extract it presented at 1.162 mg/g DW. Flavonoid myricetin was also present in both extracts, but with a four times higher concentration in the acetone extract. The acetone extract contained a higher amount of chlorogenic acid than the ethyl acetate extract (1.249 and 0.398 mg/g DW, respectively), while an inconsiderable amount of caffeic acid was found in the ethyl acetate extract only (Table 2).

GC-FID and GC-MS analyses. A detailed chemical compositional analysis of the metabolites in the ethyl acetate extract of the dry aerial parts of *A. ageratifolia* subsp. *serbica* allowed for the identification of thirty-two compounds, accounting for almost 80% of the peak areas in the gas chromatogram (Table 3). The volatile fraction of the extract was dominated by common wax constituents. The *n*-alkanes comprised more than half of the composition, with nonacosane (37.3%) and hentriacontane (9.8%) as the most abundant. The *n*-alkane distribution followed the usual pattern found in higher plants (tracheophytes) – of high carbon preference index (CPI) with odd-numbered carbon dominance centered at n-C_{29} of a Gaussian-like curve (Radulović *et al.* 2012).

The analysis also showed the presence of steroid compounds with a stigmastane nucleus and of triterpenes widespread in terrestrial plants – stigmasta-5,22-dien-3β-ol (stigmasterol, 1.0%), stigmast-5-en-3β-ol (β-sitosterol, 3.4%) and α-amyrin (2.9%).

Antioxidant activity of the extracts. The highest antioxidant activity was detected in the water extract from the aerial parts of the plant (IC_{50} = 641.06 μg/ml) and the ethyl acetate extract of the root (IC_{50} = 675.33 μg/ml) (Table 1). The lowest activity was detected for the ethyl acetate extract from the aerial parts of the plant (IC_{50} = 988.78 μg/ml). Compared to the positive control (vitamin C IC_{50} = 5 μg/ml), the tested extract showed limited antioxidant activity.

Antimicrobial activity. The intensity of the antibacterial activity of the tested extracts depended on the species of bacteria and the type of extract (Table 4). The values of MIC and MBC were in the range from 39 μg/ml to >5 000 μg/ml. Gram-positive bacteria showed higher sensitivity to the tested extracts than Gram-negative bacteria. Clinical isolates of *B. subtilis* and *S. aureus*, as well as *S. aureus* ATCC 25923 were sensitive to the tested extracts (MIC 39-156 μg/ml, MBC 39-317 μg/ml), with the exception of the water extract from the aerial parts. The ethyl acetate extract of the root showed the highest effect on *B. subtilis* (MIC 39 μg/ml), while the acetone extract from the aerial parts showed the same effect on both *B. subtilis* and *S. aureus*. The ethyl acetate extracts of the inflorescence and root showed an inhibitory effect on *S. aureus* and *S. aureus* ATCC 25923 (MIC from 78 to 156 μg/ml). The acetone extract from the aerial parts of the plant exhibited an effect on certain Gram-negative bacteria. For *E. coli* ATCC 25922, the MIC value was 156 μg/ml, while for *E. coli* and *S. enterica*, MIC was 317 μg/ml.

The MIC and MFC values for the tested yeasts were in the range from 156 μg/ml to 5 000 μg/ml (Table 4). *Rhodotorula mucilaginosa* showed sensitivity to the ethyl acetate extract from the aerial parts and the root of the plant (MIC 156 μg/ml), while *C. albicans* ATCC 10231 was sensitive to the ethyl acetate extract of the inflorescence and root (MIC 317 μg/ml). When comparing the results with the positive control, the ethyl acetate extract from the aerial parts, the inflorescence, and the root, as well as the acetone extract from the aerial parts of the plant showed noticeable antifungal activity.

Table 2. The composition of the ethyl acetate extract from the aerial parts of *Achillea ageratifolia* subsp. *serbica*.

