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SECONDARY METABOLITE CONTENT AND *IN VITRO* BIOLOGICAL EFFECTS OF *AJUGA CHAMAEPITYS* (L.) SCHREB. SUBSP. *CHAMAEPITYS*

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Abstract: The antioxidant and antimicrobial activities and contents of total phenolics and flavonoids of *Ajuga chamaepitys* (L.) Schreb. subsp. *chamaepitys* (Lamiaceae) were investigated. Five different extracts from aboveground flowering plant parts were obtained by extraction with water, methanol, acetone, ethyl acetate and petroleum ether. The total phenolic content was determined spectrophotometrically using the Folin-Ciocalteu reagent and expressed as the gallic acid equivalent (mg GA/g of extract). The highest value was obtained in the ethyl acetate extract (57.02 mg GA/g). The concentration of flavonoids, determined using a spectrophotometric method with aluminum chloride and expressed as the rutin equivalent (mg RU/g of extract), was highest in the ethyl acetate extract (91.76 mg RU/g). The antioxidant activity was determined *in vitro* using 2,2-diphenyl-1-picrylhydrazyl (DPPH) reagent. The highest antioxidant activity was detected in the acetone extract (SC_{50} value = 330.52 μg/mL). *In vitro* antimicrobial activities were determined using a microdilution method, and the minimum inhibitory concentration (MIC) and minimum microbicidal concentration (MMC) were determined. The most effective antimicrobial activity against *Bacillus cereus* was demonstrated by the acetone extract, with MIC and MMC values of 1.25 mg/mL. Based on the results of this study, *A. chamaepitys* subsp. *chamaepitys* could be considered as a valuable source of natural compounds with important biological activities.

Key words: *Ajuga chamaepitys*; phenolics; flavonoids; antioxidants; antimicrobial

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INTRODUCTION

The harmful effects and problems caused by oxidative stress and an increased production of reactive oxygen species (ROS) can be ameliorated by antioxidants. For this purpose, synthetic antioxidants are commonly used. However, concern about the safety of these compounds has led to increased interest in natural antioxidants (Yanishlieva and Marinova, 2001), which resulted in replacing these synthetic chemicals with those of natural origin (Amri et al., 2013). Plants produce a great variety of organic compounds with a very restricted and specific distribution, usually found in a single plant species or a taxonomically related group (Mazid et al., 2011). For some of these plant secondary metabolites and their sources, humans have found applications as food ingredients, pharmaceuticals or medicinal products.

Plants of the genus *Ajuga* L. (Lamiaceae) have been used in medicine since ancient times. These flowering species of the mint family are evergreen, clump-forming annual or perennial herbaceous plants, native to Europe, Asia and Africa (Çali et al., 2014). Most of the recent investigations of these plants have been related to their health-beneficial properties. The antioxidant, hypoglycemic, cytotoxic and antimicrobial activities of *A. turkestanica* has been reported by Kutepova et al. (2001) and Mamadalieva et al. (2013). *A. remota* extracts showed antimalarial and diuretic activities (Hailu and Engidawork, 2014; Gitua et al., 2012), while extracts from *A. bracteosa* and *A. iva* have demonstrated anti-inflammatory, antimicrobial, antioxidant and analgesic activities, as well as activity against pests (Aly et al., 2011; Makni

et al., 2013; Mothana et al., 2012; Pal and Pawar, 2011; Singh et al., 2012). The ethnopharmacology of the *Ajuga* species is well documented by Israili and Lyoussi (2009).

