Original article

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CHEMICAL VARIABILITY AND ANTIMICROBIAL ACTIVITY OF *AJUGA LAXMANNII* (L.) BENTH. (*LAMIACEAE*) ESSENTIAL OIL

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Ajuga plants have had a long history of ethnopharmacological use worldwide. Based on the papers published so far, it seems that there has been a great interest in isolation, structural elucidation and testing of non-volatile Ajuga phytochemicals.

The composition of hydrodistilled aerial part volatiles obtained from six populations of wild-growing A. laxmannii was investigated by means of GC and GC-MS analysis. The oils were screened for in vitro antibacterial and antifungal activity against a panel of laboratory control strains using the broth microdilution assay. The analyses resulted in the identification of one hundred fourteen constituents, accounting for 79.6-97.3% of the total composition of the oils. The main components of the analyzed samples were (E)-phytol (5.3-26.1%), nonacosane (2.3-25.6%), coumarin (tr-22.7%), 1-octen-3-ol (0-21.2%), (Z)-3-hexen-l-ol (0-20.5%), linalool (0-13.7%) and heptacosane (0.6-10.5%), which all together contributing more than two thirds to the compounds detected. Among the microorganisms tested the most susceptible strain was Pseudomonas aeruginosa (minimal inhibitory/bactericidal concentration = 1.25/2.5 mg mL-1). *Acta Medica Medianae 2017;56(2):92-101.*

Key words: *Ajuga laxmannii, essential oil composition, coumarin, antimicrobial activity*

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Introduction

The genus *Ajuga L*. (*Lamiaceae*), commonly known as bungle or bungleweed, ″ivica″ or ″krnjavica″ in Serbian, is comprised of more than 40 species widely distributed in temperate regions of both hemispheres (1). Ten species of the genus Ajuga are spread across Europe (2) and five are represented in the flora of Serbia (3). *Ajuga laxmannii* (L.) Benth. is a perennial, herbaceous plant that belongs to *Lamiaceae* family (subfamily Ajugoideae, tribe Ajugeae). The native range of A. laxmannii overlaps Central, Eastern and South-eastern Europe, Siberia, Caucasus and Asia Minor. It mainly inhabits clearings and edges of oak forests (or its degradation stages) of the lowland and mountain region. The plant also occupies herbaceous steppe-like slopes and limestone or siliceous bedrocks.

Many of Ajuga plants have been used in traditional medicine as a remedy for fever, tooth-ache, dysentery, malaria, high blood pressure, diabetes, gastrointestinal disorders, as anthelmintic, diuretic and antifungal, anti-inflammatory, and antimycobacterial agents (4 and the references cited therein). A large number of compounds isolated from Ajuga plants have been shown to possess a broad spectrum of in vitro biological and pharmacological activities and some of the most important due to economical aspects are antifeedant, insect growth-inhibitory and anti-malarial properties. Several literature sources are referring to A. laxmannii as an ethnomedicinal plant, reporting the above ground parts (herba) application in cancer (5) and in respiratory infections treatments (6). However, A. laxmannii was the subject of only one previous study, resulted in the isolation of seven compounds: two phenylpropanoids (free coumarin and coumarin derivative, melilotic acid methyl ester), two neo-clerodane type diterpenes (diasteroisomers of 14,15-dihydro-15-hydroxyajugahin), one phyto-

ecdysteroid (makisterone), and two iridoids (harpagide and 8-acethylharpagide) (7).

In spite of a great number of papers reporting the isolation of potentially bioactive compounds a little attention has been given to the analysis of Ajuga volatiles. Only six Ajuga species: A. austro-iranica (8), A. bombycina (9), *A. Bracteosa* (10, 11), A. chamaecistus (12, 13), A. Chamaepitys (14, 15, 16, 17) and *A. orientalis* (18) were investigated with this respect and no previous reports dealing with the analysis of *A. laxmannii* volatiles could be found in scientific literature. Hence, the aim of present study was to provide compositional analysis of the volatiles isolated from aerial parts of *A. laxmannii*. Samples were gathered from six different geographical localities in central parts of Balkan Peninsula with intention to explore chemical diversity of the species and to discuss the relationship between the oils' composition and the ecological and geographical distribution of the populations. Furthermore, having in mind ethnomedicinal application of the species in respiratory infections treatment, the final aim was to determine antimicrobial activity of the extracted oils and one of their main constituents against a panel of standard microorganisms.

