Journal of the Serbian Chemical Society
JSCS-info@shd.org.rs • www.shd.org.rs/JSCS
J. Serb. Chem. Soc. 75 (10) 1347–1359 (2010)
JSCS–4057
UDC 582.623–035.27:547.384:
547.431.4:615.28
Original scientific paper

Chemical composition and screening of the antimicrobial and antioxidative activity of extracts of *Stachys* species

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(Received 3 June, revised 24 August 2010)

Abstract: GC and GC/MS analyses of the diethyl ether and ethyl acetate extracts obtained from the aerial parts of *Stachys germanica* subsp. *heldreichii* (Boiss) Hayek, *Stachys iva* Griseb., *Stachys plumosa* Griseb. and *Stachys scardica* Griseb., Balkan peninsula endemics, were performed. One hundred and seventy-nine constituents, accounting for 88.8–98.1% of the total composition of the extracts, were identified. The common feature of the diethyl ether extracts was the high content of terpenoids and fatty acid-derived compounds, while the common feature of the ethyl acetate extracts was the prevalence of fatty acid-derived compounds. A disk diffusion method was used for the evaluation of the antimicrobial activities of the extracts against a panel of microorganisms (bacteria: *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Salmonella enteritidis*; fungi: *Aspergillus niger* and *Candida albicans*). The total antioxidant capacity of the extracts was evaluated by the phosphomolybdenum method. The preliminary bioassay results indicated that the diethyl ether extract of *S. plumosa* could be a possible source of antioxidant and antimicrobial compounds.

Keywords: *Stachys*; Lamiaceae; diethyl ether and ethyl acetate extracts; antimicrobial activity, antioxidant capacity.

INTRODUCTION

Stachys L., one of the largest genera of the Lamiaceae family, contains more than 270 species. This sub-cosmopolitan genus has two main centers of diversity in the Old World area.[1] One is confined to S. and E. Anatolia, Caucasia, N.W.

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doi: 10.2298/JSC100601117L

Iran and N. Iraq, and the other to the Balkan Peninsula. Serbia is an area that is moderately rich in taxa belonging to the genus (seventeen species are acknowledged), however, eight species are recognized as endemic to the Balkans, or even narrower regions.[2]

Although genus *Stachys* is considered to be one of the largest genera in the Lamiaceae family, little attention has been paid to the elucidation of its pharmacological properties. These facts stand as a surprise, especially when it is known that data exist concerning the traditional application of many *Stachys* species for genital and inflammatory tumors, cancerous ulcers and sclerosis of the spleen.[3] Teas prepared from the whole part or leaves are used in phytotherapy, possessing sedative, antispasmodic, diuretic and emmenagogue activities. In Turkish folk medicine, species of the *Stachys* genus are used in the same way as sage.[4] Several *Stachys* species are recognized in Iranian folk medicine,[5] especially *S. inflata* which is used to treat various inflammatory disorders.[6] In hitherto undertaken pharmacological studies, *Stachys* species showed a variety of effects (anti-allergic,[7] anti-anoxic,[8] antibacterial,[9–12] anxiolytic,[13] anti-inflammatory,[6,14] antinephritic,[15] antioxidant,[16–19] antihepatitis and choleretic[20,21]), confirming a wide spectrum of application and an extensive range of traditional usage. The compositions of essential oils, as well as the preliminary screening using disk diffusion method for the *Stachys* species under study (*S. germanica* subsp. *heldreichii* (Boiss) Hayek, *S. iva* Griseb., *S. plumosa* Griseb. and *S. scardica* Griseb.) have been the subject of several previous publications,[10,22–25] but to the best of our knowledge, the chemical composition of extracts and the evaluation of their antimicrobial and antioxidant activities have never been studied previously (except for the antioxidant activities of a methanol extract of *S. plumosa*[16]).

In view of the growing interest in the application of natural products in the food, cosmetics and pharmaceutical industries and bearing in mind the scarcity of previous work on the bioassay of Balkan *Stachys* species, the subject of this study was to determine the chemical composition of diethyl ether and ethyl acetate extracts and to evaluate the antimicrobial and antioxidant activities of four Balkan endemic *Stachys* species: *S. germanica* subsp. *heldreichii* (Boiss) Hayek, *S. iva* Griseb., *S. plumosa* Griseb. and *S. scardica* Griseb.

