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Chemical composition, antimicrobial, antioxidative and anticholinesterase activity of *Satureja montana* L. ssp *montana* essential oil

Research Article

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Abstract: The present study investigates the chemical compositions of three *Satureja montana* L. ssp *montana* essential oils and correlates chemical variability with biological activities. GC/MS analysis showed that with an increase in altitude (100-500-800 m), a higher content of linalool, terpinen-4-ol and *cis*-sabinene hydrate was found, while the percentage of phenolic compounds, thymol and carvacrol decreased. Antimicrobial activity of the essential oils was tested against 7 fungal and 23 bacterial strains. The essential oil characterized by the highest content of phenols and alcohols exhibited the highest antimicrobial potential. The correlation analysis showed that the major carriers of the obtained antioxidant activity are oxygenated monoterpenes. All essential oils inhibited human serum cholinesterase activity. High antimicrobial potential, together with moderate antioxidant capacity and strong inhibition of human serum cholinesterase, classifies *S. montana* essential oil as a natural source of compounds that can be used in the treatment of foodborne and neurological diseases, wound and other infections, as well as for general health improvement.

Keywords: *Satureja montana L. ssp montana • Essential oil • Antimicrobial activity • Antioxidant activity • Cholinesterase inhibiton*

1. Introduction

Satureja montana L. (winter savory) is an aromatic plant, traditionally used on the Balkan peninsula as a spice and a natural food preservative [1]. Savory honey is a very common ingredient in folk remedies for the treatment of bronchitis. Also, it is used as choleretic, stimulant, digestive, antiseptic for gastrointestinal tract [2] and for the treatment of premature ejaculation [3]. A number of investigations justified traditional utilization and have confirmed various biological effects of this plant species namely, antioxidant [4,5], antibacterial [4,6-10] and antifungal [11]. *Satureja montana* essential oil showed antiproliferative activity on human erythroleukemic K562 cells [12]. Also, this essential oil showed the ability to prevent *in vitro* peroxynitrite oxidation better than antioxidants of reference such as ascorbic acid [13].

According to the avaliable literature, there are chemical differences of this plants' essential oils, obtained from specimens collected at different localities [4,14-16] and in different ontogenetic stages [7,15,17]. It is important to note that in the mentioned investigations, their antimicrobial activities were either not tested or the authors didn't investigated the relation between chemical composition and the obtained activity of the essential oil [8-11]. Some of these studies used only disc diffusion method [4] or did not specify which subspecies of the investigated plant [4,6,7] was used for the essential oil isolation. On the Balkan peninsula, genus *Satureja* is represented by nine species. Species

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S. montana has three subspecies - ssp. *variegata* (Host) P.W. Ball; ssp. *montana* L. and ssp. *pisidica* (Wettst.) [18]. The studies investigating the chemical composition of this species' essential oils showed that there are significant differences at the subspecies level [19], which affects their biological properties.

All mentioned facts prompted us to investigate the chemical composition of this plant at the subspecies level and to correlate differences in the chemical composition with different biological activities: antimicrobial, antioxidative and anticholinesterase.

2. Experimental Procedures

2.1 Plant Material

The aerial parts of wild growing *S. montana* ssp *montana* were collected during the flowering stage from the three different localities in Montenegro: Sample 1 - near the city of Budva - 100 m; Sample 2 - Cetinje road - 500 m and Sample 3 - Orjen Mt. - 800 m a.s.l.). Voucher specimens No. 2-1930 were confirmed and deposited at the Herbarium of the Department of Biology and Ecology (BUNS Herbarium), Faculty of Natural Sciences, University of Novi Sad.

2.1.1 Isolation of the essential oil

Air-drying of plant material was performed under shade at room temperature for 10 days. Dried aerial parts (100 g) were cut and subjected to hydro-distillation for 3 h, using a Clevenger-type apparatus [20]. The resulting essential oil was dried over anhydrous sodium sulfate and preserved in a sealed vial at 4°C until further analysis.

2.1.2 Gas chromatography

GC (Gas Chromatography) analyses of the oils were carried out on a GC HP-5890 II apparatus equipped with the split-splitless injector, attached to HP-5 column (25 m x 0.32 mm, 0.52 μm film thickness) and fitted to FID (Flame Ionisation Detector). The carrier gas (H_2) flow rate was 1 ml min^{-1}, split ratio 1:30, injector temperature 250°C, and detector temperature 300°C, while the column temperature was linearly programmed from 40°C to 240°C at a rate of 4°C min^{-1}.