Compound	t_r (min)	Concentration*	
		Aerial parts (acetone)	Aerial parts (ethyl acetate)
Chlorogenic acid	13.00	1.249 ± 0.04[a]	0.398 ± 0.008[b]
Caffeic acid	13.58	-	0.346 ± 0.008
Myricetin	19.62	2.297 ± 0.06[c]	0.501 ± 0.009[d]
Apigenin	20.42	1.500 ± 0.06[e]	1.162 ± 0.05[f]
Luteolin	21.30	9.663 ± 0.10[g]	9.109 ± 0.11[h]

*mean value (mg/g dry extract) ± standard deviation; different letters indicate significant differences between the mean values of phenols and flavonoids for different extract types

Table 3. The phenolic composition of the aerial parts of *Achillea ageratifolia* subsp. *serbica*.

RI calc.[a]	Compound	Content [%][b]	Identification Method
801	Hexanal	T	RI,MS,Co-GC
1040	Lavender lactone	T	RI,MS
1148	Camphor	1.4	RI,MS,Co-GC
1168	Borneol	T	RI,MS,Co-GC
1288	Bornyl acetate	T	RI,MS,Co-GC
1587	Caryophyllene oxide	T	RI,MS,Co-GC
1592	1-Hexadecene	T	RI,MS
1792	1-Octadecene	T	RI,MS
1845	Hexahydrofarnesyl acetone	0.5	RI,MS
1966	Hexadecanoic acid	2.9	RI,MS,Co-GC
1993	1-Eicosene	0.6	RI,MS
2137	(*Z*,*Z*)-9,12-Octadecadienoic acid	1.2	RI,MS,Co-GC
2143	(*Z*)-9-Octadecenoic acid	1.8	RI,MS,Co-GC
2245	Unidentified component	3.9	
2300	Tricosane	T	RI,MS,Co-GC
2327	Unidentified component	2.1	
2337	Unidentified component	2.0	
2353	5-Methyl-5-(4,8,12-trimethyltridecyl) dihydro-2(3*H*)-furanone	T	RI,MS
2400	Tetracosane	T	RI,MS,Co-GC
2410	Eicosanyl acetate	0.7	RI,MS
2500	Pentacosane	1.0	RI,MS,Co-GC
2529	Unidentified component	2.5	
2554	Unidentified component	1.1	
2600	Hexacosane	T	RI,MS,Co-GC
2612	Docosyl acetate	1.1	RI,MS
2618	Unidentified component	1.3	
2700	Heptacosane	4.6	RI,MS,Co-GC
2800	Octacosane	1.3	RI,MS,Co-GC
2813	Tetracosyl acetate	0.4	RI,MS
2862	Unidentified component	1.8	
2900	Nonacosane	37.3	RI,MS,Co-GC
3000	Triacontane	1.0	RI,MS,Co-GC
3100	Hentriacontane	9.8	RI,MS,Co-GC
3141	α-Tocopherol	T	RI,MS,Co-GC
3157	Sesamin	3.5	RI,MS
3211	Octacosyl acetate	1.4	RI,MS
3255	Stigmasta-5,22-dien-3β-ol	1.0	RI,MS,Co-GC
3304	Stigmast-5-en-3β-ol	3.4	RI,MS,Co-GC
3360	α-Amyrin	2.9	RI,MS
	Identified	77.8	
	Unidentified (>1%)	14.7	
	Total	**92.5**	