EXPERIMENTAL

Plant material

The plant material of the four species was collected in June 2003 from natural populations, in the blooming stage as follows: *S. germanica* subsp. *heldreichii*, Galičica Mountain pastures at an altitude of 1600 m, Former Yugoslavian Republic of Macedonia; *S. iva*, Bislim-Kumanovo, the slopes of the Pčinja Gorge, Former Yugoslavian Republic of Macedonia; *S. plumosa*, along the road side Bosilegrad–Izvor, Serbia and *S. scardica*, Kozjak Mountain, Former Yugoslavian Republic of Macedonia. The material was air-dried until constant weight. Voucher specimens are deposited in the Herbarium of the Faculty of Biology, University of

Belgrade, under the accession numbers: BEOU 16024, 16017, 16018 and 16063, respectively. The plant species were identified by Dr Vladimir Ranđelović, Department of Biology and Ecology, Faculty of Science and Mathematics, University of Niš.

Preparation of the extracts

Ground samples of air-dried aerial parts (50 g per extract preparation) were extracted in sealed vessels with 250 ml of the appropriate solvent (diethyl ether or ethyl acetate) in an ultrasonic bath (Bandelin Electronics, Germany) for 5 h at room temperature. The obtained extracts were gravity filtered through small columns packed with 1 g of Celite® (Merck, Germany) in order to remove all the insoluble material and then concentrated to 10 mL using a steam of nitrogen before GC and GC/MS analysis. The yields of the dry extracts (% w/w, dry plant material), obtained by complete evaporation of the solvents *in vacuo* are given in Table I-S in the Supplementary material. The dried extracts were dissolved in the solvents used for extraction to a final concentration of 1 mg mL^{-1} and used as such for the antimicrobial and antioxidant testing.

Gas chromatography and gas chromatography/mass spectrometry

The GC/MS analyses (three repetitions for each sample) were preformed using a Hewlett Packard gas chromatograph, Model 5890, Series II equipped with an SPB-1 capillary column (Supelco Inc., Bellfonte, PA; 30 m×0.25 mm, film thickness 0.25 μm) directly coupled to a mass selective detector MSD 5971A of the same company operated in the EI mode (70 eV). Helium was used as the carrier gas at a flow rate of 1 mL min^{-1}. The operating conditions were injector temperature 250 °C and the oven temperature program: isothermal at 50 °C for 3 min, then ramped to 250 °C at a rate of 5 °C min^{-1}, and finally isothermal at 250 °C for 15 min. The injection volume for all samples was 1 μL (prepared as mentioned previously), and the injector split ratio was 1:10. The extract constituents were identified by comparison of their linear retention indices (relative to C8–C31 alkanes[26] on the SPB-1 column) with literature values[27] and their mass spectra with those of authentic standard, as well as those from Wiley 6, NIST02, MassFinder 2.3 and a home-made MS library with spectra corresponding to pure substances and components of known oils. Wherever possible, the identity of the constituents was verified by co-injection with an authentic sample. GC (FID) analysis was performed under the same experimental conditions using the same column as described for the GC/MS measurements, except that H_2 was used as the carrier gas. The FID temperature was 300 °C. Percentage area values were obtained electronically from the GC–FID response, without the use of an internal standard or correction factors.

Antimicrobial activity

The *in vitro* antimicrobial activity of the extracts against a panel of laboratory control strains belonging to the American Type Culture Collections, Maryland, USA; Gram-positive: *Staphylococcus aureus* (ATCC 6538), Gram-negative: *Klebsiella pneumoniae* (ATCC 10031), *Pseudomonas aeruginosa* (ATCC 9027), *Salmonella enteritidis* (ATCC 13076); fungal organisms *Aspergillus niger* (ATCC 16404) and *Candida albicans* (ATCC 10231) and the Gram-negative bacteria *Escherichia coli* 95 (Institute of Immunology and Virology "Torlak", Serbia) was determined using the disc diffusion assay recommended by NCCLS.[28]

