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Chemical composition and antimicrobial activity of the essential oil from *Satureja horvatii* Šilić (Lamiaceae)

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Abstract: The present paper describes the chemical composition and antimicrobial activity of the essential oil of the endemic species *Satureja horvatii* Šilić, collected in Montenegro. The essential oil was obtained from the aerial parts of the plant by hydrodistillation and analyzed by GC–MS. From the 34 compounds representing 100 % of the oil, the major compound was the phenolic monoterpene thymol (63.37 %). The oil contained smaller amounts of γ-terpinene (7.49 %), carvacrol methyl ether (4.92 %), carvacrol (4.67 %), *p*-cymene (4.52%), α-terpinene (1.81 %), borneol (1.58 %), α-thujene (1.56 %), β-caryophyllene (1.55 %) and β-myrcene (1.44 %). The antimicrobial activity of the essential oil of *S. horvatii* was evaluated using the agar diffusion and broth microdilution methods. The essential oil exhibited antimicrobial activity to varying degrees against all the tested strains. The maximum activity of *S. horvatii* oil was observed against Gram-positive bacteria (*Micrococcus luteus, Staphylococcus epidermidis, Staphylococcus aureus* and *Enterococcus faecalis*) and against the yeast (*Candida albicans*). The oil exhibited moderate activity against the Gram-negative bacteria *Escherichia coli* and *Klebsiella pneumoniae* and weak activity against *Pseudomonas aeruginosa*. This study confirms that the essential oil of *S. horvatii* possesses antimicrobial activities *in vitro* against medically important pathogens.

Keywords: *Satureja horvatii*; endemic species; essential oil; GC–MS; antimicrobial activity.

INTRODUCTION

The genus *Satureja* L. includes about 200 species of herbs and shrubs, often aromatic, with a centre of distribution in the Mediterranean Basin. In the area of the central and western Balkans, nine species of this genus have been registered.[1]

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Most of them are the well-known species *S. montana* L., *S. hortensis* L., *S. kitaibelii* Wierzb. Ex Heuff. and *S. cuneifolia* L., and all are used in traditional medicine on the Balkan Peninsula.

Satureja horvatii Šilić is an endemic relict species of the Orjen–Lovćen mountain massive (Montenegro), with a high content of the essential oil of up to 4 %.[2,3] To the best of our knowledge, there are published reports only about the chemical composition of its essential oil.

In parallel with the rapid development of a wide range of antibacterial agents since the 1940s, bacteria have proved extremely adept at developing resistance to each new employed agent. The rapidly increasing incidence of bacterial resistance to antimicrobial agents has become a serious problem worldwide. Resistance mechanisms have been identified and described for all the known antibiotics currently available for clinical use.[4] The antimicrobial activity of plant oils and extracts has been recognised for many years. With the increasing tendency for the use of volatile oils in the pharmaceutical industries, a systematic examination of essential oils for antimicrobial activity has become important.

The essential oils isolated from various *Satureja* species have been shown to have biological and pharmacological activities, such as antibacterial, fungicidal and antiviral,[5–7] anti-oxidant,[8] antispasmodic and antidiarrhoeal.[9]

This paper reports a phytochemical analysis and the *in vitro* antimicrobial activity of the essential oil of the endemic species *S. horvatii*.

EXPERIMENTAL

Plant material

The plant material of *S. horvatii* was collected on Mount Orjen (Montenegro), in Orjenske lokve locality (1580 m a. s. l.), in July 2006. The sample was gathered before the flowering period. A voucher specimen is kept at the Institute of Botany Herbarium, Faculty of Pharmacy, University of Belgrade.

Isolation of the essential oil

The aerial parts of the plant were dried at room temperature and hydrodistilled (100 g) for 3 h, using a Clevenger-type apparatus. The oil yield was 4.2 %.

Gas chromatography–mass spectrometry

*Gas chromatography***.** A Hewlett Packard, HP-5890 gas chromatograph, equipped with a split-splitless injector, a fused silica capillary column HP-5 (25 m×0.32 mm; 0.5 μm film thickness), and FID, was employed. Oil solutions in ethanol (≈ 1 %) were injected in the split mode (1:30). The injector and detector (FID) temperatures were 250 and 300 °C, respectively, while the column temperature was linearly programmed from 40 to 240 °C (4.0 °C min^{-1}).