[a] Linear retention indices experimentally determined on the DB-5MS column; [b] values are mean values from three individual analyses; RI – retention indices matching with literature data; MS – mass spectra matching; Co-GC – co-injection with pure reference compound; [c] MS, 70eV, 230 °C: RI 2245, *m/z* (rel. int.): 133 (12), 105(19), 93(15), 91(20), 79(22), 71(24), 67(15), 55(16), 43(100), 41(16); RI 2327, *m/z* (rel. int.): 244 (19), 120(15), 105(14), 97(14), 91(29), 77(21), 67(14), 43(100), 41(22), 39(14); RI 2337, *m/z* (rel. int.): 148(34), 121(25), 120(95), 91(74), 92(40), 77(32), 67(22), 43(100), 41(25), 39(23); RI 2529, *m/z* (rel. int.): 165(41), 164(71), 150(43), 98(100), 70(50), 69(49), 67(43), 66(77), 55(82), 41(85); RI 2554, *m/z* (rel. int.): 164(19), 150(100), 98(23), 81(51), 70(28), 66(27), 67(21), 55(37), 43(41), 41(37); RI 2618, *m/z* (rel. int.): 152(94), 124(42), 113(100), 98(51), 81(53), 70(47), 68(42), 67(63), 43(54), 41(70); RI 2862, *m/z* (rel. int.): 91(54), 85(49), 77(19), 70(22), 57(95), 56(20), 55(41), 43(100), 42(20), 41(56); t – Trace amounts (<0.05%).

Table 4. The antibacterial and antifungal activity of the *Achillea ageratifolia* subsp. *serbica* extracts.

Type of extracts /positive controls	Aerial parts (water)		Aerial parts (acetone)		Aerial parts (ethyl acetate)		Inflorescence (ethyl acetate)		Root (ethyl acetate)		Doxycicline / Fluconazole	
Species	MIC*	MMC*	MIC	MMC	MIC	MMC	MIC	MMC	MIC	MMC	MIC	MMC
B. pumilus NTCC 8241	>5000	>5000	2500	5000	1250	1250	2500	2500	5000	5000	0.112	7.81
B. subtilis	156	156	39	39	39	78	156	156	39	39	0.112	1.953
B. subtilis ATCC 6633	>5000	>5000	317	2500	5000	5000	5000	>5000	>5000	>5000	1.953	31.25
S. aureus	>5000	>5000	39	39	156	317	156	317	78	156	0.448	7.81
S. aureus ATCC 25923	5000	5000	156	156	156	317	156	317	78	156	0.224	3.75
P. aeruginosa ATCC 27853	>5000	>5000	2500	>5000	2500	5000	5000	>5000	5000	>5000	62.5	125
P. mirabilis ATCC 12453	2500	>5000	2500	5000	2500	5000	5000	5000	5000	5000	15.625	62.5
E. coli	>5000	>5000	317	5000	5000	5000	>5000	>5000	>5000	>5000	7.81	15.63
E. coli ATCC 25922	>5000	>5000	156	2500	5000	5000	5000	>5000	>5000	>5000	15.625	31.25
S. enterica	>5000	>5000	317	5000	5000	5000	5000	>5000	>5000	>5000	15.625	31.25
R. mucilaginosa	2500	5000	625	2500	156	1250	625	5000	156	2500	62.5	1000
C. albicans ATCC 10231	5000	5000	625	1250	625	625	317	2500	317	1250	31.25	1000

*minimal inhibitory concentration (MIC) and minimal microbiocidal concentration (MMC). The values are given in μg/ml

Antibiofilm activity. The results of *in vitro* antibiofilm activity indicated that the tested extracts of *A. ageratifolia* subsp. *serbica* showed no effect on the biofilm formation ability of *S. aureus* ATCC 25923 (BIC_{50} over 10000 μg/ml). The exception was the ethyl acetate extract from the aerial parts of the plant, with BIC_{50} at 1770.7 μg/ml, thus marking the highest score in the research of antibiofilm activity. All the tested extracts 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). Tetracycline (positive control) showed activity on the biofilm formation of *S. aureus* ATCC 25923 (BIC_{50} at 250 μg/ml) and on *P. aeruginosa* ATCC 27853 (BIC_{50} at 156 μg/ml).

DISCUSSION

In this study, the chemical composition, antioxidant activity, and antimicrobial properties of different *A. ageratifolia* subsp. *serbica* extracts were analyzed for the first time. In addition, the *in vitro* activity of *A. ageratifolia* subsp. *serbica* extracts on the biofilm formation and formed biofilm of *S. aureus* ATCC 25923 and *P. aeruginosa* ATCC 27853 was also examined for the first time.