When it comes to *A. chamaepitys*, the majority of the investigations have addressed antifeedant activity and larval inhibition (Kutas and Nádasy, 2005; Kutepova et al., 2001, Pavela, 2011), as well as antimicrobial (Ulukanli et al., 2005), antiviral, cytotoxic and antioxidant activity (Delazar et al., 2012; Turkoglu et al., 2010; Orhan et al., 2009). In addition, several qualitative phytochemical analyses resulted in the identification of clerodane diterpenes and steroidal glycosides from the aerial parts of *A. chamaepitys* subsp. *laevigata* from Turkey (Topçu et al., 2004) and *A. chamaepitys* subsp. *chia* from Bulgaria (Boneva et al., 1990). Furthermore, *β*-pinene and germacrene D have been identified as major constituents of the essential oils of *A. chamaepitys* subsp. *chia* from Turkey (Baser et al., 1990), while according to Mitić et al. (2012), in the case of *A. chamaepitys* subsp. *chia* from Serbia, the major constituents of essential oil in the blooming stage were α-pinene, *β*-pinene, germacrene D, germacrene B and viridiflorol. Chemical investigation of the essential oil from the aerial parts of *Ajuga chamaepitys* subsp. *chamaepitys* from Spain demonstrated that γ-muurolene, limonene and germacrene B were major constituents of the oil (Velasco et al., 2004).

From the taxonomic point of view, in the flora of Serbia the genus *Ajuga* is represented by six species (Janković, 1974). According to Flora Europea (Ball, 1972), the *A. chamaepitys* species include two subspecies – *A. chamaepitys* subsp. *chia* and *A. chamaepitys* subsp. *chamaepitys*. *Ajuga chamaepitys* (L.) Schreb. subsp. *chamaepitys* is an annual herbaceous plant with upright or partially flattened shoots, 5 to 30 cm in height. The whole plant is covered with white hairs. Leaves are triform with linear segments; flowers are yellow with purple spots. Having in mind that these morphological characteristics can show a high degree of variation, they may not be sufficient for identification. For this purpose, screening of the plant secondary metabolite content and their activity is required. Furthermore, phytochemical profiling studies are of interest for the purpose of authenticating plant samples and detecting species with chemically similar profiles that may have positive outcomes (Filippini et al., 2010).

In the present paper, the secondary metabolites (total phenolic and flavonoid content) of different extracts obtained from the flowering aerial parts of *A. chamaepitys* subsp. *chamaepitys* growing in Serbia were analyzed. *In vitro* assays were performed to evaluate the antioxidant and antimicrobial activities. This paper is the first report of this subspecies' activity to date. The information could be useful in defining the biology and ecology, and suitability of this plant as a source of natural compounds, as well as for future qualitative and quantitative analysis of the secondary metabolites as active compounds and their *in vitro* and *in vivo* biological activities.

MATERIALS AND METHODS

Chemicals

Organic solvents and sodium hydrogen carbonate were purchased from Zorka Pharma, Šabac, Serbia. The antibiotic tetracycline, gallic acid, rutin hydrate, chlorogenic acid and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were obtained from Sigma Chemical Co., St Louis, MO, USA. Folin-Ciocalteu phenol reagent and aluminum chloride (AlCl3) were purchased from Fluka Chemie AG, Buchs, Switzerland. Dimethyl sulfoxide (DMSO) was purchased from Centrohem, Stara Pazova, Serbia. Resazurin was obtained from Alfa Aesar GmbH & Co., KG, Karlsruhe, Germany. Nutrient liquid medium, a Mueller-Hinton broth, was purchased from Liofilchem, Italy. All other solvents and chemicals were of analytical grade.

Plant material

In July 2012, the aerial flowering parts of *A. chamaepitys* subsp. *chamaepitys* were collected from natural populations in the region of Mt. Stara Planina in eastern Serbia (position: 43°15'452"N, 22°32'952"E, altitude: 442 m). The voucher specimen was con-

firmed 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 room temperature (20°C). Dried plant parts were finely cut and stored in tightly sealed dark containers until needed.

Preparation of plant extracts

Plant material (10 g) was transferred to dark-colored flasks with 200 mL of solvent (water, methanol, acetone, ethyl acetate and petroleum ether, respectively) and stored at room temperature. After 24 h, extracts were filtered through Whatman No. 1 filter paper and the residue was re-extracted with an equal volume of solvents. After 48 h, the process was repeated. Combined filtrates were evaporated to dryness under vacuum at 40°C using a rotary evaporator. The obtained dry extracts were kept in sterile sample tubes and stored in a refrigerator at 4°C.