Gas chromatography–mass spectrometry. The analyses were performed on a Hewlett Packard, HP G1800C gas chromatograph equipped with split-splitless injector and a HP-5MS capillary column (30 m×0.25 mm; 0,25 μm film thickness). The chromatographic conditions were as metioned in preceding paragraph. Injector was heated at 250 °C, detector (MSD) was heated at 280 °C, while the column temperature was linearly programmed from 40 to 240 °C (4.0 °C min^{-1}). The EIMS spectra (70 eV) were obtained in the scan mode in m/z range 40–400.

Component identification and quantification

Identification of individual constituents was made by comparison of their retention times with those of analytical standards of the available terpenoids and by a computer search, matching the mass spectral data with those held in the Wiley 275 library of mass spectra. Confirmation was performed using AMDIS software. For quantification purposes, relative area percentages obtained by FID were used.

Antimicrobial activity

Antibacterial and antifungal activities of the essential oil of *S. horvatii* were evaluated by the agar diffusion method[10] using a panel which included laboratory control strains obtained from the American Type Culture Collection (Rockville, MD, USA): Gram-positive bacteria *Staphylococcus aureus* (ATCC 25923), *Staphylococcus epidermidis* (ATCC 12228), *Micrococcus luteus* (ATCC 10240), *Enterococcus faecalis* (ATCC 29212), *Bacillus subtilis* (ATCC 6633BB), *Bacillus cereus* (ATCC 11778), Gram-negative *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853) and *Klebsiella pneumoniae* (NCIMB 9111) bacteria and one strain of yeast *Candida albicans* (ATCC 10259).

The active cultures for the experiments were prepared by transferring a loopful of cells from stock cultures to flasks containing Müller–Hinton broth (MHB, Torlak, Belgrade) for the bacteria and Sabouraud dextrose broth (Torlak, Belgrade) for the yeast. The cultures were incubated without agitation for 24 h at 37 and 25 °C for the bacteria and yeast, respectively. The cultures were diluted with fresh Müller–Hinton broth and Sabouraud dextrose broth to achieve optical densities corresponding to 2.0×10^6 colony forming units (CFU/ml) for the bacteria and 2×10^5 CFU/ml for *C. albicans*. A suspension of the tested microorganisms (100 μl) was distributed on the solid media, *i.e.*, Müller–Hinton agar or Sabouraud dextrose agar. One drop (15 μl) of a 4 % and one of a 2 % solution of the essential oil in absolute ethanol were poured onto the prepared agar. Ampicillin (10 μg), amikacin (30 μg), bacitracin (10 μg) and nystatin (100 units) discs were used to control the sensitivity of the tested microorganisms.

These plates were then incubated at 37 °C for 24 h for the bacteria and at 30 °C for 48 h for the *C. albicans*. The results of agar diffusion assays were evaluated by measuring zone of inhibition (in mm) after incubation. All the experiments were performed in triplicate and the average value and standard deviation (SD) were calculated for the diameters of the inhibition zone.

*Determination of the minimal inhibitory concentration (*MIC*)*

The broth microdilution method was used to determine the minimal inhibitory concentration (*MIC*) according to the National Committee for Clinical Laboratory Standards.[11] The *MIC* is defined as the lowest concentration of the essential oil at which the microorganism does not demonstrate visible growth. All tests were performed in Müller–Hinton broth supplemented with Tween 80 detergent (final concentration of 0.5 % v/v), with the exception of the yeast when Sabouraud dextrose broth supplemented with Tween 80 was used. A serial doubling dilution of the oil was prepared in a 96-well microtiter plate over the concentration range 0.02–80.00 μl ml^{-1}, including one growth control and one sterility control (MHB + Tween 80 + + test oil). Overnight broth cultures of each strain were prepared and the final concentration in each well was adjusted to 2.0×10^6 CFU/ml for the bacteria and 2.0×10^5 CFU/ml for the yeast, and these were confirmed by viable counts. The plates were incubated under normal atmospheric conditions at 37 °C for 24 h for the bacteria and at 26 °C for 48 h for the yeast. The bacterial growth was indicated by the presence of a white "pellet" on the well bottom.[12] All determinations were performed in duplicate.