The following nutrition media were used: Antibiotic Medium 1 (Difco Laboratories, Detroit, MI, USA) for growing the Gram-positive and Gram-negative bacteria and Tripton soy agar (TSA, Torlak, Belgrade, Serbia) for *A. niger* and *C. albicans*. The nutrition media were prepared according to the instructions of the manufacturers. All agar plates were prepared in 90 mm Petri dishes with 22 mL of agar, giving the final depth of 4 mm. A suspension of the

tested microorganisms (0.1 mL, 10^8 cells per mL) was spread on the solid media plates. Sterile filter paper disks ("Antibiotica Test Blättchen", Schleicher and Schuell, Dassel, Germany, 6 mm in diameter) were impregnated with 20 μL of the extracts and placed on the inoculated plates. These plates, after standing at 4 °C for 2 h, were incubated at 37 °C for 24 h for the bacteria, and at 30 °C for 48 h for the fungi. Standard disks of tetracycline, gentamicin, ampicillin and nystatin (Institute of Immunology and Virology "Torlak", 30 μg of the active component, diameter 6 mm) were individually used as the positive controls, while disks imbued with 20 μL of pure diethyl ether or ethyl acetate were used as the negative controls. The diameters of the inhibition zones were measured in millimeters using a "Fisher-Lilly Antibiotic Zone Reader" (Fisher Scientific Co., USA). Each test was performed in triplicate. Mean values are presented.

Antioxidant capacity

The total antioxidant capacity of the extracts was evaluated by the method of Prieto, Pineda, and Aguilar.[29] The antioxidant capacity of the extracts was measured spectrophotometrically using the phosphomolybdenum method, based on the reduction of Mo(VI) to Mo(V) by the sample analyte and the subsequent formation of green phosphate/Mo(V) compounds with a maximum absorption at λ = 695 nm. A 0.1 mL aliquot of sample solution (100 μg mL^{-1}) was combined in an Eppendorf tube with 1 mL of the reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were capped and incubated in a boiling water bath at 95 °C for 90 min. After the samples had cooled to room temperature, the absorbance of each aqueous solution was measured at 695 nm against a blank solution, using a Perkin-Elmer Lambda 15 UV-VIS spectrophotometer. A typical blank solution contained 1 mL of reagent solution and 0.1 mL of the solvent used for the extraction (diethyl ether or ethyl acetate), which was incubated under the same conditions as for the other samples. Stock solutions of α-tocopherol acetate were prepared in diethyl ether and in ethyl acetate just prior to use. The total antioxidant capacity is expressed as equivalents of α-tocopherol acetate (l μmol g^{-1} of extract) using a standard curve at five concentrations covering the range 50–1.600 μmol. The exact concentrations were determined spectrophotometrically based on the absorption coefficients from the literature. The determination of the total antioxidant capacity was performed in triplicate.

RESULTS AND DISCUSSION

The GC and GC/MS analyses of the diethyl ether (DE) and ethyl acetate (EA) extracts of *S. germanica* subsp. *heldreichii*, *S. iva*, *S. plumosa* and *S. scardica* enabled the overall identification of 179 components, listed in Table I-S (Supplementary material). In each instance, the fraction of extracted compounds consisted of a complex mixture of different classes of compounds the identities of which are presented as a separate column in Tables I and I-S. Generally, two main classes dominated the composition of the DE extract: terpenoids (from 36.5 to 64.9%) and fatty acid derived compounds (FADs, from 33.3 to 59.9 %). The main characteristic of the EA extracts was the prevalence of FADs (from 68.4 to 85.9 %) among other classes. The common feature of the DE extracts was a relatively high content of oxygenated sesquiterpenes (from 12.2 to 33.1 %, except for *S. plumosa*, 2.1 %) and *n*-alkanes (from 14.1 to 39.3 %). *n*-Alkanes (ranging from 43.2 to 79.3 %), together with branched alkanes (from 5.9 to

17.0%) constitute considerable portions of the EA extracts. (*Z*)-Nuciferyl isobutyrate (*S. iva* DE, 15.3 %), spathulenol and caryophyllene oxide (3.8 % DE and 4.3 % EA for *S. germanica*, and 3.8 % DE and 3.6 % EA *S. iva*, respectively) and abieta-8,11,13-triene (13.6 % DE and 17.8 % EA for *S. plumosa* and 5.0 % DE *S. scardica*, Table I) were compounds of terpenoid origin detected in significant amounts. Monoterpenoids, aliphatic alcohols and aldehydes represented residual portions of the analyzed extracts, or were not even detected.