Determination of total phenolic content

The total phenolic content was determined using a modified spectrophotometric method (Singleton et al, 1999). The reaction mixture was prepared by mixing 0.5 mL of methanol solution (1 mg/mL) of each extract with 2.5 mL of 10% Folin-Ciocalteu reagent dissolved in water and 2.5 mL of 7.5% NaHCO3. The samples were incubated at 45°C for 15 min. The absorbance was measured at λmax=765 nm. The samples were prepared in triplicate and the mean value of absorbance was obtained. A blank was concomitantly prepared with methanol instead of extract solution. The same procedure was repeated for the gallic acid, and the calibration curve was constructed. The total phenolic content was expressed in terms of gallic acid equivalent (mg of GA/g of extract).

Determination of flavonoid content

The concentration of flavonoids was determined using a spectrophotometric method with some modifications (Quitter et al, 2000). The sample contained 1 mL of methanol extract in a concentration of 1 mg/mL and 1 mL of 2% $AlCl_3$ solution dissolved in methanol. The samples were incubated for 1 h at room temperature. The absorbance was measured at λ_{max}=415 nm. The samples were prepared in triplicate and the mean value of absorbance was obtained. Instead of extract solution, methanol was used in order to prepare a blank. The same procedure was repeated for rutin, and the calibration curve was constructed. Concentration of flavonoids in extracts was expressed in terms of rutin equivalent (mg of RU/g of extract).

Evaluation of DPPH-scavenging activity

The ability of the plant extract to scavenge free radicals was assessed by the standard method (Tekao et al., 1994), with suitable modifications (Kumarasamy et al., 2007). The stock solution of the plant extract was prepared in methanol to achieve a concentration of 1 mg/mL. Dilutions were made to obtain concentrations of 500, 250, 125, 62.5, 31.25, 15.62, 7.81, 3.90, 1.99 and 0.97 μg/mL. Diluted solutions (1 mL each) were mixed with 1 mL of DPPH methanolic solution (80 μg/mL). After 30 min in darkness at room temperature (23°C), the absorbance was recorded at 517 nm. The control samples contained all the reagents except the extract. The percentage of scavenging was calculated using the following equation: % scavenging=100×(A *control* – A *sample*)/A *control*). The SC_{50} values (concentration of the extract at which 50% of the DPPH radical is neutralized) were estimated from the % scavenging versus the concentration sigmoidal curve using nonlinear regression analysis. The data are presented as mean values±standard deviation (SD) (n=3).

Determination of antimicrobial activity

Antimicrobial activity of extracts was tested against 13 strains of bacteria (standard strains: *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27853, *Bacillus subtilis* IP 5832, *Bacillus pumilus* NCTC 8241, *Proteus mirabilis* ATCC 12453, and clinical isolates: *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Proteus mirabilis, Salmonella enterica, Salmonella ty-*

phimurium and *Bacillus cereus*. All clinical isolates were a generous gift from the Institute of Public Health, Kragujevac. Standard microorganisms were provided from a collection held by the Microbiology Laboratory, Faculty of Science, University of Kragujevac.

Bacterial suspensions were prepared by the direct colony method. The turbidity of initial suspension was adjusted by comparison with 0.5 McFarland's standard for bacteria (Andrews, 2005). Initial bacterial suspensions contained about 10^8 colony-forming units (CFU)/mL. 1:100 dilutions of initial suspension were additionally prepared in sterile 0.85% saline for examining G(-) bacteria, and at a 1:10 ratio for G(+) bacteria.