RESULTS AND DISCUSSION

Chemical composition of the essential oil

The chemical composition of the essential oil of *S. horvatii* is presented in Table I. The identified compounds (thirty-four components) amounted to 100 % of the oil. The dominant constituents were monoterpene compounds in an amount of 97.24 % (hydrocarbons: 19.79 %; oxygenated monoterpenes: 77.45 %), while the quantity of sesquiterpene compounds was 2.76 % (hydrocarbons: 2.53 %; oxygenated sesquiterpenes: 0.23 %).

TABLE I. Chemical composition of *S. horvatii* essential oil determined by GC and GC–MS

No.	Constituents	$I^{a,13}$	Area, %
1	*α*-Thujene	924	1.56
2	*α*-Pinene	932	0.72
3	Camphene	946	0.47
4	Sabinene	969	0.06
5	*β*-Pinene	974	0.25
6	1-Octen-3-ol	974	0.25
7	*β*-Myrcene	988	1.44
8	*α*-Phellandrene	1002	0.26
9	Δ^3-Carene	1008	0.06
10	*α*-Terpinene	1114	1.81
11	*p*-Cymene	1020	4.52
12	*β* –Phellandrene	1025	0.60
13	*cis*- *β*-Ocimene	1032	0.15
14	*trans*-*β*-Ocimene	1044	0.05
15	*γ*-Terpinene	1054	7.49
16	*cis*-Sabinene hydrate	1065	0.71
17	*α*-Terpinolene	1086	0.09
18	Linalool	1095	0.38
19	Borneol	1165	1.58
20	Terpinen-4-ol	1174	0.47
21	*α*-Terpineol	1186	0.91
22	Carvacrol methyl ether	1241	4.92
23	Thymol	1289	63.73
24	Carvacrol	1298	4.67
25	Thymol acetate	1349	0.08
26	*β*-Caryophyllene	1417	1.55
27	Aromadendrene	1439	0.10
28	*α*-Humulene	1452	0.06
29	*γ*-Muurolene	1478	0.07
30	Germacrene D	1484	0.23

[a]Kovats index

TABLE I. Continued

No.	Constituents	$I^{a,13}$	Area, %
31	Bicyclogermacrene	1500	0.45
32	δ-Cadinene	1522	0.08
33	Spathulenol	1577	0.12
34	Caryophyllene oxide	1582	0.11
Monoterpene compounds			97.24
	Monoterpene hydrocarbons	–	19.79
	Oxygenated monoterpene	–	77.45
Sesquiterpene compounds		–	2.76
Sesquiterpene hydrocarbons		–	2.53
Oxygenated sesquiterpenes		–	0.23
Total			100

[a]Kovats index

The oil of *S. horvatii* is characterized by a high content of the phenolic monoterpene thymol (63.73 %). Other important compounds were the monoterpene hydrocarbons γ-terpinene (7.49 %), *p*-cymene (4.52 %), and the oxygenated compounds carvacrol methyl ether (4.92 %), carvacrol (4.67 %) and borneol (1.58 %). The essential oil also contained smaller percentages of α-thujene (1.56 %), β-caryophyllene (1.55 %) and β-myrcene (1.44 %).

Other *Satureja* species, such as *S. montana* and *S. subspicata* have lower content of essential oil (0.3–1.8 %), the main constituent of which is carvacrol (16.8––45.7 %), an isomer of thymol. Thymol in these species was registered in concentrations of less than 5 %.[14–16]

The chemical composition of the essential oils of *Satureja* spp. shows a large interspecies and intraspecies variability, which depends upon genetic factors, environmental factors, and stage of the plant development.[17,18]