2.1.3 Gas chromatography–mass spectrometry

The same analytical conditions were employed for GC-MS analyses, where a HP G 1800C Series II GCD system was used. The transfer line was heated to 260°C. Mass spectra were acquired in EI mode (70 eV), in *m/e* range 40-400 (column HP-5MS 30 m x 0.25 mm, 0.25 μm film thickness).

2.1.4 Identification of components

Oil components were identified by comparing their mass spectra with those of Adams [21] Wiley 275 and NIST/NBS libraries [22]. The experimental values of retention indices were determined by the use of calibrated Automated Mass Spectral Deconvolution and Identification System software (AMDIS ver.2.1., DTRA/NIST, 2002). The obtained results were correlated with literature data [21] as well as by using other sources (www.flavornet.org; iowtv.pherobase.com). For quantification, area percent data obtained by FID were used.

2.2 Antimicrobial activity

2.2.1 Microbial strains

The antimicrobial activity of *S. montana* L. ssp *montana* essential oil was evaluated using three groups of microorganisms: (1) laboratory reference strains, *Bacillus subtilis* ATCC 6633, *Bacillus cereus* ATCC 10876, *Clostridium perfringens* ATCC 19404, *Listeria monocytogenes* ATCC 15313, *Staphylococcus aureus* ATCC 6538, *Staphylococcus aureus* ATCC 25923 and *Sarcina lutea* ATCC 9341 (Gram (+) bacteria), *Escherichia coli* ATCC 8739, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 9027 and *Salmonella enteritidis* ATCC 13076 (Gram (-) bacteria) and *Aspergillus niger* ATCC 16404, *Candida albicans* ATCC 10231 and *Saccharomyces cerevisiae* 112 Hefebank Weihenstephan (fungal microorganisms) obtained from the American Type Culture Collection; (2) clinical isolates from wounds: *Klebsiella pneumoniae, Klebsiella oxytoca, Escherichia coli, Proteus mirabilis, Staphylococcus aureus, Staphylococcus* sp., *Streptococcus pyogenes, Pseudomonas aeruginosa, Enterococcus* sp., *Enterobacter* sp., *Citrobacter* sp. and *Acinetobacter* sp. (Source: Center for Microbiology, Institute for Public Health of Vojvodina, Novi Sad, Serbia); and (3) fungal strains isolated from matress dust: *Aspergillus fumigatus, Aspergillus restrictus, Penicillium chrysogenum* and *Acremonium chrysogenum.*

2.3 Micro-well Dilution Assay

Minimum inhibitory (MIC) and bactericidal/fungicidal concentrations (MBC/MFC) of essential oils were determined by employing the broth micro-well dilution method as described previously [22]. Bacterial species were cultured at 37°C in Mueller Hinton agar for bacteria and Sabouraud dextrose agar for fungi (28 and 30°C). After 18 h of incubation, bacterial suspensions were made in Mueller Hinton broth and their turbidity was standardized to 0.5 McFarland.

The final density of bacterial and yeast's inoculum was 5 x 10^5. Suspensions of the molds were made in sterile saline and their turbidity was confirmed by viable counting in a Thoma chamber. Final size of the mold's inoculum in Sabouraud dextrose broth was 1 x 10^4. Dimethylsulfoxide (DMSO) was used to dissolve the essential oils. Then, serial dilutions of the oils were made in a microtiter plate (dilution factor 2). The inoculum was added to all wells and the plates were cultivated at 37°C for 24 h (bacteria) or at 30°C (*C. albicans*) and 28°C (molds) for 48 h. All experiments were performed in triplicate, where two growth controls consisting of corresponding medium with DMSO (10%) were included. Antibiotics Chloramphenicol, Streptomycin, Kanamycin and Nystatin served as positive controls. One inoculated well was included, to allow control of the adequacy of the broth for organism growth. One non-inoculated well, free of any antimicrobial agent, was included to ensure medium sterility. The bacterial growth was determined by adding 20 µl of 0.5% TTC (triphenyl tetrazolium chloride) aqueous solution. Minimal inhibitory concentration (MIC) was defined as the lowest concentration of the oil that inhibited visible growth (red colored pellet on the bottom of the wells after the addition of TTC), while minimal bactericidal concentration (MBC) was defined as the lowest oil concentration that killed 99.9% of bacterial/fungal cells. To determine MBC/MFC (minimal fungicidal concentration), broth was taken from each well without visible growth and inoculated in Mueller Hinton agar (MHA) for 24 h at 37°C for bacteria or in Sabouraud dextrose agar (SDA) for 48 h at 28°C (molds) and 30°C (yeast). The experiments were done in triplicate and the mean values are presented.

