NATURAL PRODUCT COMMUNICATIONS

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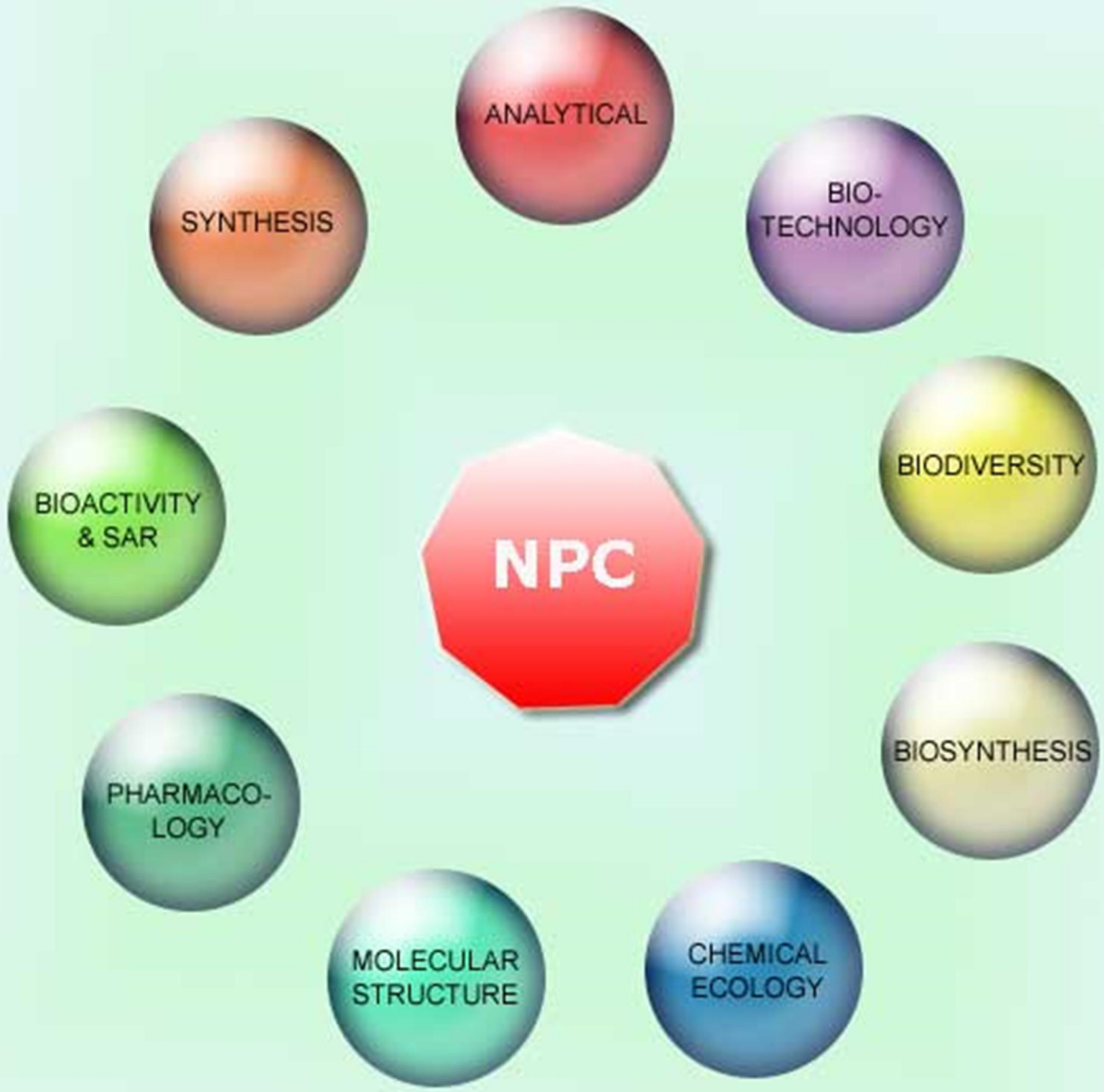

Volume 9. Issue 4. Pages 445-596. 2014
ISSN 1934-578X (printed); ISSN 1555-9475 (online)
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NPC Natural Product Communications

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NPC Natural Product Communications

2014
Vol. 9
No. 4
569 - 572

Cytotoxicity and Antimicrobial Activity of the Essential Oil from *Satureja montana* subsp. *pisidica* (Lamiceae)

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Received: March 21st, 2013; Accepted: January 13th, 2014