The total phenolic and flavonoid content investigated in the methanolic extract of *Achillea wilhelmsii* C. Koch indicated that the total phenolic content was 37.4 mg-GA/g, while the total flavonoid content was 2.5 mgRU/g (Fathi *et al.* 2011). Our results indicate that the total phenolic and flavonoid content in the acetone extract from the aerial parts of *A. ageratifolia* subsp. *serbica* was higher than the methanolic extract of *A. wilhelmsii.* Also, the total phenolic content in all the extracts from the aerial parts of *A. ageratifolia* subsp. *serbica* was lower than the total phenolic content in the methanolic extracts of the aerial parts of *Achillea coarctata* Poir., *Achillea kotschyi* Boiss., and *Achillea lycaonica* Boiss. et Heldr. (Agar *et al.* 2015). The reason for these differences may be found in the solubility of the investigated content or in the different habitats of the investigated species.

To our knowledge, there are no available data about the phenolic composition of *A. ageratifolia* subsp. *serbica*. The ethyl acetate extract of the best known and most investigated *Achillea* species, *A. millefolium,* showed the presence of some common constituents: chlorogenic acid (1.45 mg/g DW), caffeic acid (0.02 mg/g DW), and apigenin (1.03 mg/g DW) (Abou Baker 2020). Compared with the *A. ageratifolia* subsp. *serbica* extract, the *A. millefolium* extract possessed a higher content of chlorogenic acid, while the amounts of caffeic acid and apigenin were significantly lower.

Afshari *et al.* (2018) tested methanolic extracts of different *Achillea* species, e.g. *A. santolina* L., *A. millefolium*, *A. aucheri* Boiss., *A. nobilis* L., *A. filipendulina* Lam., and

A. pachycephala Rech.f. The common constituents found in all the tested extracts were chlorogenic acid, caffeic acid, luteolin, and apigenin. The ethyl acetate extract of *A. ageratifolia* subsp. *serbica* contained a higher amount of chlorogenic acid (0.398 mg/g DW) than the *A. santolina* (38.22 mg/100 g DW) and *A. millefolium* extracts (2.84 mg/100 g DW), but lower than the *A. aucheri* (84.56 mg/100 g DW), *A. nobilis* (105.17 mg/100 g DW), *A. filipendulina* (124.23 mg/100 g DW), and *A. pachycephala* (112.88 mg/100 g DW) extracts. The concentration of chlorogenic acid in the acetone extract of *A. ageratifolia* subsp. *serbica* (1.249 mg/g DW) was significantly higher compared with all the *Achillea* species tested in the above-mentioned paper. Caffeic acid was found in significantly lower concentrations in the *Achillea* species reported by Afshari *et al.* (2018) compared with the *A. ageratifolia* subsp. *serbica* ethyl acetate extract, with the exception of *A. millefolium*, where it was slightly higher (41.99 mg/100 g DW). The most significant difference among all the tested *Achillea* species can be observed in the content of the flavonoids apigenin and luteolin. A comparison of the phenolic composition of the *A. ageratifolia* subsp. *serbica* ethyl acetate and the acetone extracts from *Achillea* species examined by Afshari *et al.* (2018), showed that only the *A. filipendulina* methanolic extract contained a similar amount of apigenin (151.50 mg/g DW), while all the other species possessed significantly (up to 12-fold) lower amounts. The dominant component found in the *A. ageratifolia* subsp. *serbica* ethyl acetate and acetone extracts, luteolin (9.109 and 9.663 mg/g DW, respectively), was significantly higher compared with all the tested *Achillea* species: *A. santolina* (6.17 mg/100 g DW), *A. millefolium* (24.77 mg/100 g DW), *A. aucheri* (0.53 mg/100 g DW), *A. nobilis* (25.88 mg/100 g DW), *A. filipendulina* (2.74 mg/100 g DW), and *A. pachycephala* (56.11 mg/100 g DW), thus suggesting that *A. ageratifolia* subsp. *serbica* may be a significant source of flavonoids.