2.4 Antioxidant assays

2.4.1 1,1-diphenyl-2-picrylhydrasyl (DPPH) radical scavenging assay

Relatively stable organic radical DPPH has been widely used in the determination of antioxidant activity of single compounds as well as the different plant extracts [23]. The DPPH assay was performed as previously described [24]. Radical scavenging activity (RSC) of the essential oils was calculated applying following equation:

$$\text{DPPH RSC}(\%)=100\ (A_0-A_1/A_0)$$

Where:

A_0 - absorbance of the blank; A_1 - absorbance of the sample.

The EC_{50} values were determined by polynomial regression analysis of the obtained DPPH-RSC values (software MS Excel 07).

2.4.2 Oxygen radical absorbance capacity (ORAC) - ABTS+ radical cation decolorisation assay

Antioxidant capacity of the oils was evaluated using a Perkin Elmer Lambda 15 UV-VIS spectrophotometer, using the improved $ABTS^+$ method, as described by Re *et al.* [25]. $ABTS^+$ radical cation was generated by a reaction of 7 mM L^{-1} ABTS (Sigma Aldrich, Germany) with 2.45 mM L^{-1} potassium persulfate (Merck, Germany) until an absorbance of 0.700 ± 0.050 at 734 nm was reached. Generation of radical before the antioxidants are added prevents interference of compounds, which affect radical formation. This modification makes the assay less susceptible to artifacts and prevents overestimation of antioxidant capacity [26]. When stable absorbance is obtained, the antioxidant sample is added to the reaction medium, and the antioxidant activity is measured in terms of decolorization. Fifty microlitres of diluted sample were mixed with 1.9 ml of diluted $ABTS^+$ solution. The mixture was allowed to stand for 6 min at room temperature and the absorbance was immediately recorded at 734 nm. Trolox (Sigma Aldrich) solution (final concentration 0-15 µM L^{-1}) was used as a reference standard. The results were expressed as Trolox equivalents (µM of trolox equivalents per mg of essential oil).

2.4.3 Total reducing power assay Fe(III) to Fe(II)

Reducing power of the oils was determined as described previously [24] and expressed in relation to the reducing power of ascorbic acid as a positive control (Ascorbate Equivalent Antioxidant Capacity).

2.5 Inhibition of cholinesterase

Modified Ellman's method was applied for testing impact of essential oils on pooled human serum cholinesterase [27]. A total of 10 healthy volunteers (18-65 years old from both sexes), from the Pirot General Hospital, donated blood with written consent. According to the questionnaire, none of them had serious medical disorders, nor are or have been drug, cigarette, or alcohol abusers. At least a month before the blood donation, none of them had been taking any medication [27].

2.6 Statistical analysis of data

The experimental results were expressed as mean±standard deviation (SD) of three replicates. Correlation coefficients to determine the relationship between two variables (between different antioxidant assays; tests and content of major groups of essential oils constituents) were calculated using MS Excel software (CORREL statistical function).

3. Results

Essential oils of wild growing *S. montana* ssp. *montana*, (collected at three different locations) were isolated by hydro distillation. The yields of the essential oils were 0.42, 0.40 and 0.38% (v/w) (S1 - Budva, S2 - Cetinje and S3 – Orijen Mt., respectively). Qualitative and quantitative data on the main constituents of essential oils have been summarized in Table 1.

GC and GC/MS analyses revealed that the essential oil from the S1 possess a high content of the phenolic compounds thymol and carvacrol, followed by linalool. Chemical composition of the S2 essential oil is characterized by a high content of carvacrol, linalool and terpinen-4-ol, followed by *cis*-sabinene hydrate. Compared to the other components of the essential oils isolated from S1 and S2, *p*-cymene and borneol had significantly higher content. Contrary to the previous two samples, essential oil isolated from the S3 did not contained any phenolic compounds, but had linalool, *cis*-sabinene hydrate and terpinen-4-ol as the most abundant components. All three samples had significant amount of the sesquiterpenes β-caryophyllene and caryophyllene-oxide. Apart from the two mentioned compounds, the oil from the S3 contained a significant amount of nerolidol.