TABLE I. Chemical composition (%) of four *Stachys* species extracts

RI[a]	Compounds[b]	Class	Method[c]	*S. germanica*		*S. iva*		*S. plumosa*		*S. scardica*	
				DE[d]	EA[e]	DE	EA	DE	EA	DE	EA
947	α-Pinene	T	a,b,c	–[f]	–	–	0.1	0.1	1.4	0.1	–
1375	β-Bourbonene	T	a,b	2.2	–	–	–	–	–	0.2	–
1409	β-Caryophyllene	T	a,b,c	0.7	–	0.9	tr[g]	0.1	–	1.3	tr
1459	γ-Muurolene	T	a,b	0.6	–	–	–	–	–	2.0	–
1464	Germacrene D	T	a,b,c	1.5	–	0.3	–	–	–	–	–
1491	γ-Cadinene	T	a,b	–	–	–	–	–	–	2.0	–
1498	δ-Cadinene	T	a,b	–	–	2.7	–	0.2	–	1.4	–
1563	Spathulenol	T	a,b,c	3.8	–	3.8	–	–	–	0.4	–
1571	Caryophyllene oxide	T	a,b,c	4.3	–	3.6	0.1	0.7	–	3.1	–
1580	4(14)-Salvialen-1-one	T	a,b	1.4	–	–	–	–	–	1.3	–
1583	Viridiflorol	T	a,b	2.5	–	1.1	–	–	–	–	–
1601	Copaborneol	T	a,b	–	–	2.0	–	–	–	–	–
1623	Isospathulenol	T	a,b	–	–	–	–	–	–	1.9	–
1625	1-*epi*-Cubenol	T	a,b	–	–	1.2	–	–	–	–	–
1637	τ-Muurolol	T	a,b	0.3	–	1.2	–	0.3	–	1.6	–
1651	α-Cadinol	T	a,b	0.5	–	–	–	0.2	–	2.1	–
1669	Valeranone	T	a,b	2.1	–	1.4	0.1	–	–	–	–
1678	Cadalene	T	a,b	–	–	–	–	–	–	1.9	–
1692	3-Hydroxy-5,6-epoxy--β-ionone	CR	a,b	1.5	–	–	–	0.5	–	–	–
1731	7,8-Dihydro-3--oxo-α-ionol	CR	a,b	1.0	–	–	–	–	–	0.3	–
1844	Neophytadiene, isomer I	T	a,b	–	1.1	1.4	0.3	2.7	0.6	2.6	2.8
1845	Hexahydrofarne-sylacetone	CR	a,b	3.5	–	–	–	–	–	–	–
1900	Nonadecane	FAD	a,b,c	1.2	–	0.3	–	0.1	–	0.2	–
1910	Methyl hexadeca-noate (*syn.*[h] methyl palmitate)	FAD	a,b	1.2	0.2	0.1	–	0.3	–	0.5	–
1929	1-Methylphenantrene	O	a,b	–	–	–	–	–	1.2	–	–
1944	(*Z*)-Nuciferyl isobutyrate	T	a,b	4.5	0.2	15.3	1.2	0.1	–	–	–
1968	*n*-Hexadecanoic acid	FAD	a,b,c	–	–	–	–	6.0	–	–	–