Antimicrobial activity

Numerous studies have demonstrated that the essential oils of *Satureja* species are among the most potent essential oils with regard to antimicrobial properties.[15,19–21] This was confirmed and extended in the present study. According to the results, the essential oil had great *in vitro* antimicrobial activities against all nine bacteria and the yeast species tested (Tables II and III). These results are comparable to previously published activities for *Satureja* species.[16]

The data obtained from the agar diffusion method indicated that the inhibitory effect increased with increasing oil concentration from 2 to 4 %. The strongest inhibition zones (26–32 mm) were detected for the Gram-positive bacteria. Among them *M. luteus* was found to be the most sensitive. The oil also exhibited high antimicrobial activity against important human pathogens, such as *S. aureus* and *E. faecalis*. The Gram-negative strains displayed variable degrees of suscep-

tibility. Maximum activity was observed against *E. coli* and *K. pneumoniae* (22––28 mm), while *P. aeruginosa* exhibited weak inhibition zones (10–14 mm).

TABLE II. Antimicrobial activity of *S. horvatii* essential oil expressed by the diameter of the inhibition zone (mm)

Microorganism	Essential oil		Ampicillin	Amikacin	Bacitracin	Nystatin
	2.0 %	4.0 %	10 μg/disc	30 μg/disc	10 μg/disc	100 units/disc
S. aureus ATCC 25923	24.3±1.0	26.5±0.9	35.0±7.0	26.5±2.1	n.t.[a]	n.t.
S. epidermidis ATCC 12228	23.0±0	26.5±1.0	12.0	n.t.	n.t.	n.t.
M. luteus ATCC 10240	30.5±3.0	32.0±2.0	33.0	n.t.	16.7±1.2	n.t.
E. faecali ATCC 29212	26.0±1.0	27.5±2.4	16.0	n.t.	n.t.	n.t.
B. subtilis ATCC6633bb	23.2±1.3	30.0±0	15.0	n.t.	n.t.	n.t.
B. cereus ATCC 11778	19.0±1.2	26.0±0.8	12.0±3.4	n.t.	n.t.	n.t.
E. coli ATCC 25922	24.8±0.9	26.6±0.5	20.5±0.7	20.0	n.t.	n.t.
K. pneumoniae NCIMB 9111	22.2±2.3	24.0±0	17.0±4.2	n.t.	n.t.	n.t.
P. aeruginosa ATCC 27853	10.0±3.7	14.5±0.5	10.0	27.5±3.5	n.t.	n.t.
C. albicans ATCC 10259	23.5±1.0	26.0±2.5	n.t.	n.t.	n.t.	20.0±0.87

[a]Not tested

The *in vitro* antimicrobial activity of *S. horvatii* essential oil was evaluated by the broth microdilution method and expressed as the minimum inhibitory concentration. In liquid medium, the essential oil was active against all the test strains. The inhibitory properties of the oil were observed within a range of concentrations from 0.10 to 16.00 μl ml^{-1}. The oil exhibited the highest inhibitory effect against the Gram-positive bacteria *S. epidermidis*, *M. luteus* and *S. aureus*. The oil was effective against Gram-negative *E. coli* and *K. pneumoniae* (2.50 and 4.00 μl ml^{-1}, respectively), while *P. aeruginosa* seemed to be more resistant to the investigated oil (16.00 μl ml^{-1}).

In the present study, it was confirmed that Gram-positive bacteria are more sensitive to the investigated oil than Gram-negative bacteria (Tables II and III). The relative tolerance of Gram-negative bacteria to essential oils has been ascribed to the presence of a hydrophilic outer layer, which can block the penetration of hydrophobic components through the target cell membrane.[22] The essential oils rich in phenolic compounds are widely reported to exhibit high levels of antimicrobial activity.[20,23,24] The major component of *S. horvatii* was the phe-

nolic monoterpene thymol. Since the active antimicrobial compounds of essential oils are phenolics and terpenes, it seems reasonable that their mode of action might be similar to that of other phenolic compounds. Most of the studies on the mechanism of phenolic compounds focused on their effects on cellular membranes, altering its function, causing swelling and increasing its permeability. Increases in cytoplasmic membrane permeability appear to be a consequence of the loss of the cellular pH gradient, decreased ATP levels, and loss of the proton motive force, which lead to cell death.