Antimicrobial activity was tested by determining the minimum inhibitory concentration (MIC) and minimum microbicidal concentration (MMC) using a microdilution method with resazurin (Sarker et al, 2007). Ninety six-well plates were prepared by dispensing 100 μL of nutrient broth, Mueller-Hinton broth into each well. One hundred μL from the stock solution of tested extracts (concentration of 40 mg/mL) was added into the first row of the plate. Then, two-fold, serial dilutions were performed using a multichannel pipette. The obtained concentration range was from 20 mg/mL to 1.25 mg/mL. 10 μL of diluted bacterial suspension was added to each well to give a final concentration of 5×10^5 CFU/mL. Finally, 10 μL of resazurin solution was added to each well inoculated with bacteria. Resazurin is an oxidation-reduction indicator used for the evaluation of microbial growth. Resazurin is a blue non-fluorescent dye that becomes pink and fluorescent when reduced to resorufin by oxidoreductases within viable cells. The inoculated plates were incubated at 37°C for 24 h. MIC was defined as the lowest concentration of tested substance that prevented resazurin color change from blue to pink.

For determination of minimum microbicidal concentration (MMC), 10 μL of samples from the wells were plated and no indicator color change was recorded on nutrient agar medium. At the end of the incubation period, the lowest concentration with no growth (no colony) was defined as the minimum microbicidal concentration (MMC).

Tetracycline was used as a positive control. Stock solutions of crude extracts were obtained by dissolving in DMSO and then diluted into Mueller-Hinton broth to achieve a concentration of 10% DMSO. A solvent control test was performed to study the effects of 10% DMSO on the growth of microorganisms. It was observed that 10% DMSO did not inhibit the growth of microorganisms. In addition, the concentration of DMSO was additionally decreased because of the two-fold serial dilution assay (the working concentration was 5% and lower). Each test included growth control and sterility control. All tests were performed in duplicate and MICs were constant.

Data analysis

All data are presented as means±standard deviations (mean±SD) where appropriate. For comparison between samples, data were analyzed by Student's *t*-test and one-way analysis of variance (ANOVA). In all cases, p values <0.05 were considered statistically significant. All statistical analyses were performed using the SPSS package.

RESULTS

In order to determine the biological activity of *A. chamaepitys* subsp. *chamaepitys* extracts, the contents of total phenolics and flavonoids, the DPPH scavenging ability, MIC and MMC were investigated. To achieve the most effective extraction we used various solvents with diverse polarity, and analyzed five extracts obtained by extraction with water, methanol, acetone, ethyl acetate and petroleum ether.

The total phenolic content of the plant extracts are presented in Table 1. Extracts of *A. chamaepitys* subsp. *chamaepitys* were characterized by the content of total phenolic compounds in the range from 25.49 to 57.02 mg GA/g, with the highest content determined in the ethyl acetate extract (57.02 mg GA/g). In all other extracts, the total phenolic content was much lower.

The content of flavonoids is presented in Table 1. They ranged from 9.32 to 91.76 mg RU/g. The highest

Table 1. Total phenolic and flavonoid contents.

Type of extract	Total phenolic content (mg GA/g)	Flavonoid content (mg RU/g)
water	25.49 ± 0.50	9.32 ± 0.33
methanolic	33.70 ± 0.99	63.87 ± 0.66
acetone	32.47 ± 0.89	61.77 ± 0.51
ethyl acetate	57.02 ± 0.61	91.76 ± 0.81
petroleum ether	36.52 ± 0.77	35.24 ± 0.59

Each value in the table was obtained by calculating the average of three analyses±standard deviation (SD).

Table 2. DPPH-scavenging activity.

Type of extract	SC_{50} values (μg/mL)
water	1053.45 ± 1.28
methanolic	759.15 ± 1.21
acetone	330.52 ± 0.91
ethyl acetate	981.95 ± 1.35
petroleum ether	958.16 ± 1.49

Each value in the table was obtained by calculating the average of three analyses ch value in the table.

flavonoid content was obtained in the ethyl acetate extract (91.76 mg RU/g), followed by the methanol (63.87 mg RU/g) and acetone extracts (61.77 mg RU/g). The petroleum ether and water extracts had several-fold lower flavonoid content.