In the present paper, the essential oils were tested using a broth microdilution assay against a panel of 30 microorganisms including laboratory control strains (ATCC) of bacteria and fungi, clinical isolates from wounds and fungal isolates from matress dust (Table 2). The antimicrobial assay showed that the highest activity was exhibited by the oil from the S1, with inhibitory/bactericidal activity in the range 0.18-0.78 μL mL^{-1} (except against *P. aeruginosa*, which was much more resistant) while, the essential oils from the S2 and S3 samples showed much higher efficacy against *P. aeruginosa*. When considering Gram positive bacteria, all three investigated oils showed significant antimicrobial effect in the range from 0.18-6.25 μL mL^{-1}. The obtained activity was in the range from 0.18-1.56 μL mL^{-1} against both tested *S. aureus* strains.

All three tested oils exhibited activity against all tested multiresistant strains in the range 3.13-50.0 μL mL^{-1}, with the exception of *P. aeruginosa* and *Citrobacter sp.* (resistant to the highest tested concentrations of the S2 and S3 oils), together with *K. pneumoniae* (resistant to the highest tested concentrations of the S3 oil). Essential oil isolated from the S1 exhibited the most prominent antimicrobial activity.

No.	Constituents	Sample 1 100 m*	Sample 2 500 m*	Sample 3 800 m*
1	α-thujene	0.20	/	/
2	α-pinene	0.21	/	/
3	camphene	0.25	/	/
4	sabinene	1.04	1.05	0.26
5	1-octen-3-ol	/	0.07	0.18
6	β-myrcene	0.19	/	/
7	α-phellandrene	/	0.08	/
8	α-terpinene	0.62	0.18	0.65
9	p-cymene	6.61	5.38	1.39
10	β-phellandrene	/	0.20	0.26
11	D-limonen	0.31	/	/
12	1,8-cineole	0.13	0.13	/
13	cis-β-ocimene	0.24	0.09	0.47
14	γ-terpinene	2.11	0.06	2.39
15	cis-sabinene hydrate	3.50	14.61	23.05
16	cis-linalool oxide	0.48	0.33	/
17	terpinolene	0.60	0.35	0.27
18	trans-linalool oxide	t	0.08	/
19	linalool	15.38	17.94	32.58
20	cis-p-menth-2-en-1-ol	0.33	0.76	1.38
21	terpine-1-ol	/	0.44	0.33
22	trans-pinocarveol	0.29	/	/
23	camphor	0.19	0.40	/
24	borneol	3.87	3.62	0.69

Table 1. Phytochemical composition (%) of *Satureja montana* ssp *montana* essential oils.

No.	Constituents	Sample 1 100 m*	Sample 2 500 m*	Sample 3 800 m*
25	terpinen-4-ol	4.81	10.60	10.99
26	p-cymene-8-ol	0.30	0.69	/
27	α-terpineol	0.64	1.30	1.30
28	cis-piperitol	/	0.39	/
29	trans-piperitol	/	0.28	/
30	trans-carveol	/	0.04	/
31	trans-dihydrocarvone	0.09	/	/
32	thymol methyl ether	2.32	0.05	/
33	carvacrol methyl ether	2.30	0.69	/
34	carvone	/	0.12	/
35	geraniol	0.19	0.12	/
36	geranial	/	0.09	/
37	bornyl acetete	/	0.06	/
38	thymol	24.69	2.46	/
39	carvacrol	15.19	24.46	/
40	myrtenyl acetate	0.15	/	/
41	α-cubebene	0.08	/	/
42	α-copaene	/	0.04	/
43	β-bourbonene	0.15	0.32	0.98
44	β-elemene	/	/	/
45	β-caryophyllene	4.38	3.37	2.57
46	β-copaene	/	0.08	0.13
47	germacrene D	/	0.38	2.34
48	aromadendrene	/	0.14	/
49	cis-β-farnesene	0.12	/	/
50	α-humulene	0.17	0.13	0.14
51	bicyclogermacrene	/	/	0.71
52	γ-muurolene	0.14	0.16	/
53	γ-crene D	0.24	/	/
54	leden-viridiflorene	/	0.32	/
55	β-bisabolene	1.19	0.37	/
56	β-curcumene	0.14	0.14	/
57	δ-cadinene	0.27	0.25	0.27
58	elemol	0.24	0.47	2.72
69	nerolidol	/	/	9.36
60	spathulenol	0.39	1.32	/
61	caryophyllene oxide	3.96	3.30	1.27
62	maalinen	/	0.14	0.37
63	β-eudesmol	/	0.82	0.38
64	β-oplopenone	0.13	/	/
65	τ-cadinol	0.40	/	/
66	τ-muurolol	0.55	/	/
67	β-bisabolol	0.26	/	/
	Monoterpene hydrocarbons	11.78	7.04	5.42
	Oxygenated monoterpenes	75.53	80.08	70.77
	Sesquiterpene hydrocarbons	11.39	10.93	20.86
	Oxygenated sesquiterpenes	2.72	0.82	0.38
	Identified (%)	47 compounds (95.80%)	49 compounds (98.80%)	27 compounds (97.25%)
	Oil yield (%)	0.42	0.40	0.38
	SD - standard deviation	0.03	0.02	0.02