Materials and methods

Plant material

Sweet and strong-smelling, glandular-pubescent above ground parts of A. laxmannii were collected from natural populations at six localities (samples A1-A6, 200 g per each sample). The material was sampled from spatially remote populations growing in different habitats in the nature. Each site of collection was chosen to represent original complex of ecological conditions. A detailed list concerning position, phenophase, plant associations and geological substrate data on the collected plants (among other generalities given) is presented in Table 1. Voucher specimens have been deposited in the Herbarium of the Institute of Botany and Botanical Garden "Jevremovac", University of Belgrade (BEOU). The acquisition numbers of the herbarium deposited plants are given in Table 1, as well.

Table 1. Data on collected *A. laxmannii* samples

Sample	BEOU acquisition number	Date of collection	Phenophase	Locality	Altitude m a.s.l.	Habitat and vegetation	UTM 10x10 square	GPS Position	Geological substrate
A1	16 279	30.05.2008.	blossoming	E Serbia, Pirot (Sarlak)	612	hornbeam shrub formations, (ass. *Carpinetum orientalis serbicum*)	FN28	N_lat: 43°10'19,5" E_long 22°33'23,5"	limestone
A2	16 646	06.06.2009.	blossoming	S Serbia, Rujan Mt. (Slavujevac)	717	termophilous forest ass. *Orno-Quercetum pubescentis*	EM67	N_lat: 42°15'36,6" E_long 21°46'29,7"	silicate
A3	16 654	28.07.2009.	fructification	E Serbia, Bela Palanka (Kremenica)	582	steppe-like vegetation ass. (ass. *Potentillo-Caricetum humilis*)	FN08	N_lat: 43°12'21,3" E_long 22°20'58,2"	limestone
A4	16 647	21.06.2010.	fructification	E Serbia, Kamenički Vis (Kamenica)	611	termophilous forest (ass. *Quercetum frainetto-cerris*)	EP70	N_lat: 43°23'40,3" E_long 21°56'18,2"	limestone
A5	16 666	08.05.2012.	blossoming	E Serbia, Kravljansko topilo	380	hornbeam shrub formations (ass. *Carpinetum orientalis serbicum*)	EP71	N_lat: 43°26'39,7" E_long 21°52'24,6"	limestone
A6	16 648	26.05.2012.	blossoming	S Macedonia, Vitološte (Golema Skrka)	1164	termophilous forest (ass. *Querco-Carpinetum orientalis macedonicum*)	EL65	N_lat: 41°10'456,6" E_long 21°47'38,8"	marble

Table 2. Percentage composition of essential oils obtained from aerial parts of six *Ajuga laxmannii* (L.) Benth. samples originating from Central Balkan