TABLE I. Continued

RI[a]	Compounds[b]	Class	Method[c]	*S. germanica*		*S. iva*		*S. plumosa*		*S. scardica*	
				DE[d]	EA[e]	DE	EA	DE	EA	DE	EA
1979	Ethyl hexadecanoate (*syn.* ethyl palmitate)	FAD	a,b	1.7	0.1	0.3	–	6.1	–	4.8	–
2000	Eicosane	FAD	a,b,c	0.5	–	–	–	–	–	1.9	–
2007	13-*epi*-Manoyl oxide	T	a,b	–	–	–	–	2.4	–	–	–
2039	Abieta-8,11,13-triene	T	a,b	0.3	–	0.1	–	13.6	17.8	5.0	0.8
2073	Methyl-(*Z*,*Z*,*Z*)--9,12,15-octadecatrienoate (*syn.* methyl linolenate)	FAD	a,b	1.5	–	0.1	–	1.1	–	0.8	–
2083	(*Z*)-9-Octadecenoic acid (*syn.* oleic acid)	FAD	a,b	–	0.6	–	–	1.7	–	0.2	0.5
2084	Methyl-(*Z*)-9-octadecenoate (*syn.* methyl oleate)	FAD	a,b	1.0	–	0.2	–	–	–	0.4	–
2093	Isoabienol	T	a,b	–	–	3.6	1.2	–	–	–	–
2100	Heneicosane	FAD	a,b,c	2.2	0.4	–	0.4	0.2	–	0.5	0.1
2105	*trans*-Phytol	T	a,b	5.2	0.7	4.0	0.8	7.9	–	3.8	0.9
2127	Methyl octadecanoate (*syn.* methyl stearate)	FAD	a,b	1.5	–	3.2	–	0.4	0.9	0.4	0.1
2140	Ethyl (*Z*,*Z*)-9,12-octadecadienoate (*syn.* ethyl linoleate)	FAD	a,b	1.4	–	0.3	–	2.9	–	0.4	–
2145	Ethyl (*Z*,*Z*,*Z*)-9,12,15--octadecatrienoate (*syn.* ethyl linolenate)	FAD	a,b	0.5	–	0.3	–	4.4	–	0.2	–
2170	Thunbergol	T	a,b	–	–	–	–	2.3	–	0.9	–
2178	Ethyl octadecanoate	FAD	a,b	0.2	–	0.2	–	1.5	–	0.4	–
2200	Docosane	FAD	a,b,c	1.6	0.6	0.3	tr	–	–	0.6	0.2
2209	Sclareol	T	a,b	–	–	0.3	0.1	–	1.5	–	–
2223	*cis*-Totarol	T	a,b	–	–	–	0.9	3.0	–	–	–
2270	Dehydroabietal	T	a,b	–	–	–	–	3.5	–	0.9	–
2271	1-Eicosanol	FAD	a,b	2.8	0.4	–	–	–	–	–	–
2300	Tricosane	FAD	a,b,c	5.5	1.8	3.8	0.5	6.6	–	17.6	1.4
2328	11-Methyltricosane	FAD	a,b	–	–	–	–	–	–	–	4.4
2338	Dehydroabietic acid methyl ester	T	a,b	–	–	–	–	2.8	–	1.4	1.2
2347	Dehydroabietol	T	a,b	–	–	–	–	1.3	6.2	–	–
2361	Butyl octadecanoate	FAD	a,b	–	–	0.5	0.2	1.4	–	0.8	–
2378	Ethyl eicosanoate	FAD	a,b	0.6	–	tr	–	0.4	–	2.1	–
2398	Labd-13(*E*)-en--8α,15-diol	T	a,b	–	–	14.3	–	–	–	–	–
2400	Tetracosane	FAD	a,b,c	1.0	0.6	tr	–	0.9	–	0.8	0.5
2431	Methyl neoabietate	T	a,b	–	–	0.3	9.6	0.2	–	–	–
2468	1-Docosanol	FAD	a,b	1.2	0.4	–	–	–	–	–	–

TABLE I. Continued

RI[a]	Compounds[b]	Class	Method[c]	*S. germanica*		*S. iva*		*S. plumosa*		*S. scardica*	
				DE[d]	EA[e]	DE	EA	DE	EA	DE	EA
2500	Pentacosane	FAD	a,b,c	3.6	2.5	0.6	0.8	1.2	2.6	1.3	1.4
2564	2-Methylpentacosane	FAD	a,b	0.1	–	–	–	–	13.2	–	–
2573	3-Methylpentacosane	FAD	a,b	–	0.3	–	1.5	–	–	–	–
2576	Ethyl docosanoate	FAD	a,b	–	–	–	–	0.8	6.1	0.2	–
2700	Heptacosane	FAD	a,b,c	4.9	7.2	2.9	5.3	4.3	27.7	4.3	5.0
2771	3-Methylheptacosane	FAD	a,b	0.2	1.0	–	0.1	0.1	–	–	–
2800	Octacosane	FAD	a,b,c	0.8	1.9	0.8	1.4	0.5	2.8	0.3	1.1
2808	Squalene	T	a,b	0.8	1.1	0.1	0.2	0.2	–	0.5	1.6
2859	10-Demethylsqualene	T	a,b	0.1	0.2	0.5	–	0.3	–	1.0	0.4
2900	Nonacosane	FAD	a,b,c	17.0	36.9	18.0	28.3	–	6.5	3.6	19.3
2934	10-Methylnonacosane	FAD	a,b	–	2.7	–	12.0	–	–	–	–
2973	3-Methylnonacosane	FAD	a,b	–	0.3	–	–	–	3.2	–	0.6
3000	Triacontane	FAD	a,b,c	0.6	2.1	–	2.1	–	3.6	8.0	5.9
3100	Hentriacontane	FAD	a,b	–	20.9	–	23.3	–	–	–	43.7
Total				96.4	88.8	98.1	93.2	91.9	96.7	97.5	94.7
Yield, % (w/w)				1.4	10.0	1.5	4.0	1.4	8.0	1.5	5.8
Grouped components											
Terpenoids (T)				36.5	3.9	64.7	14.7	47.3	28.3	43.0	8.8
Monoterpene hydrocarbons				–	–	tr	0.1	0.6	2.2	0.3	–
Oxygenated monoterpenes				0.4	–	–	–	2.5	–	0.1	–
Sesquiterpene hydrocarbons				6.5	–	6.0	tr	0.8	–	12.6	tr
Oxygenated sesquiterpenes				22.5	0.2	32.9	1.4	2.1	–	12.7	0.1
Diterpenes				6.0	2.4	25.2	13.0	40.8	26.1	15.7	6.5
Triterpenes				1.1	1.3	0.6	0.2	0.5	–	2.1	2.2
Fatty acid derived compounds (FAD)				59.9	84.9	33.4	78.5	44.6	68.4	54.5	85.9
n-Alkanes				39.2	74.9	26.9	62.1	14.1	43.2	39.3	79.3
Branched alkanes				0.3	7.1	–	15.4	0.2	17.0	0.8	5.9
Alcoholes				4.0	0.8	–	–	–	–	–	–
Aldehydes				–	–	tr	0.1	0.5	–	–	0.1
Fatty acids and fatty acid esters				10.4	1.7	6.4	0.9	28.3	7.0	13.8	0.6
Carotenoid derived compounds				6.0	0.4	0.1	–	1.3	–	0.3	–
Others (O)				–	–	–	–	0.2	1.2	0.3	–