continued **Table 1.** Phytochemical composition (%) of *Satureja montana* ssp *montana* essential oils.

Microorganisms	Essential oil (MIC/MBC in μL mL^{-1})			Antibiotics (MIC/MBC in μg mL^{-1})			
	Sample 1 100 m*	Sample 2 500 m*	Sample 3 800 m*	Strept.	Chlor.	Nyst.	Kan.
Gram (-) sorce No.							
Escherichia coli ATCC 8739	0.78/0.78	0.78/3.13	3.13/6.25	8.0/8.0	nt	nt	nt
Escherichia coli ATCC 25922	0.78/0.78	1.56/3.13	6.25/12.5	16.0/16.0	nt	nt	nt
Pseudomonas aeruginosa ATCC 9027	11.25/11.25	0.20/0.78	3.13/6.25	8.0/8.0	nt	nt	nt
Salmonella enteritidis ATCC 13076	0.78/0.78	0.78/1.56	6.25/6.25	4.0/4.0	nt	nt	nt
Gram (+) source No.							
Bacillus subtillis ATCC 6633	0.35/0.35	1.56/3.13	0.78/3.13	nt	8.0/8.0	nt	nt
Bacillus cereus ATCC 10876	0.35/6.25	1.56/6.25	0.78/6.25		4.0/16.0		
Clostridium perfringens ATCC 19404	0.18/0.18	0.39/0.39	0.39/0.39	nt	1.0/8.0	nt	nt
Listeria monocytogenes ATCC15313	0.35/0.78	0.78/1.56	0.78/1.56	nt	8.0/16.0	nt	nt
Staphylococcus aureus ATCC 25923	0.35/0.35	0.78/1.56	1.56/1.56	nt	1.0/8.0	nt	nt
Staphylococcus aureus ATCC 6538	0.18/0.18	0.39/0.78	1.56/1.56	nt	2.0/16.0	nt	nt
Sarcina lutea ATCC 9341	0.18/0.18	0.10/0.10	0.39/0.39	nt	0.5/2.0	nt	nt
Clinical isolates of bacteria from wounds							
Klebsiella pneumoniae	25.0/25.0	6.25/25.0	>50.0/>50.0	nt	nt	nt	>100/>100
Klebsiella oxytoca	12.5/12.5	12.5/12.5	25.0/25.0	nt	nt	nt	12.5/12.5
Escherichia coli	12.5/12.5	12.5/12.5	25.0/50.0	nt	nt	nt	>100/>100
Proteus mirabilis	12.5/12.5	12.5/12.5	25.0/50.0	nt	nt	nt	>100/>100
Staphylococcus aureus	6.25/12.5	12.5/12.5	12.5/12.5	nt	nt	nt	>100/>100
Staphylococcus sp.	12.5/12.5	12.5/12.5	12.5/12.5	nt	nt	nt	>100/>100
Streptococcus pyogenes	12.5/12.5	6.25/25.0	12.5/25.0	nt	nt	nt	25.0/25.0
Pseudomonas aeruginosa	50.0/50.0	>50.0/>50.0	50/>50.0	nt	nt	nt	>100/>100
Enterococcus sp.	12.5/25.0	25.0/25.0	25.0/50.0	nt	nt	nt	>100/>100
Enterobacter sp.	25.0/25.0	25.0/25.0	50.0/>50.0	nt	nt	nt	12.5/12.5
Citrobacter sp.	50.0/50.0	>50.0/>50.0	>50.0/>50.0	nt	nt	nt	25.0/25.0
Acinetobacter sp.	3.13/3.13	6.25/12.5	12.5/12.5	nt	nt	nt	>100/>100
Fungi source No.							
Candida albicans ATCC 10231	0.35/0.35	0.78/1.56	3.13/3.13	nt	nt	16.0/16.0	nt
Saccharomyces cerevisiae 112. Hefeb. Weihenstephan	0.09/0.09	0.09/1.56	3.13/3.13	nt	nt	16.0/16.0	nt
Aspergillus niger ATCC 16404	0.18/0.18	1.56/1.56	3.13/3.13	nt	nt	8.0/8.0	nt
Fungal isolates							
Aspergillus fumigatus	0.04/0.38	0.09/0.75	0.09/0.38	nt	nt	0.04/0.04	nt
Aspergillus restrictus	0.09/0.75	0.09/0.75	0.09/0.75	nt	nt	0.08/0.08	nt
Penicillium chrysogenum	0.09/0.38	0.19/0.38	0.09/0.19	nt	nt	0.04/0.04	nt
Acremonium chrysogenum	0.04/0.04	0.04/0.04	0.04/0.04	nt	nt	0.04/0.04	nt