RI[a]	Components	%[b]						MI [c]
		A_1	A_2	A_3	A_4	A_5	A_6	
764	(*Z*)-2-Penten-1-ol	0.4	0.2	0.4	- [d]	-	-	RI, MS
765	3-Methyl-2-buten-1-ol (*syn.*[e] Prenol)	0.2	tr [f]	0.6	-	-	-	RI, MS
778	3-Methyl-2-butenal (*syn.* Prenal)	0.1	-	tr	-	-	-	RI, MS
802	Hexanal	0.1	-	-	-	-	-	RI, MS
835	Furfural	0.2	tr	1.1	-	-	-	RI, MS, Co-GC
847	(*E*)-3-Hexen-1-ol	tr	0.2	0.3	-	-	-	RI, MS
851	(*Z*)-3-Hexen-l-ol	14.1	20.5	18.7	-	-	-	RI, MS
860	(*Z*)-2-Hexen-l-ol	tr	2.7	0.7	-	-	-	RI, MS
862	1-Hexanol	tr	tr	0.6	-	-	-	RI, MS, Co-GC
935	(*E*)-3-Hepten-2-one	0.1	tr	tr	-	-	-	RI, MS
937	*α*-Pinene	tr	-	-	0.1	-	-	RI, MS, Co-GC
964	Benzaldehyde	0.2	tr	0.4	-	-	-	RI, MS, Co-GC
976	1-Octen-3-ol	15.5	21.2	13.1	-	0.3	-	RI, MS
982	*β*-Pinene	tr	-	-	0.1	-	-	RI, MS, Co-GC
991	(*E*)-3-Octen-2-ol	0.1	-	-	-	-	-	RI, MS
992	*β*-Myrcene	-	-	-	0.3	-	-	RI, MS, Co-GC
995	3-Octanol	0.6	0.7	tr	-	-	-	RI, MS
1006	(*E*)-3-Hexenyl acetate	0.1	tr	-	-	-	-	RI, MS
1012	(*E,E*)-2,4-Heptadienal	0.1	-	tr	-	-	-	RI, MS
1026	2-Ethylhexanol	0.2	-	-	-	-	-	RI, MS
1028	*p*-Cymene	-	-	-	0.1	-	-	RI, MS, Co-GC
1031	Limonene	-	-	-	0.2	-	-	RI, MS, Co-GC
1032	Carvomenthene (*syn*. *p*-Ment-1-ene)	0.1	-	-	-	-	-	RI, MS
1035	Benzyl alcohol	1.0	0.6	1.7	-	tr	-	RI, MS, Co-GC
1046	Phenylacetaldehyde	1.5	0.6	1.2	-	-	-	RI, MS, Co-GC
1047	Salicylaldehyde	tr	-	0.6	-	-	-	RI, MS, Co-GC
1055	5-Methyldecane	0.1	-	-	-	-	-	RI, MS
1060	3-Methylbenzaldehyde	-	-	-	-	0.3	-	MS
1066	(*E*)-2-Octen-l-ol	0.4	0.6	tr	-	-	-	RI, MS
1070	Acetophenone	0.6	-	tr	-	-	-	RI, MS, Co-GC
1095	l-(2,5-Dimethyl-3-furyl)-ethanone	0.4	tr	0.6	-	-	-	RI, MS
1101	Linalool	13.7	9.1	5.0	-	3.3	-	RI, MS, Co-GC
1105	Nonanal	0.1	-	0.4	-	-	-	RI, MS
1117	2-Phenylethanol	1.1	0.4	0.6	-	-	-	RI, MS, Co-GC
1149	*trans*-Verbenol	0.1	-	-	-	-	-	RI, MS, Co-GC
1154	1-Phenylethanol	0.3	tr	-	-	-	-	RI, MS
1167	2-Hydroxyacetophenone	0.2	-	1.1	-	-	-	RI, MS, Co-GC
1176	2- Methylbenzofuran	0.1	-	-	-	-	-	RI, MS
1190	(*Z*)-3-Hexenyl butanoate	-	-	-	-	0.2	-	RI, MS
1194	*α*-Terpineol	0.5	0.4	1.0	-	0.3	-	RI, MS, Co-GC
1200	Dodecane	-	0.6	tr	-	-	-	RI, MS, Co-GC
1252	Phenylacetic acid	0.1	-	-	-	-	-	RI, MS, Co-GC
1272	1-Decanol	-	0.4	tr	-	-	-	RI, MS
1279	3-Methyldodecane	0.1	-	-	-	-	-	RI, MS
1298	Bornyl acetate	-	0.4	-	-	-	-	RI, MS
1300	Tridecane	-	3.2	0.6	-	-	-	RI, MS
1317	*p*-Vinylguaiacol	0.8	tr	2.1	-	0.3	-	RI, MS
1360	Eugenol	1.0	tr	tr	-	0.4	-	RI, MS, Co-GC
1426	*β*-Caryophyllene	0.1	3.3	tr	tr	-	-	RI, MS, Co-GC
1443	Coumarin (*syn.* 2*H*-1-Benzopyran-2-one)	22.7	6.2	11.8	tr	14.6	3.6	RI, MS, Co-GC
1461	4-Methyltetradecane	0.4	0.5	0.3	-	0.2	-	RI, MS
1463	Geranyl acetone	-	-	0.4	-	-	-	RI, MS