The analysis of *A. ageratifolia* subsp. *serbica* could be important in understanding its potential biological activities and possible pharmaceutical usage. The *n*-alkane distribution followed the usual pattern found in higher plants (tracheophytes) – of high carbon preference index (CPI) with odd-numbered carbon dominance centered at $n\text{-}C_{29}$ of a Gaussian-like curve (Radulović *et al.* 2012). Further, the presence of steroid compounds with a stigmastane nucleus and of triterpenes, widespread in terrestrial plants, was also detected (Hernández Vázquez *et al.* 2012; Valitova *et al.* 2016).

Fathi *et al.* (2011) investigated and demonstrated the antioxidant activity of the methanolic extract of *A. wilhelmsii* (IC_{50} = 58.9 ± 2.7 μg/ml). Agar *et al.* (2015) researched the antioxidant activity of methanolic extracts of *A. coarctata*, *A. kotschyi,* and *A. lycaonica* grown in Turkey, suggesting the strong radical scavenging activity of this plant species. In the aforementioned study by Afshari *et al.* (2018), the IC_{50} values in the samples ranged from 365.5 μg/ml (*A. pachycephala*) to 854.1 μg/ml (*A. millefolium*). Comparing the activities of the investigated plant extracts, it can be seen that the extracts of *A. ageratifolia* subsp. *serbica s*howed moderate antioxidant activity. However, some extracts of *A. ageratifolia* subsp. *serbica s*howed higher antioxidant activity than the methanolic extract of *A. millefolium* from the study by Afshari *et al.* (2018).

Some researchers have shown that extracts from the aerial parts of the following plants: *A. clavennae*, *A. holosericea*, *A. lingulata* and *A. millefolium,* exhibit a wide spectrum of antimicrobial activity related to certain bacteria (*S. aureus*, *E. coli*, *K. pneumoniae*, *P. aeruginosa* and *S. enteritidis*) and fungi (*Aspergillus niger* and *C. albicans*) (Stojanović *et al.* 2005; Si *et al.* 2006). Mohammadi-Sichani *et al.* (2011) demonstated the inhibitory effects of the methanolic extract from the aerial parts of *A. wilhelmsii* on the growth of *S. aureus*, *B. cereus*, *E. coli* and *P. aeroginosa* (MIC values from 6.25 to 25 mg/ml). Fierascu *et al.* (2015) showed the very important *in vitro* antifungal activity of the hydroalcoholic extract of *A. millefolium*, which is in correlation with the results from *A. ageratifolia* subsp. *serbica.*

It is known that plant extracts can inhibit the biofilm formation of *P. aeruginosa* ATCC 27853 and *S. aureus* ATCC 25923 (Perumal & Mahmud 2013; Muruzović *et al.* 2016). There are some data about the effects of *Achillea ageratum* L. essential oil on the biofilm formation of *C. albicans* (BIC 60% was 9 μg/ml) (Vavala *et al.* 2009). However, the antibiofilm activity of extracts of *A. ageratifolia* subsp. *serbica* has not been investigated to date.

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 specialized metabolites, especially chlorogenic acid, myricetin, apigenin and luteolin. Thirty-two compounds were identified using GC-FID and GC-MS analyses. However, the antioxidant activity of the tested extracts was moderate. The antibacterial activity of the tested extracts was shown against *B. subtilis* and *S. aureus,* and the antifungal activity of the extracts was significant. The tested extracts showed moderate antibiofilm activity on the biofilm formation ability. Based on the results, it could be concluded that the tested extracts of *A. ageratifolia* subsp. *serbica* may be a significant source of flavonoids, which is important due to their high biological importance and proven health benefits. Also, the marked extracts showed a potential for application as biofungicides. Since the collection of this species from natural habitats is not allowed in our country, collection and potential usage should be based on plants cultivated in nurseries, and not on those collected from the wild. The additional effects of *A. ageratifolia* subsp. *serbica* extracts and their fine mechanisms of action will be disclosed in future studies.