Table 2. Antimicrobial activity of *Satureja montana* ssp *montana* essential oils.

**altitude*
Strept. – Streptomycin; Chlor. – Chloramphenicol; Nyst. – Nystatin; Kan. - Kanamycin, nt – not tested

It showed high activity against *Acinetobacter* sp. (MIC=MBC=3.125 μL mL^{-1}) and *S. aureus* (MIC/MBC=6.25/12.5 μL mL^{-1}), moderate activity against *K. oxytoca, P. mirabilis, Staphylococcus* sp., *S. pyogenes* and *E. coli* (MIC=MBC=12.5 μL mL^{-1}) and the weakest activity against *Citrobacter* sp. and *P. aeruginosa* (MIC=MBC=50.0 μL mL^{-1}). The same concentration exhibited both inhibitory and bactericidal effect in most strains tested, except in the cases of *S. aureus* and *Enterococcus* sp. (MIC/MBC=12.5/25.0 μL mL^{-1}) (Table 2). Comparison of the obtained results with the antimicrobial activity of the reference antibiotics (positive controls) showed that all tested essential oils possess generaly high antimicrobial activity. High activity against all tested fungal strains in the range of 0.09-3.13 μL mL^{-1} (Table 2) was observed with the tested essential oils. The special significance of this activity can be observed against pathogenic yeast *C. albicans* and fungal filamentous strains from the genus *Aspergillus* (*A. niger, A. fumigatus* and *A. restrictus*).

Antioxidant properties tested using three different assays are presented in Table 3. Antioxidant (radical scavenging) activity of all tree samples, obtained by DPPH assay, was estimated as weak (approximatively 10-fold weaker than BHT as reference standard). Results obtained by ORAC assay exibited relatively poor antioxidant capacity, which was in very good agreement with DPPH assay (correlation coefficient of -0.9689, $p<0.05$). Total reducing power estimated as Fe(III) to Fe(II) for all samples can also be considered as weak. The best antioxidant properties were exibited by S2, followed by S1, while S3 showed the lowest antioxidant capacity among the tested oil samples. With an aim to elucidate which group of essential oil constituents is responsible for specific antioxidant activity, correlation analysis was performed and the results are presented in Table 4. The correlation analysis showed that the major carriers of the antioxidant activity are oxygenated monoterpenes, which showed the highest correlation with DPPH method.

In addition to antimicrobial activity, determination and estimation of the antioxidant activity, anticholinesterase activities of the essential oils were measured. All of the tested oils displayed anticholinesterase activity, where S1 exhibited the strongest inhibition of human serum cholinesterase (-85.71±0.24%), while S2 and S3 had similar inhibition percents (-51.16±0.13 and 49.17±0.11%, respectively).