1481	(*E*)-*β*-Ionone	0.2	-	-	-	tr	-	RI, MS, Co-GC
1486	Germacrene D	-	2.0	0.4	tr	-	-	RI, MS, Co-GC
1495	2-Tridecanone	0.1	-	-	-	-	-	RI, MS
1523	3,4-Dimethyl-5-pentyl-5*H*-furan-2-one	0.2	-	-	-	-	-	RI, MS
1595	2-Tetradecanone	-	1.0	0.4	-	-	-	RI, MS
1608	Vanillic acid	-	-	-	-	0.2	-	RI, MS
1662	(*E*)-4-Oxo-β-ionone	0.2	-	-	-	-	-	RI, MS
1695	2-Pentadecanone	-	0.9	0.4	0.7	0.2	-	RI, MS
1705	2-Pentadecanol	-	0.5	-	0.3	0.2	-	RI, MS
1782	1-Pentadecanol	-	-	-	0.6	0.2	-	RI, MS
1795	2-Hexadecanone	-	-	-	0.5	-	-	RI, MS
1823	Isopropyl myristate (*syn.* Isopropyl tetradecanoate)	-	-	0.3	tr	-	-	RI, MS
1845	Hexahydrofarnesyl acetone	1.7	1.7	3.0	6.3	3.4	1.2	RI, MS
1863	Neophytadiene, Isomer II	-	-	-	0.4	-	-	RI, MS
1882	1-Hexadecanol	-	tr	-	0.4	-	-	RI, MS
1895	2-Heptadecanone	-	-	-	0.2	-	-	RI, MS
1900	Neophytadiene, Isomer III	-	-	-	0.2	-	-	RI, MS
1900	Nonadecane	0.5	0.6	tr	0.2	tr	-	RI, MS, Co-GC
1956	(*Z*)-9-Hexadecenoic acid (*syn.* Palmitoleic acid)	-	0.7	-	-	0.4	-	RI, MS
2000	Eicosane	0.1	tr	tr	0.2	tr	-	RI, MS, Co-GC
2012	Manoyl oxide	-	-	-	-	-	0.3	RI, MS
2023	Isopropyl palmitate (*syn.* Isopropyl hexadecanoate)	-	0.4	0.4	-	-	-	RI, MS
2051	16-Kaurene	-	-	-	-	0.3	-	RI, MS
2066	*ar*-Abietatriene	0.1	tr	tr	1.5	0.5	tr	RI, MS
2086	Abietadiene	-	-	-	-	0.9	tr	RI, MS
2082	1-Octadecanol	-	-	0.4	1.3	0.7	tr	RI, MS
2100	Heneicosane	0.6	0.9	1.7	-	0.1	-	RI, MS, Co-GC
2106	γ-Hexadecalactone	-	-	-	0.2	-	-	RI, MS
2118	(*E*)-Phytol	5.3	9.5	6.2	16.6	26.1	16.0	RI, MS
2140	(*Z,Z,Z*)-9,12,15-Octadecatrienoic acid (*syn*. Linolenic acid)	0.2	-	-	0.8	-	-	RI, MS
2146	(*Z*)-9-Octadecenoic acid (*syn*. Oleic acid)	0.1	-	-	-	-	-	RI, MS, Co-GC
2200	Docosane	0.1	tr	-	0.3	0.2	tr	RI, MS, Co-GC
2222	(*E*)-Phytyl acetate	0.1	-	-	0.8	0.4	-	RI, MS
2240	Dehydroabietal	-	-	-	-	0.4	-	RI, MS
2300	Tricosane	0.8	1.6	0.3	1.2	1.7	3.7	RI, MS, Co-GC
2322	*trans*-Ferruginol	-	-	-	1.1	0.6	0.4	RI, MS
2354	5-Methyl-5-(4,8,12-trimethyltridecyl)dihydro-2(3*H*)-furanone	-	-	-	1.6	0.2	tr	RI, MS
2400	Tetracosane	0.1	tr	-	0.6	0.6	0.7	RI, MS, Co-GC
2500	Pentacosane	0.6	1.5	0.4	2.4	3.4	4.9	RI, MS, Co-GC
2600	Hexacosane	-	-	-	0.6	0.5	0.7	RI, MS, Co-GC
2700	Heptacosane	1.1	1.2	0.6	5.6	6.0	10.5	RI, MS, Co-GC
2762	4-Methylheptacosane	-	-	-	0.4	-	-	RI, MS
2800	Octacosane	-	-	-	1.2	0.9	1.3	RI, MS, Co-GC
2834	Squalene	-	-	-	0.3	0.2	1.2	RI, MS, Co-GC
2900	Nonacosane	4.0	2.3	8.6	17.8	17.5	25.6	RI, MS, Co-GC
3000	Triacontane	0.1	-	-	1.4	0.9	1.0	RI, MS, Co-GC
3100	Hentriacontane	1.4	0.7	1.9	9.9	6.8	9.9	RI, MS, Co-GC
3200	Dotriacontane	-	-	-	0.4	0.3	0.5	RI, MS, Co-GC
3300	Tritriacontane	-	-	-	2.8	2.0	2.8	RI, MS, Co-GC
	Yield (%, w/w)	0.012	0.010	0.010	0.010	0.012	0.010	
	Total identified	95.0	97.3	88.3	79.6	95.7	84.3	
	Number of components	63	49	50	41	42	22	
	Compound classes							
	Terpenoids	20.3	24.7	13.2	21.7	33.0	17.9	
	hydrocarbons	0.3	5.3	0.4	3.2	1.9	1.2	

	oxygenated	20.0	19.4	12.8	18.5	31.1	16.7	
	Phenylpropanoids	23.7	6.2	11.8	tr	15.0	3.6	
	Fatty acid derivatives	44.5	64.8	54.5	57.9	47.2	62.8	
	n-alkanes	9.4	12.6	14.1	44.6	40.9	61.6	
	branched alkanes	0.6	0.5	0.3	0.4	0.5	-	
	alcoholes	31.2	47.0	34.2	2.6	1.6	-	
	aldehydes	0.3	-	0.4	-	-	-	
	ketones	0.6	1.9	1.4	1.4	0.2	-	
	Ili carbonyl compounds	0.9	1.9	1.8	1.4	0.2	-	
	fatty acids and fatty acid esters	0.3	1.1	0.7	1.0	0.4	-	
	carotenoid derived compounds	2.1	1.7	3.4	7.9	3.6	1.2	
	Others	6.5	1.6	8.8	-	0.5	-	