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REFERENCES

Abou Baker DH. 2020. *Achillea millefolium* L. ethyl acetate fraction induces apoptosis and cell cycle arrest in human cervical cancer (HeLa) cells. *Annals of Agricultural Sciences* **65**: 42–48.

Adams RP. 2007. *Identification of essential oil components by gas chromatography/mass spectrometry, 4th ed.* Allured Publishing Corp., Carol Stream, IL.

Afshari M, Rahimmalek M & Miroliaei M. 2018. Variation in polyphenolic profiles, antioxidant and antimicrobial activity of different *Achillea* species as natural sources of antiglycative compounds. *Chemistry & Biodiversity* **15**: e1800075.

Agar OT, Dikmen M, Ozturk N, Yilmaz MA, Temel H & Turkmenoglu FP. 2015. Comparative studies on phenolic composition, antioxidant, wound healing and cytotoxic activities of selected *Achillea* L. species growing in Turkey. *Molecules* **20**: 17976–18000.

Chaieb K, Kouidhi B, Jrah H, Mahdouani K & Bakhrouf A. 2011. Antibacterial activity of thymoquinone, an active principle of *Nigella sativa* and its potency to prevent bacterial biofilm formation. *BMC Complementary and Alternative Medicine* **11**: 29.

Fathi H, Aghaee L & Ebrahimzadeh MA. 2011. Antioxidant activity and phenolic contents of *Achillea wilhemsii*. *Pharmacology Online* **2**: 942–949.

Fierascu I, Ungureanu C, Avramescu SM, Fierascu RC, Ortan A, Soare LC & Paunescu A. 2015. *In vitro* antioxidant and antifungal properties of *Achillea millefolium* L. *Romanian Biotechnological Letters* **20**: 10626–10636.

Gajić M. 1974. *Achillea* L. In: Josifović M (ed.), *Flora SR Srbije* **7**, pp. 90–110, Serbian Academy of Sciences and Arts, Belgrade.

Georgieva L, Gadjalova A, Mihaylova D & Pavlov A. 2015. *Achillea millefolium* L. - phytochemical profile and *in vitro* antioxidant activity. *International Food Research Journal* **22**: 1347–1352.

Hernández Vázquez L, Palazon J & Navarro-Ocaña A. 2012. *The pentacyclic triterpenes α, β-amyrins: A review of sources and biological activities*. Phytochemicals, Venketeshwer Rao, Intech Open.

Mihailović NR, Mihailović VB, Ćirić AR, Srećković NZ, Cvijović MR & Joksović LG. 2019. Analysis of wild raspberries (*Rubus idaeus* L.): Optimization of the ultrasonic-assisted extraction of phenolics and a new insight in phenolics bioaccessibility. *Plant Foods and Human Nutrition* **74**: 399–404.

Mladenović MZ & Radulović NS. 2017. The essential oil of *Achillea ageratifolia* (Sm.) Boiss. var. *serbica* (Nyman) Heimerl (Asteraceae) revisited: the stereochemical nomenclature issues, structural elucidation and synthesis of (new) sabinyl esters. *Flavour and Fragrance Journal* **32**: 5–23.

Mohammadhosseini M, Sarker SD & Akbarzadeh A. 2017. Chemical composition of the essential oils and extracts of *Achillea* species and their biological activities: A review. *Journal of Ethnopharmacology* **199**: 257–315.

Mohammadi-Sichani M, Amjad L & Mohammadi-Kamalabadi M. 2011. Antibacterial activity of methanol extract and essential oil of *Achillea wilhelmsii* against pathogenic bacteria. *Zahedan Journal of Research in Medical Sciences* **13**: 9–14.

Muruzović M, Mladenović K, Stefanović O, Vasić S & Čomić L. 2016. Extracts of *Agrimonia eupatoria* L. as sources of biologically active compounds and evaluation of their antioxidant, antimicrobial and antibiofilm activity. *Journal of Food and Drug Analysis* **24**: 539–547.