4. Discussion

Literature data concerning *S. montana* showed differences in the chemical composition of essential oils with change in altitude of the plant (growth) collection [4,12,16]. Beside this, it was found that the specimens collected during the flowering season had the highest content of the carvacrol and thymol and their precursors *p*-cymene and γ-terpinene [7,15,17]. Finally, differences in the chemical compositions not only reflects the genetic variability of the genus *Satureja* [19], but also the variability within the same species, as in the case of the *S. hortensis* [28]. Unfortunately, only two studies using plant material have identified the plant to its subspecies level [14,29], which makes it difficult to compare data from literature. All mentioned facts prompted us to collect plant material

	DPPH(EC50)* (μg/mL)	ORAC (TE, μg/mL per mg of essential oil)	Total reducing power (ascorbate equivalents - μg/mL of ascorbic acid per mg of essential oil)
S1	494.1±2.5	101.1±0.9	120.7±0.4
S2	426.3±1.4	106.3±0.9	211.4±1.2
S3	593.2±2.9	76.8±0.8	166.2±0.8

Table 3. Antioxidant properties of *Satureja montana* essential oils estimated by DPPH, ORAC and total reducing power Fe (III) to Fe (II).

*EC50 (BHT)=41.6±0.4 μg/mL

	Monoterpens	Oxigenated monoterpens	Sesquiterpens	Total monoterpens
TRP	-0.8932*	0.2043	0.2608	-0.3144
DPPH	-0.3480	-0.9955*	0.9306*	-0.9087*
ORAC	0.5690	0.9412*	-0.9922*	0.9837*

Table 4. Relation between percentage of major groups of essential oil constituents and antioxidant activities.

Asterics mark significant correlatons at P<0.05

from the same *Satureja* subspecies (*S. montana* ssp. *montana*), in the same developmental stage (flowering period), from three localities at different altitudes and to investigate the relation between chemical composition and biological activity of essential oils from S. *montana* ssp. *montana* essential. Yields of essential oils isolated from plant material were between 0.38 - 0.42% (v/w), which is relatively lower compared to other reports in literature where the yield of this oil was reported to range between 0.22% and 2.8% [16,29]. It can be noted that the essential oils collected from all three localities had similar yields, but number of constituents showed evident decrease, which was related to the increase in altitude. Monoterpene hydrocarbons (oxygenated monoterpenes) were the dominant class of compounds in the oils of all three samples. Although the oil from the sample S1 has the lowest content of sesquiterpenes, this class of compounds is more diverse than in the other two samples. Obtained data showed the correlation between altitude increment and higher content of alcohols linalool (15.38%; 17.94% and 32.58%, respectively) and terpinen-4-ol (0%, 10.60% and 10.99%, respectively). Also, a similar correlation was found in the case of the aldehide *cis*-sabinene hydrate (0%, 14.61% and 23.05%, respectively), but the content of the major phenol compounds (thymol and carvacrol) significantly decreased with the increase of the altitude. Similar trends were also present in the study of Slavkovska [14], where increasing the altitude (200-600 m) caused a reduction of the phenol compounds by two fold, as well as the increase in the content of alcohols (linalool 8.1-2.8% and borneol 7.1-10.6%) in the composition of the S. *montana* ssp. *montana* essential oil. Decrease of the carvacrol content in the essential oil of *S. montana* collected in Albania (55.95%-39.53%-40.51%-21.07%-2.21%) was also corelated with increase in altitude (4 m-15 m-207 m-750 m-800 m a.s.l.) [16]. Essential oil from a sample collected in Italy (150 m a.s.l., La Spezia Province [30] was characterized by very high content of the carvacrol, while the oil collected from Sannine Mountain in Lebanon (1800 m) had no any phenols but was characterized by very high amounts of alcohols 1,8-cineole (8.87%), linalool (11.41%) and γ-terpineol (12.66%) [12]. Decrease of the phenol content, followed by the increase of alcohols was also recorded in the chemical compositions of the oils from two different localities in Bosnia and Herzegovina, Trebinje - Konjic (carvacrol 23.3-10.6%; thymol 31.7-3.8%; geraniol 0.1-22.3%, terpinen-4-ol 0.8-10.3%, borneol 2.9-4.8%) [4].