[a]Compounds listed in order of elution from a HP-5 MS column with retention indices (RI) determined experimentally by co-injection of a homologous series of C7-C33 n-alkanes; bPercentage values present the mean of three individual analyses per each sample; cMethods of identification: RI, constituent identified by retention index matching; MS, constituent identified by mass spectra comparison; Co-GC, constituent identity confirmed by co-injection of an authentic sample; d -, not detected; e tr-trace (<0.05%); f syn. – synonym; A1-A6, samples of A. laxmannii collected from six different localities (Table 1 contains detailed description for each sample).

Isolation of the essential oil

Immediately after the collection, the fresh above ground parts of the plants were subjected to hydrodistillation for 2.5 hours in the original Clevenger-type apparatus. The obtained greenish and fragrant oils were acquired in the yield 0.010-0.012% (w/w, based on weight of fresh plant material, Table 2). The oils were separated, dried over anhydrous magnesium sulfate and immediately analyzed.

Gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) analyses

Chemical composition of the oil was investigated by GC and GC-MS. The GC-MS analysis (three repetitions) was performed using an Hewlett-Packard 6890N gas chromatograph equipped with a fused silica capillary column (HP-5MS, 250 μm x 25 m, film thickness 0.25 μm, Agilent Technologies, USA) and coupled with a 5973 inert mass selective detector of the same company, recording at 70 eV. Full scan spectra were acquired over the range 35-500 amu (scan time - 5 scans sec-1). GC-MS was operated under the following conditions- injector temperature: 250°C; GC-MS interface temperature: 250°C; oven temperature: programmed from 70-225°C at 5°C min-1, then isothermal for 10 min; carrier gas: He, 1.0 mL min-1, constant flow mode, vacuum outlet (37 cm/sec linear velocity); injected volume: 1 μL of 1/100 diluted solution in diethyl ether, split ratio 40:1. Oil constituents were identified by comparison of their linear retention indices (relative to n-alkanes (19) on the HP-5MS column) with literature values (20) and their mass spectra with those of authentic standards, as well as those from Wiley 6, NIST02, MassFinder 2.3, and a homemade MS library with the spectra corresponding to pure substances and components of known essential oils, and wherever possible, by co-injection with an authentic sample. GC (FID) analysis was carried out under the same experimental conditions using the same column as described for the GC-MS. The percentage composition of the oil was computed from the GC peak areas without any corrections.

Antimicrobial activity

Microbial strains

The in vitro antimicrobial activity of essential oils was tested against a panel of laboratory control strains belonging to the American Type Culture Collection Maryland, USA: Gram-positive: Bacillus subtilis ATCC 6633 and Staphylococcus aureus ATCC 6538; Gram-negative: Escherichia coli ATCC 8739, Pseudomonas aeruginosa ATCC 9027 and Salmonela typhimurium ATCC 14028; fungal organisms: Aspergillus niger ATCC 16404 and Candida albicans ATCC 10231. All microorganisms were maintained at -20ºC under appropriate conditions and regenerated twice before use in the manipulations.

Broth microdilution assay

The minimal inhibitory concentration (MIC) of the samples was determined by using a broth microdilution method according to the recommendations of the National Committee for Clinical Laboratory Standards (21). After overnight cultivation, microbial suspensions were made in Mueller Hinton broth or Sabouraud Dextrose broth. Cell suspensions were adjusted with sterile saline solution to obtain a turbidity of 0.5 Mc Farland. Dimethyl sulphoxide (10%, v/v aqueous solution) was used to dissolve and to dilute the samples. The highest concentration to be tested was 10 mg mL-1 for the oils and for coumarin. A serial double dilution of the samples was prepared in 96 well microtiter plates, using the method of Sarker et al. (22) with slight modifications. The lowest concentration of the sample that inhibited visible growth was taken as the MIC value. One row was used as a positive control and contained a broad-spectrum antibiotic (doxycycline in a serial dilution of 200-0.05 μg mL-1) to determine the sensitivity of Gram-negative and Gram-positive

bacteria and an antimycotic (nystatin in a serial dilution of 50-0.02 μg mL-1) to determine the sensitivity of fungi. The other row contained the solvent as negative control. Tests were carried out in triplicate.