O'Toole G, Kaplan HB & Kolter R. 2000. Biofilm formation as microbial development. *Annual Review of Microbiology* **54**: 49–79.

Perumal S & Mahmud R. 2013. Chemical analysis, inhibition of biofilm formation and biofilm eradication potential of *Euphorbia hirta* L. against clinical isolates and standard strains. *BMC Complementary and Alternative Medicine* **13**: 346.

Quettier-Deleu C, Gressier B, Vasseur J, Dine T, Brunet C, Luyckx M, Cazin M, Cazin JC, Bailleul F & Trotin F. 2000. Phenolic compounds and antioxidant activities of buckwheat (*Fagopyrum esculentum* Moench) hulls and flour. *Journal of Ethnopharmacology* **72**: 35–42.

Radulović N, Denić M, Stojanović-Radić Z & Skropeta D. 2012. Fatty and volatile oils of the gypsywort *Lycopus europaeus* L. and the Gaussian-like distribution of its Wax Alkanes. *Journal of the American Oil Chemists' Society* **89**: 2165–2185.

Richardson IB. 1976. *Achillea* L. In: Tutin TG, Heywood VH, Burges NA, Moore DM, Valentine DH, Walters SM & Webb DA (eds.), *Flora Europaea* **4**, pp. 159–165, University Press, Cambridge.

Saeidnia S, Gohari AR, Mokhber-Dezfuli N & Kiuchi F. 2011. A review on phytochemistry and medicinal properties of the genus *Achillea*. *DARU Journal of Pharmaceutical Sciences* **19**: 173–186.

Sarker SD, Nahar L & Kumarasamy Y. 2007. Microtitre plate-based antibacterial assay

incorporating resazurin as an indicator of cell growth, and its application in the in vitro antibacterial screening of phytochemicals. *Methods* **42**: 321–324.

SGRS. 2010-2016. Pravilnik o proglašenju i zaštiti strogo zaštićenih i zaštićenih divljih vrsta biljaka, životinja i gljiva. *Službeni Glasnik RS* 5/2010, 47/2011, 32/2016, 98/2016**.**

Shah R & Peethambaran B. 2017. Anti-inflammatory and anti-microbial properties of *Achillea millefolium* in acne treatment. In: Chatterjee S, Jungraithmayr W & Bagchi D (eds.), *Immunity and inflammation in health and disease: emerging roles of nutraceuticals and functional foods in immune support*, pp. 241–248, Academic Press.

Si XT, Zhang ML, Shi QW & Kiyota H. 2006. Chemical constituents of the plants in the genus *Achillea*. *Chemistry and Biodiversity* **3**: 1163–1180.

Simonetti G. 1990. *Simon & Schuster's Guide to Herbs and Spices*. Simon & Schuster, Inc. New York.

Stojanović G, Radulović N, Hashimoto T & Palić R. 2005. *In vitro* antimicrobial activity of extracts of four *Achillea* species: The composition of *Achillea clavennae* L. (Asteraceae) extract. *Journal of Ethnopharmacology* **101**: 185–190.

Takao T, Watanabe N, Yagi I & Sakata K. 1994. A simple screening method for antioxidant and isolation of several antioxidants produced by marine bacteria from fish and shellfish. *Bioscience, Biotechnology, and Biochemistry* **58**: 1780–1783.

Tuberoso CIG, Montoro P, Piacente S, Corona G, Deiana M, Dessi MA & Cabras P. 2009. Flavonoid characterization and antioxidant activity of hydroalcoholic extracts from *Achillea ligustica* All. *Journal of Pharmaceutical and Biomedical Analysis* **50**: 440–448.

Valant-Vetschera KM & Wollenweber E. 2001. Exudate flavonoid aglycones in the alpine species of *Achillea* sect. *Ptarmica*: Chemosystematics of *A. moschata* and related species (Compositae–*Anthemideae*). *Biochemical Systematics and Ecology* **29**: 149–59.