[a]Components listed in order of elution from SPB-1 column (RI – experimentally determined retention indices on the mentioned column by co-injection of a homologous series of *n*-alkanes C_8–C_{31}); [b]major components are given in Table I (components having relative abundances ≥1.0 % in at least one sample), while the other identified components (minor) can be found in Supplementary material (see Supplementary material for detail composition on extracted volatiles); [c]a – constituent identified by retention index matching; b – constituent identified by mass spectra comparison; c – constituent identified by co-injection of an authentic sample; [d]diethyl ether extract; [e]ethyl acetate extract; [f]not detected; [g]trace (<0.05 %); [h]synonym

As already mentioned, an interesting feature of the extracts was the occurrence of branched alkanes, detected in relatively significant amounts in the EA

extracts (from 5.9 to 17.0 %) and as minor constituents in the DE extract (from 0.2 to 0.4 %). Until now, branched alkanes were considered to have limited occurrence in the plant kingdom, and their distribution was assumed to be a characteristic of evolutionary primitive and old higher plant taxa.[30] However, it might be just as possible that these compounds may be taken into account as common constituents of epicuticular waxes of evolutionary and philogenetically more complex plants, although present in lower amounts (distribution of long chain *n*-alkanes is dominant[31]). Publications dealing with other members of the Lamiaceae family, *i.e.*, *Marrubium*,[32] *Micromeria*[33] and *Scutellaria*,[34] also reported the identification of branched alkenes among the components detected during analyses of epidermal tissue hydrocarbons. This provides more reason to speculate and employ this observation as a hypothesis for future work (extending analyzes to related families).

An additional characteristic of the analyzed extracts was the ubiquitous presence of diterpenoids in all samples. Among the samples analysed, *S. plumosa* contained the highest portion (DE 40.8 % and EA 26.1 %), while *S. germanica* (DE 6 % and EA 2.4 %) had the smallest portion of the diterpene moieties detected. The distribution of the extractable compounds within the diterpenoid fraction was in favor of abietanes, labdanes and phytanes. Conservation of the diterpenoid skeleton has proven to be useful in reflecting chemotaxonomic features within the Lamiaceae family.[35,36] Concerning the taxonomical problems within the *Stachys* genus and several papers dealing this matter,[37–40] it might be valuable to perceive if diterpenes (and if, which of the skeletal types is the most prominent one) can be considered as biomarkers reflecting potential infrageneric chemotaxonomic relations.

Comparing the composition of the obtained extracts (DE and EA, present study) with previously published results for the essential oils[22] of the *Stachys* species under study (collected from the same localities), it can be observed that FAD constituents were present in high amounts in the extracts (Table I), while only in the oil of *S. germanica* subsp. *heldreichii* were FADs identified (*n*-alkanes 0.2 %).