Statistical analyses

All data were expressed as means±standard deviation of triplicate measurements. The confidence limits were set at $P < 0.05$. Standard deviations (SD) did not exceed 5% for the majority of the values obtained.

Principal component analysis (PCA) and agglomerative hierarchical clustering (AHC) were performed using the Excel program plug-in XLSTAT version 2011.3.01. Both methods were applied utilizing percentages of constituents of six samples that exceeded 5% of the total oil contribution in at least one of the six A. laxmannii oils. AHC was performed using Pearson dissimilarity (as aggregation criteria simple linkage, unweighted pair-group average and complete linkage were used) and Euclidean distance (aggregation criterion: weighted pair-group average, unweighted pair-group average and Ward's method). The definition of the groups was based on Pearson correlation, using complete linkage and unweighted pair-group average method.

Results and discussion

Results of the GC and GC-MS analyses of the hydrodistilled essential oils obtained from A. laxmannii samples collected from six different localities are given in Table 2. One hundred fourteen identified constituents accounted for 79.2-97.3% of the total composition of the volatiles. The main components of the analyzed samples were (E)-phytol (5.3-26.1%), nonacosane (2.3-25.6%), coumarin (tr-22.7%), 1-octen-3-ol (0-21.2%), (Z)-3-hexen-l-ol (0-20.5%), linalool (0-13.7%) and heptacosane (0.6-10.5%). The oil profile was characterized by the presence of the fatty acid metabolism derived compounds (FAD fraction, as shown in Table 2, as the most abundant compound class. Additionally, two other classes can be mentioned as the ones with significant relative percentages: phenylpropanoids (PP, 0-24.5%, detected for the first time in Ajuga oil sample, with a considerable amount of coumarin (tr-22.7%)) and terpenoids (13.2-33.2%, class that was further characterized by a higher content of oxygenated in comparison to hydrocarbons counterparts, Table 2). The terpenoid fraction of all six samples was a relatively simple one (with a few compounds contributing to the overall oil composition, Table 2) and consisting mostly of mono (acyclic) and diterpenoids (phytanes and abietanes).

In order to facilitate the discussion of a possible ecological significance (environmental and geographical factors) of the summarized data on A. laxmannii volatiles, as well as the interpretation and the conclusions to be statistically supported,

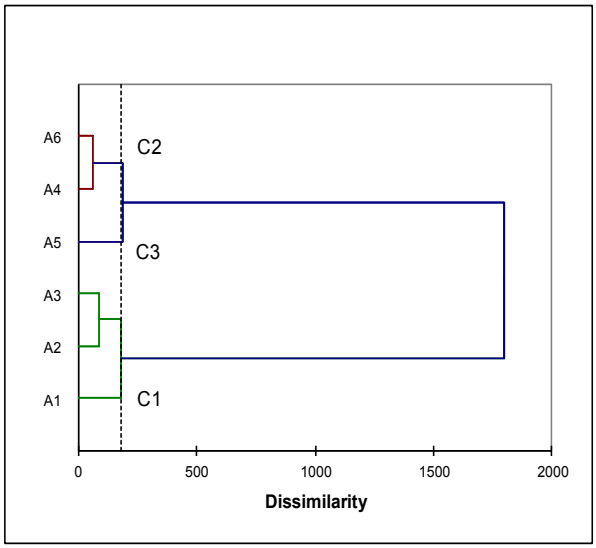

Figure 1. Dendrogram obtained by agglomerative hierarchical clustering (AHC; constituent contents that exceeded 5% of the total oil composition in at least one of the 6 oils used as original variables) and representing the chemical composition dissimilarity relationships of the 6 *A. laxmannii* essential oil samples. The AHC was performed with Euclidean distances as metric (dissimilarity within the interval [0, 1799]) and using Ward's method as aggregation criterion. Three groups of oils were found. For the sample designation (A1–A6), see Table 2.