The antimicrobial and cytotoxic activities of the essential oil of *Satureja montana* ssp. *pisidica* from two localities (mountains Korab and Galičica) were studied. Forty-nine components were identified in the each sample. Oxygenated monoterpene hydrocarbons were the major compounds: carvacrol, thymol, carvacrol methyl ether and β-linalool. Both tested essential oils showed very high and similar antimicrobial activity. Minimal inhibitory concentrations ranged from 12.5 µg/mL against *S. epidermidis* to 50 µg/mL against *P. aeruginosa* and *C. albicans*. The cytotoxic effect of the essential oils was tested against MDA-MB-361, MDA-MB-453, HeLa, LS174 and MRC5 cells. The essential oil from Korab demonstrated significantly better results than the oil from Galičica, particularly against HeLa and MDA-MB-453 cell lines, with IC_{50} values of 63.5 and 72.3 µg/mL, while the oil from Galičica was the most active on the human epithelial cervical cancer HeLa cells (IC_{50} 99.7 µg/mL).

Keywords: *Satureja montana* ssp. *pisidica*, Essential oil, Cytotoxicity, Antimicrobial activity, Carvacrol.

The genus *Satureja* L. includes over 30 species of herbs and shrubs, often aromatic, with a centre of distribution in the Mediterranean Basin. In the area of the Balkan Peninsula nine species of this genus have been registered. *S. montana* contains three subspecies: ssp. *montana*, ssp. *variegata* (Host) P.W.Ball, and ssp. *pisidica* (Wettst.) Šilić [1].

S. montana ssp. *pisidica* (syn. *S. macedonica* Formanek, *S. montana* L. var. *pisidica* (Wettst.) Hal., *S. olympica* Hal.) is widespread in Macedonia (FYRM), but sporadically in Serbia and Montenegro. The chemistry of *S. montana* ssp. *montana* essential oil has been well-studied [2,3] and several biological activities, like antimicrobial, antiviral, antiparasitic, immunostimulative and antioxidative have been shown [3-7]. Research data for the essential oil composition of *S. montana* ssp. *pisidica* are scarce, while the biological significance of its essential oil has not yet been investigated [2,7]. This paper aims to describe the composition of the essential oils of *S. montana* ssp. *pisidica* from two localites, as well their antimicrobial properties and cytotoxicity against five cancer cell lines.

The aerial parts, before the flowering period, of *S. montana* spp. *pisidica* yielded a moderate content of essential oil (0.9%, v/w; Korab; 1.1%, v/w; Galičica; light yellow calculated on dry weight basis). The chemical composition of the oils from the two localities is summarized in Table 1. Forty-eight components were identified in the samples of the essential oils, representing almost 100% of the oil (Korab: 94.1%; Galičica: 98.7% respectively). The composition of the oils obtained from the different localities was qualitatively the same but some quantitative differences could be seen.

Oxygenated monoterpene hydrocarbons were the major compounds (Korab: 58.4%; Galičica: 80.3% respectively). Carvacrol was the most dominant compound in both oils (Korab: 20.9%; Galičica: 37.6%, respectively). Also, a high content of thymol was determined in the essential oil from Galičica. Carvacrol methyl ether was detected in very high content in the sample from Korab (11.8%), as well as *p*-cymene (17.1%), but in very low content in the essential oil from the plant from Galičica (0.5% and 6.8%, respectively). γ-Terpinene was identified in both samples (5-8%). Sesquiterpene hydrocarbons (Korab: 8.8%; Galičica: 5.7%) and oxygenated sesquiterpenes (Korab: 7.0%; Galičica: 1.6%) was low in both samples. Spathulenol and β-caryophyllene were the most abundant sesquiterpenes. The major difference in composition between the two oils was in the content of thymol, carvacrol methyl ether and ß-linalool.

The only paper dealing with the composition of the essential oil of *S. montana* spp. *pisidica* was that of Slavkovska *et al*. from the locality of Galičica, but with *p*-cymene as a major compound (29.3%), followed by a high content of linalool (24%) and carvacrol (18.3%) [2]. This could be explained by the fact that the plant was collected in the flowering period, while our samples were collected before flowering. Such differences in the amounts of the main components in the essential oil, depending on the stage of plant development, were found for *S. cuneifolia* Ten. [8], *S. montana* L., *S. subspicata* Bartl. ex Vis. [9] and *S. horvatii* Šilić [10].