Satureja montana essential oils showed very high antimicrobial activity against all tested strains. Our results concur with previous investigations studying the antimicrobial activity of *S. montana* against *P. aeruginosa,* wherein the said strain showed higher resistance when compared to other tested microorganisms [4,7]. The study by Nedorostova *et al.* [10] demonstrated complete resistance of this strain to *S. montana* essential oil. In our studies, the obtained activity of essential oils from the S2 and S3 samples showed much higher effect against *P. aeruginosa* while S1 was not as effective. This difference in activity can be attributed to the higher alcohol content in the of these oils [31]. The activity of *S. montana* essential oil (carvacrol-50.2% and thymol-11.0%) against clinical enteropathogens *Escherichia coli*, *Plesiomonas shigelloides*, *Shigella flexneri*, *Salmonella enterica* serovar *typhimurium*, *Yersinia enterocolitica* and *Vibrio parahaemolyticus* was previously investigated and confirmed to be in the range of MIC/MBC=0.02-0.32/0.04-0.64% (w/v), as well as against much more resistant *E. coli* O157:H7 (thymol (43.0%); MIC/MBC = 0.05/0.013 (vol/vol)) [8]. In the case of gram positive bacteria, previous investigations of *S. montana* essential oil against *S. aureus* were confirmed by our results, showing high sensitivity of this strain to the tested essential oils [4,7,8,10]. Altogether our results confirmed the previously reported data on the eficacy of *S. montana* essential oil in controling growth and survival of *Listeria monocytogenes* [9] as well as against food contaminant *B. cereus* [7]. During the last decades, the main issue in microbiology has been growing number of multiresistant strains and, at the same time, decline in the number of the useful antibiotics without toxic or carcinogenic effect. All three tested oils exhibited activity against all tested multiresistant strains, with the exception of *P. aeruginosa*, *Citrobacter sp.* and *K. pneumoniae*. Among multiresistant strains tested, *K. pneumoniae* was also the more resistant strain in the study performed by Skočibušić and Bežić [7]. *Satureja montana* essential oils generaly exhibited very high antifungal activity, regardless of the individual components' percentage values, which confirms previous results on this subject [7]. Beside this, the oil from the material collected in Italy (Malba Mt.) was active against 46 species from 23 genera of pathogenic fungi at very low concentrations from 0.10 to 0.25 μL mL^{-1} [11]. Among the tested essential oils, the S1 showed the most prominent antimicrobial activity, where it is very clear that high phenol and alcohol content in the chemical composition were the most efficient antimicrobial combinations. Generally high antimicrobial activity of the *S. montana* ssp *montana* essential oil, reported in previous and present studies can be contributed to its major compounds carvacrol, thymol, terpinen-4-ol and linalool [8,32,33]. Mentioned results not only suggest synergistic activity of the individual oil components, but also points to the

significance of their percentage ratio in the antimicrobial mixture. Antioxidant activity testing showed relatively low antioxidant potential of the tested essential oils. One can clearly note that antioxidant activity of all samples showed maximum activity at the medium altitude of the collection, which is in accordance with oxygenated monoterpenes content in the S2, confirmed by correlation analysis. In the case of sesquiterpenes, the situation is reversed, so we can state as a conclusion that total reducing power may in general be attributed to the monoterpene fraction of the examined essential oils. Many neurological disorders, associated with increased activity of cholinesterase could be prevented by its inhibition. In that way, examination of widely applied natural products such as essential oils as a potential source of cholinesterase inhibitors, can play an important role. All three essential oils, especially S1, qualify in that way as potent cholinesterase inhibitors with prospective application in treatment of neural diseases.

The results of the present study show a correlation between the chemical composition of the *S. montana* ssp *montana* essential oil and altitude of growth of the plant material. With increase of the altitude, the content of phenol compounds (carvacrol and thymol) are either decreased, or their ratio is changed. The same change in altitude is related to the increase in the alcohol content in the essential oil's composition. High inhibitory and microbicidal effect of *S. montana* ssp *montana* essential oil on human pathogens and food contaminants together with moderate antioxidative potential, confirms traditional usage of this plant species for the treatment of the respiratory and intestinal infections. Beside this, the tested oils were active against multiresistant clinical isolates from wounds, which is very significant considering their general resistance and difficulties in the treatment of these infections. This fact can be used for further researches with focus on the development of the drugs that contain active constituents from these oils. Finally, the investigated oils could be used for the preparation of aerosols and disinfectants of the closed areas for the purpose of reduction and prevention of the spore germination from spoilt food or allergic fungal species. All tested samples proved themselves to be a cholinesterase inhibitors, justifying their wide usage in folk medicine and nutrition. As a final recommendation derived from the present study, commercial cultivation of the species *S. montana* ssp *montana* should be done at lower altitudes. In this way, higher content of antibacterial and antifungal compounds in the essential oil isolated from this plant material will be achieved, while at the same time, the other benefits such as antioxidative properties and anticholinesterase action would not be diminished.

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