The results of the antimicrobial assay showed that the DE extracts of *Stachys* inhibited the growth of all the tested bacteria (Table II), but only *S. plumosa* exhibited significant antimicrobial activity against the most persistent human pathogens *E. coli*, *P. aeruginosa* and *S. aureus* (comparable to antibiotics used as the positive controls). The obtained results seem to be in agreement with the activity that can be expected when referring to the chemical composition of the extracts, since *S. plumosa* DE components (oxygenated [3.1.1] bicyclic (pinane type) monoterpenoides *cis*/*trans*-verbenol, myrtenal, pinocarvone) have been shown to act as good antimicrobial agents.[41] However, concerning the hitherto reported publications, diterpenes (identified in the present extracts in significant amounts,

TABLE II. The antimicrobial activity (diameters of growth inhibition zones measured in mm) of the extracts of four *Stachys* species

Sample	Microorganism										
	E. coli[a]		*S. enteritidis*	*P. aeruginosa*		*K. pneumoniae*		*S. aureus*		*C. albicans*	*A. niger*
	C[b]	S[b]	S	C	S	C	S	C	S	S	S
						S. iva					
DE	–	22.7±0.09	22.7±0.67	–	–	19.9±0.16	21.1±0.64	–	–	–	–
EA	–	19.0±0.11	15.8±0.15	–	17.8±0.11	–	15.8±0.23	–	17.9±0.66	–	–
						S. germanica					
DE	–	24.1±0.19	24.0±0.84	–	29.4±0.28	–	20.7±0.05	–	22.0±0.61	–	28.6±0.37
EA	–	15.6±0.23	15.4±0.61	–	15.8±0.10	–	–	–	–	–	–
						S. plumosa					
DE	23.8±0.04	31.8±0.13	31.8±1.23	20.4±0.10	31.0±0.01	–	20.2±0.35	23.1±0.84	30.0±0.61	–	24.1±0.10
EA	–	15.8±0.31	16.2±0.37	–	16.1±0.51	–	–	–	14.8±0.03	–	–
						S. scardica					
DE	–	23.8±0.09	25.9±0.07	–	25.0±0.08	–	–	–	27.0±0.94	23.0±0.77	24.7±0.70
EA	–	15.2±0.08	17.8±0.12	–	14.7±0.26	–	–	–	14.9±0.21	–	–
Tetracycline[c]	30.6±0.53		31.3±0.73	29.3±0.63		30.3±0.77		30.7±0.42		nt[d]	nt
Gentamicine[c]	24.2±0.99		22.2±0.35	19.8±0.87		16.5±0.62		19.2±1.23		nt	nt
Ampicillin[c]	12.2±0.61		11.2±0.14	–		14.1±0.09		–		nt	nt
Nystatin[c]	nt		nt	nt		nt		nt		17.2±0.34	19.0±0.27

[a] Mean value ±*SD* (in mm) of 5 experiments, including disk diameter, 6 mm (20 μg of extract per disk). Values for static zones represent the extra millimeters around the cidal zone (or the sole disk if no cidal activity) in which the growth of microorganisms was inhibited but in which the microorganisms were not killed; [b] bacteri- and fungicidal zones; [c] positive control cidal activity (30 μg per disk); [d] not tested

Table I) might be acting as antimicrobial agents in these plants as well.[42,43] Labdane type diterpenes detected in the DE extracts of *S. iva* and *S. plumosa*, 13--*epi*-manoyl oxide (*S. plumosa* DE, Table I) and labd-13(*E*)-ene-8*α*,15-diol (*S. iva* DE, Table I), have been tested previously and found to possess significant antimicrobial activity.[44,45] However, although *S. iva* contained significant amount of labd-13(*E*)-ene-8*α*,15-diol (14.3 %), the activity of the extract was a selective one and affected the growth of *K. pneumoniae* only. The EA extracts exhibited no significant antimicrobial activity. The low activity could be correlated with the high content of alkane fraction (alkanes are known as substances that are not active against microorganisms).

The results of the total antioxidant capacity assay (Table III) showed that the extracts possessed antioxidant activity. The antioxidant capacity of the DE extracts was approximately ten times higher than the capacity of the EA extracts. The values for the capacity of EA extracts were comparable for all species under study, ranging between 4 and 5 μmol g^{-1} of the EA extracts (expressed as equivalents of *α*-tocopherol acetate, rounded numbers). The highest antioxidant capacity was measured for the DE extract of *S. plumosa*, and was approximately two times higher than the activity of DE extracts of the other examined species (Table II). A previously published paper reported significant antioxidant activities for the methanol extract of *S. plumosa* (tested for its ferric reducing antioxidant power assay, 1,1-diphenyl-2-picryl-hydrazyl free radical and OH radical scavenging activity, and in lipid peroxidation assays), which were attributed to the large amount of total phenolics in the methanol extract of the examined species (reported in this paper as well).[16] The value obtained in the total antioxidant capacity assay for the DE extract of *S. plumosa* (present work, no polyphenols detected) indicates that apart from polyphenols, this species might be having other