The antimicrobial activity results of *S. montana* ssp. *pisidica* essential oils, presented in Table 2, are expressed as minimal inhibitory concentrations (MICs). These are the first results of antimicrobial activity of essential oil of *S. montana ssp. pisidica*. Both tested essential oils showed very high and similar antimicrobial activity, no matter the difference in carvacrol and thymol contents. The MIC ranged from 12.5 µg/mL against *S. epidermidis* (Galičica) to 50 µg/mL against *P. aeruginosa* and *C. albicans* (Korab). The essential oil from Galičica showed slightly better activity, especially against *S. epidermidis*, which could be

Table 1: Composition of essential oils of *Satureja montana* ssp. *pisidica*.

Components	Area (%)		RI exp[a]
	Korab	Galičica	
α-Thujene	0.6	0.4	930
α-Pinene	0.4	0.3	937
Camphene	0.2	0.2	952
1-octen-3-ol	-	0.8	975
Sabinene	0.1	-	976
ß-Pinene	0.2	-	980
ß-Myrcene	0.6	0.8	992
α-Phellandrene	0.1	0.1	1006
α-Terpinene	0.6	1.4	1018
p-Cymene	17.1	6.8	1032
(-)-limonene	-	0.3	1033
1,8-Cineol	0.3	0.1	1035
(*Z*)-β- Ocimene	1.6	0.9	1037
(*E*)-β- Ocimene	0.3	0.5	1047
γ-Terpinene	5.0	8.2	1063
cis-Sabinene hydrate	0.7	1.4	1070
cis-Linalool oxide	0.1	0.3	1074
α-Terpinolene	0.2	0.2	1090
ß-Linalool	15.2	0.6	1112
endo-Borneol	1.3	1.3	1170
Terpinene-4-ol	0.5	1.5	1181
α-Terpineol	-	0.3	1189
Thymol methyl ether	-	1.9	1236
p-Cymen-8-ol	0.1	-	1194
Carvacrol methyl ether	11.8	0.5	1251
(+)-Carvone	0.1	-	1259
Thymol	0.2	24.5	1290
Carvacrol	20.9	37.6	1295
Thymol acetate	-	0.2	1359
Carvacrol acetate	0.1	0.3	1379
α-Copaene	0.1	-	1384
ß-Caryophyllene	3.3	3.5	1428
ß-Copaene	0.1		1436
Aromadendrene	0.1	0.3	1446
α-Humulene	0.1	0.1	1462
Aloaromadendren	0.2	-	1470
γ-Muurolene	0.1	-	1487
Germacrene D	1.2	-	1493
α-Amorphene	-	0.1	1490
Viridiphlorene	-	0.4	1501
Bicyclogermacrene	1.7	-	1509
ß-Bisabolene	1.5	0.9	1520
γ-Cadinene	0.1	0.1	1521
δ-Cadinene	0.3	0.3	1532
Spathulenol	4.0	0.5	1589
Caryophyllene oxide	2.3	1.1	1592
iso-Spathulenol	0.5	-	1621
α-Cadinol	0.2	-	1669
Grouped components			-
Monoterpene hydrocarbons	19.9	11.2	
Oxygenated monoterpenes	58.4	80.2	
Sesquiterpene hydrocarbons	8.8	5.7	
Oxygenated sesquiterpenes	7.0	1.6	
Other compounds	-		
Total (%)	94.1	98.8	

[a]RI_{exp}-Retention indecies relative to C_9-C_{23} *n*-alkanes on HP 5MS.

attributed to a high content of phenolic monoterpenes. As the essential oils from *Satureja* species are known as antimicrobial agents, our results were in accordance with the most recent paper of Marin *et al*. [3], as well as with others [9,11].

Carvacrol, a monoterpenic phenol present in very high content in the tested essential oils, possesses a wide spectrum of antimicrobial activity, extended to food born pathogenic fungi, yeasts and bacteria. The mechanism of antimicrobial activity could be connected with the lipophilicity of carvacrol and its effects on the structural and functional properties of the cytoplasmatic membrane [12].

To determine the cytotoxic effect of the essential oils, MDA-MB-361 (estrogen-dependant) and MDA-MB-453 (estrogen-nondependant) breast cancer cell lines, a human epithelial cervical cancer cell HeLa, a human colon cancer cell line LS174, as well as healthy MRC-5 human embryonic lung fibroblast cell lines were treated with compounds, and cell survival was determined using the

Table 2: Antimicrobial activity of *Satureja montana* spp. *pisidica* essential oil.