The antioxidant activity of *A. chamaepitys* subsp. *chamaepitys* extracts was determined by the DPPH method, and the results are shown in Table 2. The SC_{50} value of different extracts from *A. chamaepitys* subsp. *chamaepitys* ranged from 1053.45 to 330.52 μg/mL. The highest anti-DPPH radical ability was manifested by the acetone extract (SC_{50} value at 330.52 μg/mL).

The antibacterial results are presented in Table 3. The obtained values for MIC and MMC were in the same range (from 1.25 mg/mL to more than 20 mg/mL). The extracts displayed weak effects on Gram-negative bacteria. The strongest defect was that exhibited by the acetone extract against *Proteus mirabilis* and *P. mirabilis* ATCC 12453 (MIC = 10 mg/mL). As for Gram-positive bacteria, the antibacterial effects were much better, and the methanol, acetone and ethyl acetate extracts stood out. The acetone extract possessed the strongest activity, with the overall best antibacterial result on *Bacillus cereus* (MIC and MMC = 1.25 mg/mL). The acetone extract also substantially inhibited *Staphylococcus aureus* ATCC 25923 and *Bacillus subtilis* IP 5832 growth (MIC and MMC = 2.5 mg/mL).

Table 3. Antibacterial activities of A. chamaepitys subsp. chamaepitys extracts.

Bacteria	Water extract		Methanolic extract		Acetone extract		Ethyl acetate extract		Petroleum ether extract		Tetracycline	
	MIC[1]	MMC	MIC	MMC	MIC	MMC	MIC	MMC	MIC	MMC	MIC	MMC
Gram (–) bacteria												
Salmonella enterica	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	1.95	3.91
Salmonella typhimurium	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	/	/
Proteus mirabilis	20	>20	20	>20	10	>20	20	>20	20	>20	500	500
Proteus mirabilis ATCC 12453	20	>20	20	>20	10	>20	20	>20	20	>20	125	125
Escherichia coli	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	1.95	3.91
Escherichia coli ATCC 25922	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	0.98	3.91
Pseudomonas aeruginosa	>20	>20	20	>20	20	>20	20	>20	20	>20	500	1000
Pseudomonas aeruginosa ATCC 27853	20	20	20	>20	20	>20	>20	>20	>20	>20	7.81	62.5
Gram (+) bacteria												
Bacillus subtilis IP 5832	20	20	5	20	2.5	2.5	5	10	/	/	>0.5	>0.5
Bacillus cereus	20	20	5	5	1.25	1.25	5	5	/	/	>0.5	3.91
Bacillus pumilus NCTC 8241	>20	>20	5	20	2.5	5	5	10	/	/	>0.5	>0.5
Staphylococcus aureus	20	20	20	>20	5	20	20	>20	5	20	0.98	15.62
Staphylococcus aureus ATCC 25923	20	20	20	20	2.5	2.5	10	>20	5	20	0.24	1.95

[1]Minimum inhibitory concentration (MIC) and minimum microbicidal concentration (MBC) values are given as mg/mL for plant extract and μg/mL for antibiotic (tetracycline); / means not determined.

DISCUSSION

As highly unstable and reactive molecules, free radicals cause oxidative damage and in the end participate in cell death (Abbas et al., 2013). Phenolic compounds are ubiquitous in plants, possessing many biological activities due to their capability to act as free-radical scavengers (Holderbaum et al., 2014). The beneficial properties and importance of phenolic compounds may be linked to the well-recognized role of these substances as substrates for oxidative reactions (by both enzymatic and chemical mechanisms), as well as protective agents against the oxidative damage caused by free radicals (Lombardo et al., 2010). The mechanisms responsible for the phenolic toxicity to microorganisms include enzyme inhibition by the oxidized compounds through specific or nonspecific interactions with proteins (Biradar et al, 2008). Flavonoids are hydroxylated phenolic substances, widely distributed in plants (Khoddami et al., 2013). They can be synthesized by plants in response to microbial infection, and can consequently act as effective antimicrobial substances against a wide array of microorganisms.