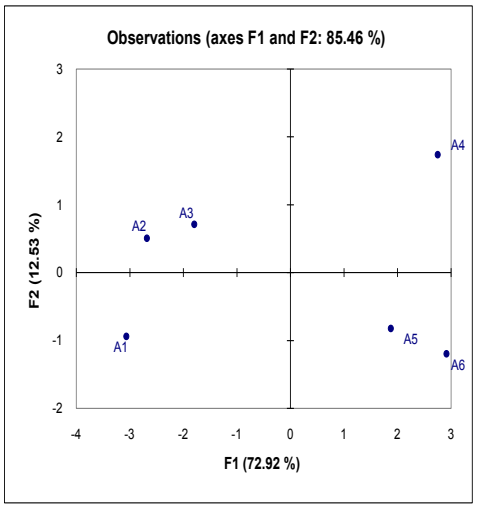

Figure 2. Principal Component Analysis (PCA; using the sums of constituent percentages that exceeded 5% of the total oil contribution in at least one of the six oils as original variables). Axes (F1 and F2 factors: the first and second principal component) refer to the ordination scores obtained from the samples. Axis F1 accounts for almost 72.92% and axis F2 accounts for 12.53% of the total variance. For sample designation see Table 2.

we have applied multivariate analyses: agglomerative hierarchical cluster analysis (AHC) and principal component analysis (PCA). Both methods were applied by considering as original variables without any recalculation all the constituents with contents that exceed 5% of the total oil composition in at least one of the 6 oil samples. The results of AHC and PCA analyses are depicted in Figures 1 and 2, respectively. The dendrogram in Figure 1, obtained as the result of AHC analysis, indicates the existence of three statistically different classes C1- C3. The first clade C1 grou-ped samples A1-A3, the second clade C2 consisted of samples A4 and A6, while the third was separating sample A5 from the rest of A. Lax-man-nii oils. All of the samples grouped under the C1 have (Z)-3-hexen-1-ol, 1-octen-3-ol, linalool and coumarin as most abundant components, all together representing more than 40% of the total oils which make them more related to each other than to the rest of A. laxmannii accessions. A noteworthy content of alkanes and diterpenoids made the samples under the clusters C2 (A4 and A6) and C1 (A5) more similar to each other. In the PCA, the horizontal and vertical axes accounted for 72.92 and 12.53% of the variation, respec-tively (Figure 2). The results of both statistical analyses were mostly in agreement and nearly the same clustering can be seen in PCA analysis (Figure 2) indicating the existence of at least 3 different essential oil chemical profiles. Even though the influence of environmental factors in the composition of essential oils is well known for *Lamiaceae* (23-27) in our analysis samples were grouped together with no apparent geographical or geological correlation which could only pinpoint the complexity of factors that may influence the chemotypification. Climatic factors during the time (five years) over which our material was sampled were not taken into consideration, and it may be assumed that these may be at certain extent related to compositional variation within species studied (28). In addition, concerning morphological polymorphism that could be useful in determining whether the accumulation of specific metabolites is related to infraspecies variability at extent significant enough for establishing varieties or even subspecies levels, the fieldwork repeated by the botanist in sampling sites did not reveal this type of incidence within populations under study. However, without further work that would encompass more comprehensive study, it cannot be clearly stated whether genetic or environmental factors were the determinants underlying the chemical polymorphism observed in *A. laxmannii*. Results of antimicrobial activity of A. laxmannii oils, evaluated using broth-microdilution assay, are presented in Table 3. The determinations of the minimal inhibitory concentration (MIC) and minimal bactericidal or fungicidal concentration (MBC or MFC, respectively) were carried out in triplicate, and consistent values were obtained

Table 3. Minimum inhibitory concentrations (MIC) and minimum bactericidal and fungicidal concentrations (MBC/MFC) of *A. laxmannii* essential oil samples

Microorganism	Oil samples (mg/mL)														Positive control (referent standard)				Negative control	
	A1		A2		A3		A4		A5		A6		Coumarin		Doxycycline (μg/mL)		Nystatin (μg/mL)		DMSO (10% aqueous solution)	
	MIC	MBC/MFC	MIC	MBC/MFC	MIC	MBC/MFC	MIC	MBC/MFC	MIC	MBC/MFC	MIC	MBC/MFC	MIC	MBC/MFC	MIC	MBC	MIC	MFC	MIC	MBC/MFC
B. subtilis ATCC 6633	2.50	10.00	2.50	10.00	2.50	10.00	na	na	2.50	10.00	na	na	na	na	1.56	1.56	nt	nt	na	na
E. coli ATCC 8739	na	na	na	na	na	na	na	na	na	na	na	na	na	na	0.78	0.78	nt	nt	na	na
P. aeruginosa ATCC 9027	1.25	2.50	2.50	10.00	2.50	10.00	2.50	10.00	2.50	2.50	na	na	na	na	12.5	12.5	nt	nt	na	na
S. typhimurium ATCC 14028	na	na	na	na	na	na	na	na	na	na	na	na	na	na	6.25	> 50.00	nt	nt	na	na
S. aureus ATCC 6538	na	na	na	na	na	na	na	na	na	na	na	na	na	na	6.25	0.78	nt	nt	na	na
A. niger ATCC 16404	2.50	10.00	na	na	na	na	2.50	10.00	na	na	na	na	na	na	nt	nt	0.78	0.78	na	na
C. albicans ATCC 10231	2.50	10.00	2.50	10.00	2.50	10.00	2.50	10.00	2.50	10.00	1.25	10.00	5.00	10.00	nt	nt	6.25	6.25	na	na