Microorganisms	MIC (μg/mL)				
	S. montana ssp. *pisidica*		Ampicillin	Amikacin	Nystatin
	Korab	Galičica			
Staphylococcus aureus ATCC 25923	25.0	25.0	0.5	n.t.*	n.t.
Staphylococcus epidermidis ATCC 12228	25.0	12.5	1.5	n.t.	n.t.
Micrococcus luteus ATCC 3341	25.0	25.0	2.0	n.t.	n.t.
Bacillus subtilis ATCC 6633	25.0	25.0	1.8	n.t.	n.t.
Escherichia coli ATCC 25922	25.0	25.0	2.0	1.5	n.t.
Klebsiella pneumoniae ATCC 13883	25.0	25.0	2.8	2.0	n.t.
Pseudomonas aeruginosa ATCC 27853	50.0	25.0	n.t.	2.5	n.t.
Candida albicans ATCC 10231	50.0	25.0	n.t.	n.t.	3.8
Candida albicans ATCC 10259	50.0	25.0	n.t.	n.t.	4.2

*n.t.- not tested

MTT assay. The cytotoxicity of the oils on human cancer cell lines is shown in Figure 1 and the IC_{50} values are given in Table 3.

Essential oil from Galičica was the most active on HeLa cancer cells (IC_{50} 99.7 μg/mL), with lower activity against the other cell cultures. The essential oil from Korab demonstrated significantly better results, particularly for HeLa and MDA-MB-453 cell lines (IC_{50} 63.5 and 72.3 μg/mL). Also, it was observed that at concentrations of 100 μg/mL and higher (Korab), or at 200 μg/mL (Galičica), the essential oils induced a dramatic drop in the survival of malignant cells (Figure 1.).

The *S. montana* ssp. *pisidica* essential oil from Galičica showed no adverse effect on the MRC-5 cell line (IC_{50} 297.4 μg/mL). In contrast, the sample from Korab displayed a comparable IC_{50} value on malignant and MRC 5 cells (Table 3). The small differences between IC_{50} values led to our conclusion that the sample from Korab, due to a cytotoxic effect on healthy MRC-5 cells, needs further consideration for its toxicity. The cytotoxicity of *S. montana* essential oil has not been studied before, but the cytotoxicity of essential oils rich in carvacrol, as well as carvacrol itself has been studied in detail. Četojević-Simin *et al*. showed that the methanol extract of *S. montana* stimulated proliferation of HT-29 cells, and inhibited proliferation of HeLa cells with no activity against MCF-7 cells [13].

Interestingly, as carvacrol was recognized as a major and lipophilic compound, the essential oil with less carvacrol (Korab) possessed higher cytotoxic activity against the tested cell lines, which could be attributed to other important monoterpenes like *p*-cymene, γ-terpinene and β-linalool. A recent paper by Yousefzadi *et al*. has shown the significant antiproliferative effects of *S. sahendica* essential oil rich in thymol, γ-terpinene and *p*-cymene against MCF7, Vero, SW480 and JET 3 cell lines, in a dose-dependent manner [14].

Several studies have shown significant cytotoxic activity of carvacrol against A549 cell line [15], and myoblast cells, even after activation of mutated N-ras oncogene [16], human metastatic breast cancer cells MDA-MB 231 [17], as well anti-proliferative and anti-cancinogenic activity *in vivo* [18]. A recent study by Liang and Lu

Table 3: Cytotoxicity of the essential oils of *Satureja montana* ssp. *pisidica* from Korab and Galičica against MDA-MB 361, MDA-MB-453, HeLa, LS174 and MRC5 cell lines (expressed as IC_{50}).

Essential oil	IC_{50} (μg/mL)*				
	MDA-MB-361	MDA-MB-453	HeLa	LS174	MRC5
S. montana ssp. *pisidica* (Galičica)	234.6±0.11	240.3±0.31	99.7±0.11	189.8±0.31	297.4±0.11
S. montana ssp. *pisidica* (Korab)	109.0±0.21	72.3±0.11	63.5±0.31	99.4±0.22	102.8±0.11

*IC_{50} values were expressed as the mean±SD determined from the results of MTT assay in three independent experiments.

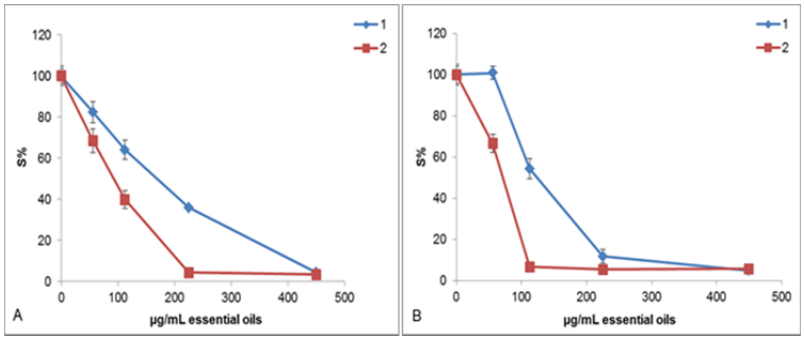

Figure 1: Representative graphs: the dose-dependent cytotoxic effect on (A) MDA-MB-361 cell line and (B) MDA-MB-453 cell line of the essential oils of *S. montana* ssp. *pisidica* from Galičica (1) and Korab (2).