According to the obtained results, *A. chamaepitys* subsp. *chamaepitys* extracts have shown considerable total phenolic and flavonoid contents. However, the concentration of these substances depends on the polarity of the solvent used. The high concentrations in the acetone and ethyl acetate extracts are the result of the high solubility of these metabolites, which is consistent with their chemical characteristics (Chebil et al., 2007; Min and Chun-Zao, 2005). Similar results were obtained in many previous studies (Anagnostopoulou et al., 2006; Anokwuru et al., 2011; Ćurčić et al., 2012; Matejić et al. 2014; Radojević et al., 2012).

To increase defenses and survival in response to biotic and abiotic stresses, antioxidants provide substantial information on the cellular redox state, as well as gene expression (Shao et al., 2008). By scavenging free radicals, antioxidants can terminate or retard the oxidation process (Mandal et al., 2009). Due to the great variability among antioxidant compounds and their complex structure-activity relationships, studies involving secondary metabolite production should incorporate antioxidant activity evaluation (Amoo et al., 2012). In our work, five different extracts of *A. chamaepitys* subsp. *chamaepitys* have demonstrated unequal DPPH radical-scavenging abilities. Considerable antioxidant activity was recorded only with the acetone extract, while in case of all the other extracts a significantly lower capability to neutralize free radicals is noticeable. Regarding the results for secondary metabolite extraction with ethyl acetate as most suitable solvent for phenolic and flavonoid extraction, neither direct nor strong relations can be found between the antioxidant activity of the plant and the phenolic and flavonoid content. This may suggest that the major role in *A. chamaepitys* subsp. *chamaepitys* free radical scavenging activity belongs to non-phenolic compounds. It was reported by Terpinc et al. (2012) that no significant correlation could be found between the total phenolic content and antioxidant activity of various plant extracts.

Plant extracts have been for some time of interest to scientists as possible new substances for treating various bacterial infections. In the study of Turgoklu et al. (2010), methanol, water and chloroform extracts of *A. chamaepitys* subsp. *euphratica* were investigated. According to the authors, this subspecies may be considered as a promising source of natural substances. However, major differences can be noticed when comparing these two subspecies. In the case of the antioxidant activity of *A. chamaepitys* subsp. *euphratica*, a higher value was recorded in the water extract, followed by the methanol extract, which is contrary to our results for *A. chamaepitys* subsp. *chamaepitys*. The same applies to the results for phenols and flavonoids. Differences between these two subspecies can be noticed also in the case of antimicrobial activity. The antimicrobial activity of *A. chamaepitys* subsp. *euphratica* was examined using the disc-diffusion method and Gram-negative bacteria were more sensitive than Gram-positive bacteria –opposite to the case of *A. chamaepitys* subsp. *chamaepitys*. The same method for evaluating antimicrobial activity was used in the study conducted by Sarac and Ugur (2007) with *A. chamaepitys* subsp. *chia* ethanolic extract from aerial parts, and the results indicated no inhibition of selected Gram-positive bacteria. They tested *S. aureus* ATCC 25923, which we have also

tested, and observed no influence. This could be compared with our results where only the acetone extract provided higher activity (MIC and MMC at 2.5 mg/mL). Comparing the results for antimicrobial activity of *A. chamaepitys* subsp. *chamaepitys* extracts with the standard antibiotic tetracycline, methanol, ethyl acetate and particularly acetone extracts showed partially high antimicrobial effects toward the *B. cereus*, *B. subtilis* IP 5832, *B. pumilus* NCTC 8241, *S. aureus* and *S. aureus* ATCC 25923. Thus, the antimicrobial activity of the acetone extract of *A. chamaepitys* subsp. *chamaepitys* could be considered as good.

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