TABLE II. The total antioxidant capacity of the extracts of *Stachys* species from the Balkans expressed as equivalents of *α*-tocopherol acetate (μmol g^{-1} of extract)

Sample	Antioxidant activity (the mean values±*SD* of five experiments)
S. iva	
DE	38.1±0.35
EA	4.4±0.77
S. germanica	
DE	47.0±0.31
EA	4.0±0.11
S. plumosa	
DE	75.3±1.94
EA	5.1±0.59
S. scardica	
DE	34.0±0.54
EA	4.9±0.51

compound(s) that could act as antioxidant agent(s). However, due to the complexity of the extract analyzed (more than 80 compounds detected), it seems difficult to explain which component of this complex mixture may be responsible for the expressed activity.

CONCLUSIONS

The performed GC and GC/MS analyses enabled the identification of 179 components obtained from four Balkan endemic *Stachys* species (*S. germanica* subsp. *heldreichii* (Boiss) Hayek, *S. iva* Griseb., *S. plumosa* Griseb. and *S. scardica* Griseb) extracts. Terpenoids (from 36.5 to 64.9 %) and fatty acid derived-compounds (FADs from 33.4 to 59.9 %) were the main compound classes detected among the extractable matter obtained when diethyl ether was used as the extracting solvent. The main characteristic of the ethyl acetate extracts was the prevalence of FADs (from 68.4 to 85.9 %), with its most dominant subfraction represented by *n*- and branched alkanes. An additional characteristic of the analyzed extracts was the presence of diterpenoids in all samples (from 40.8 to 2.4 %). The results of the antimicrobial assay showed that DE extracts of *Stachys* inhibited the growth of all tested bacteria (*S. plumosa* activity was comparable to those of the antibiotics used as positive controls), while the activities of the EA extracts could be considered weak. The results of the total antioxidant capacity assay showed that the studied species possessed antioxidant activity, which was found to be the greatest in the case of the DE extract of *S. plumosa*.

In conclusion, the DE extract of *S. plumosa* exhibited good antimicrobial and antioxidant activity and could be considered as a good candidate for raw material phyto-preparations.

SUPPLEMENTARY MATERIAL

The yields of the dry extracts of plant material are available electronically from http://www.shd.org.rs/JSCS/, or from the corresponding author on request.

Acknowledgement. This work was funded by the Ministry of Science and Technological Development of the Republic of Serbia, Project No. 142054B.

И З В О Д

ХЕМИЈСКИ САСТАВ, АНТИМИКРОБНА И АНТИОКСИДАНТНА АКТИВНОСТ ЕКСТРАКАТА ОДАБРАНИХ БИЉНИХ ВРСТА РОДА *Stachys*

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Диетил-етарски и етил-ацетатни екстракти надземних делова ендемичних балканских биљних врста, *Stachys germanica* ssp. *heldreichii* (Boiss) Hayek, *Stachys iva* Griseb., *Stachys*

plumosa Griseb. и *Stachys scardica* Griseb, анализирани су комбинацијом GC и GC/MS. У испитиваним екстрактима идентификовано је укупно 179 компонената, које су чиниле од 88,8 до 98,1 % екстраката. Етарски екстракти анализираних биљака садрже висок проценат терпеноида (од 36,5 до 64,7 %) и деривата масних киселина (од 33,4 до 59,9 %), док су се етил-ацетатни одликовали високим процентом деривата масних киселина (од 68,4 до 85,9 %). Антимикробна активност добијених екстраката испитана је диск дифузионом методом користећи лабораторијски контролисане сојеве бактерија (*Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa* и *Salmonella enteritidis*) и гљивица (*Aspergillus niger* и *Candida albicans*). Антиоксидантни капацитет екстраката одређен је фосфомолибденском методом. Прелиминарни резултати in vitro спроведених биотестова указују на значајну антимикробну активност и антиоксидантни капацитет етарског екстракта биљне врсте *S. plumosa*, те да ову биљну врсту треба узети у обзир за даља испитивања у циљу практичне примене.

(Примљено 3. јуна, ревидирано 24. августа 2010)

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