"nt" not tested, "na" not active at the concentration(s) tested

against each microorganism tested. The assayed samples showed no activity (at the concentrations tested in the range from 0.02 to 10 mg/ mL) against the following strains: E. coli ATCC 8739, S. aureus ATCC 6538 and S. typhimurium ATCC 14028, and only two samples (A1 and A4) were active against A. niger ATCC 16404 (Table 3). Oil sample with the broadest activity spectrum was A1 (active against 4 out of 7 strains), while the sample A6 was effective only against C. albicans ATCC 10231. When was present, the activities of the inspected samples were consistent with respect to the MIC and MBC/MFC values, ranging from 2.50 and 10.00 mg mL-1 in most of the cases, except for the sample A1 where MIC=1.25 mg mL-1 was observed against P. aeruginosa. All of the tested samples were less effective than the antibiotic or antimycotic used as reference standard (Table 3). Additionally, we have tested one of the dominant compounds detected in our samples, the coumarin alongside with essential oils, to screen if this component might attribute to the observed antimicrobial properties. The results have shown that C. Albicans was the only strain susceptible to the pure compound tested and that moderate anticandidal activity of the assayed samples might be associated to the coumarin itself (MIC = 5 mg mL-1; MFC = 10 mg mL-1), which is in agreement with the findings previously published by Montagner et al. (29). Additionally, the resulting activity could be related to the presence of other oils' main components, renowned as antimicrobial agents, such as linalool (30, 31), nonacosane (32) or (E)-phytol (33). How-ever, it is difficult to attribute the activity of a complex mixture such as essential oils to a single or particular constituent, and possible synergistic and/or antagonistic activity of the components should be also taken into consideration.

Conclusion

To the best of our knowledge, the composition and antimicrobial activity of A. laxmannii essential oil have not been reported so far and therefore our results can be viewed as the first investigation of the biological activity of this oil related to its chemical composition. The results of the performed statistical analyses (AHC and PCA) show that among the studied plant populations a degree of chemical variability can be observed. The samples characterized by a high percentage of coumarin could be of interest from the pharmaceutical and perfume industry points of view.

Acknowledgment

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ANALIZA VARIJABILNOSTI HEMIJSKOG SASTAVA I ANTIMIKROBNE AKTIVNOSTI ETARSKOG ULJA *AJUGA LAXMANNII* (L.) BENTH. (*LAMIACEAE*)

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Biljke roda Ajuga imaju dugu istoriju etnofarmakološke primene. Na osnovu publikovanih radova proisteklih iz dosadašnjih istraživanje hemije biljaka roda Ajuga, može se zaključiti da je gotovo sva pažnja fitohemičara bila usmerena isključivo na hemiju neisparljivih komponenata: izolovanje, strukturna karakterizacija izolovanih jedinjenja i testiranje biološke aktivnosti izolovanih supstanci.

Sastav etarskog ulja izolovanog metodom hidrodestilacije iz nadzemnih delova 6 uzoraka samonikle biljke *Ajuga laxmannii* sakupljenih sa šest lokaliteta ispitivan je gasnom (GC) i gas-masenom (GC-MS) analizom. Antimikrobna aktivnost dobijenih uzoraka etarskog ulja testirana je in vitro primenom mikrodilucione metode. Analizom ulja identifikovano je 114 jedinjenja koja su činila 79,6-97,3% ukupnog sastava ulja. Glavne komponente analiziranih uzoraka etarskih ulja bile su (E)-fitol (5,3-26,1%), nonakozan (2,3-25,6%), kumarin (tr-22,7%), 1-okten-3-ol (0-21,2%), (Z)-3-heksen-1-ol (0-20,5%), linalool (0-13,7%) i heptakozan (0,6-10,5%). Najosetljiviji mikro-organizam na dejstvo ulja bila je bakterija Pseudomonas aeruginosa (minimalna inhibitorna/baktericidna koncentracija = 1,25/2,5 mg mL-1). *Acta Medica Medianae 2017;56(2):92-101.*

Ključne reči: *Ajuga laxmannii, sastav etarskog ulja, kumarin, antimikrobna aktivnost*