Valitova JN, Sulkarnayeva AG & Minibayeva FV. 2016. Plant sterols: Diversity, biosynthesis, and physiological functions. *Biochemistry* (Moscow) **81**: 819–834.

van den Dool H & Kratz PD. 1963. A generalization of the retention index system including linear temperature programmed gas—liquid partition chromatography. *Journal of Chromatography A* **11**: 463–471.

Vasić SM, Stefanović OD, Ličina BZ, Radojević ID & Čomić LR. 2012. Biological activities of extracts from cultivated Granadilla *Passiflora alata*. *Excli Journal* **11**: 208–218.

Vavala E, Ragno R, Sivric S, Sartorelli G, Filippi A, Palamara AT & Angiolella L. 2009. Antimycotic activity of *Achillea ageratum* L. essential oil. *International Journal of Essential Oil Therapy* **3**: 101–105.

Wootton-Beard PC, Moran A & Ryan L. 2011. Stability of the total antioxidant capacity and total polyphenol content of 23 commercially available vegetable juices before and after in vitro digestion measured by FRAP, DPPH, ABTS and Folin-Ciocalteu methods. *Food Research International* **44**: 217–224.

REZIME

Botanica SERBICA

Fitohemijski sastav i biološka aktivnost različitih vrsta ektrakata *Achillea ageratifolia* subsp. *serbica*

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Achillea ageratifolia subsp. *serbica* je endemit sa područja Balkana koji je slabo istražen i gotovo da nema podataka o potencijalnoj primeni ove biljke. Svrha ove studije bila je istraživanje fitohemijskog sastava kao i antioksidativne, antimikrobne i antibiofilm aktivnosti vodenog, acetonskog i etil acetatnog ekstrakta spomenute biljke. Sastav fenola i flavonoida je utvrđen korišćenjem HPLC-PDA analize, dok je hemijski sastav etil acetatnog ekstrakta nadzemnog dela biljke istražen korišćenjem GC i GC-MS. Ispitivana je antioksidativna i antimikrobna aktivnost, kao i inhibicija inhibicija sposobnosti formiranja i uticaj na formirani biofilm *Staphilococcus aureus* ATCC 25923 i *Pseudomonas aeruginosa* ATCC 27853.Ukupni sadržaj fenola kretao se u rasponu od 18,61 mgGAE/g ekstrakta do 43,78 mgGAE/g ekstrakta. Ukupni sadržaj flavonoida kretao se u rasponu od 10,79 mgRUE/g ekstrakta do 34,02 mgRUE/g ekstrakta. Acetonski i etil acetatatni ekstrakti nadzemnog dela biljke sadrže hlorogenu kiselinu, miricetin, apigenin i luteolin. Analiza isparljivih sekundarnih metabolita etil acetatnog ekstrakta nadzemnog dela biljke omogućila je identifikaciju trideset i dva sastojka. Najbolju antioksidativnu aktivnost pokazao je vodeni ekstrakt nadzemnog dela biljke (IC_{50} = 641,06 μg/ml) i etil acetatatni ekstrakt korena (IC_{50} = 675,33 μg/ml). *Bacillus subtilis* i *S. aureus* su pokazali osetljivost na testirane ekstrakte, dok je antifungalna aktivnost svih ekstrakata bila značajna. Ekstrakti su pokazali umeren inhibitorni efekat na sposobnost formiranja biofilma *P. aeruginosa* ATCC 27853, dok je samo etil acetatni ekstrakt biljne cvasti pokazao uticaj na formirani biofilm *P. aeruginosa* ATCC 27853. Testirani ekstrakti pokazali su potencijal za dalje ispitivanje i potencijalnu primenu kao biofungicidi.

Ključne reči: antimikrobna aktivnost, antibiofilm aktivnost, antioksidativno delovanje, fenoli, flavonoidi, Asteraceae