TABLE III. Antimicrobial activity of *S. horvatii* essential oil expressed as *MIC* (μl/ml)

Microorganism	Essential oil	Ampicillin	Amikacin	Bacitracin	Nystatin
S. aureus (ATCC 25923)	0.20	0.50	n.t.[a]	n.t.	n.t.
S. epidermidis (ATCC 12228)	0.10	1.0	n.t.	n.t.	n.t.
M. luteusi (ATCC 10240)	0.10	0.50	n.t.	n.t.	n.t.
E. faecali (ATCC 29212)	0.40	0.50	n.t.	n.t.	n.t.
B. subtilis (ATCC6633bb)	2.00	1.00	n.t.	n.t.	n.t.
B. cereus (ATCC 11778)	2.50	1.00	n.t.	n.t.	n.t.
E. coli (ATCC 25922)	2.50	2.00	0.50	n.t.	n.t.
K. pneumoniae (NCIMB 9111)	4.00	2.00	n.t.	n.t.	n.t.
P. aeruginosa (ATCC 27853)	16.00	n.t.	1.00	n.t.	n.t.
C. albicans (ATCC 10259)	0.40	n.t.	n.t.	n.t.	0.50

[a]Not tested

A number of reports indicate that essential oils containing carvacrol, eugenol or thymol have the highest antimicrobial properties.[25] However, the antimicrobial activities of *Satureja* species do not arise only from the thymol and carvacrol content since the oil of *S. cuneifolia*, which is relatively rich in β-cubebene, limonene, α-pinene, spathulenol and β-caryophyllene also displayed relatively good activity.[15] Some studies reported that whole essential oils have a greater antibacterial activity than the major components mixed,[26,27] which suggests that the minor components are critical to the activity and could also affect the antimicrobial properties.

CONCLUSIONS

The essential oil of *S. horvatii* exhibited antimicrobial activity to varying degrees against all the tested strains. The maximum activity was observed against Gram-positive bacteria (*M. luteus*, *S. epidermidis*, *S. aureus* and *E. faecalis*) and against the yeast (*Candida albicans*). The oil exhibited moderate activity against the Gram-negative bacteria *E. coli* and *K. pneumoniae* and weak activity against *P. aeruginosa.* This study confirms that the essential oil of *S. horvatii* possesses antimicrobial activities *in vitro* against medically important pathogens.

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ИЗВОД

ХЕМИЈСКИ САСТАВ И АНТИМИКРОБНА АКТИВНОСТ ЕТАРСКОГ УЉА ВРСТЕ *Satureja horvatii* Šilić (Lamiaceae)

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У раду је дат хемијски састав и антимикробна активност етарског уља, ендемичне врсте *Satureja horvatii* Šilić, сакупљане у Црној Гори. Етарско уље је дестилацијом воденом паром изоловано из надземног дела биљке и анализирано методом GC–MS. 34 компоненте чине 100 % уља, а главна компонента је тимол (63,37 %). Уље је садржало и мањи проценат γ-терпинена (7,49 %), карвакрол-метил-етра (4,92 %), карвакрола (4,67 %), *p*-цимена (4,52 %), α-терпинена (1,81 %), борнеола (1,58 %), α-тујена (1,56 %), β-кариофилена (1,55 %) и β-мирцена (1,44 %). Антимикробна активност етарског уља *S. horvatii* је испитивана коришћењем агар дифузионе и бујон микродилуционе методе. Етарско уље је показало различит степен антимикробне активности на тестиране организме. Уље *S. horvatii* је испољило максималну активност на грам-позитивне бактерије *Micrococcus luteus, Staphylococcus epidermidis, Staphylococcus aureus* и *Enterococcus faecalis* и на гљивицу *Candida albicans*. Уље је показало умерену активност на грам-негативне бактерије *Escherichia coli* и *Klebsiella pneumoniae* и слабу на *Pseudomonas aeruginosa*. Истраживањем је утврђено да етарско уље ендемичне врсте *S. horvatii* поседује антимикробну активност у *in vitro* условима на значајне медицинске патогене.

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