has shown that carvacrol is cytotoxic to human glioblastoma cells in a concentration-dependent manner, influences Ca^{2+} rise, as well the production of reactive oxygen species (ROS) in human glioblastoma cells [19]. The authors concluded that carvacrol induced cell death through apoptosis mediated by ROS. Similar results were obtained by Hsu *et al.* for thymol, the second major compound in the essential oil of *S. montana* from Galičica [20]. Huang *et al*. tested the anti-proliferative effects of carvacrol on MDA-MB 231 cells, and showed induction of apoptosis in MDA-MB 231 cells with an IC_{50} of 100 μM [21].

In conclusion, our results have shown strong antimicrobial and cytotoxic activities, which could be attributed to the major phenolic monoterpene, carvacrol. Concerning the difference in cytotoxicity of the two tested oils against healthy human fibroblast cell line MRC-5 with IC_{50} values of 297.4 μg/mL (Galičica) and 102.8±0.11 (Korab), as well as recent findings of apoptotic effects of thymol and carvacrol, the cytotoxicity to MRC-5 cells needs further research on the underlying molecular mechanisms of action.

Experimental

Plant material: The aerial parts, before the flowering period, of *S. montana* ssp. *pisidica* were collected from mountains Korab (1370 m a. s. l.), and Galičica (1596 m a. s. l.) (FYRM) in July 2011. A voucher specimen was deposited at the Department of Botany, University of Belgrade, Faculty of Pharmacy, Belgrade, Serbia.

Isolation of the essential oil: The plant material was air-dried at room temperature for 3 days and the oil isolated (Korab; 50 g; Galičica: 58 g) by hydrodistillation for 2 h using a Clevenger-type apparatus, according to the Ph. Eur. 6.0 [22].

Essential oil analysis: Volatile constituents were determined by GC and GC-MS. GC analysis was performed on an Agilent 6890N GC system equipped with 5975 MSD and FID, using a HP-5 MS column (30 m x 0.25 mm x 0.25 μm). Injection volume was 2 μL and injector temperature was 200°C with a 10:1 split ratio. Helium was the carrier gas at a flow rate of 1.0 mL/min (constant flow mode). Column temperature was linearly programmed in the range 60-280°C at a rate of 3°C/min and held at 280°C for 5 min. The transfer line was heated at 250°C. The FID detector temperature was 300°C. EI mass spectra (70 eV) were acquired in the m/z range 35-550. Identification of the compounds was based on comparison of their retention indices (RI), their retention times (t_R) and mass spectra with those obtained from authentic samples and/or data bases and literature [23]. Relative percentages of the identified compounds were computed from the GC-FID peak area.

Antimicrobial activity: The antimicrobial activity was evaluated using 7 different laboratory control strains of bacteria: *Staphylococcus aureus* (ATCC 25923), *S. epidermidis* (ATCC 12228), *Micrococcus luteus* (ATCC 9341), *Bacillus subtilis* (ATCC 6633), *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 13883), *Pseudomonas aeruginosa* (ATCC 27853), and two strains of yeast *Candida albicans* (ATCC 10231 and ATCC 10259). The broth microdilution method was used to determine minimal inhibitory concentrations (MIC_s) of essential oils according to Clinical and Laboratory Standards Institute and procedure given by Kundaković et al. [24,25]. Samples of essential oils were dissolved in dimethyl sulfoxide (DMSO) in concentrations of 1.0 mg/mL. All microbial tests were performed in duplicate and 2 positive growth controls were included. Ampicillin, Amikacin and Nystatin were used as standard substances.

Cytotoxicity assay

Cell lines: MDA-MB-361 (estrogen-dependant) and MDA-MB-453 (estrogen-nondependant) breast cancer cell lines, HeLa - human epithelial cervical cancer cells, LS174 - a human colon cancer cell line and MRC-5 human embryonic lung fibroblast cell lines were grown in RPMI-1640 medium (Sigma) at 37°C. Media were supplemented with 10% fetal bovine serum, L-glutamine, and penicillin-streptomycin (Sigma).

Treatment of cell lines: Stock solutions (100 mg/mL) of essential oils, made in DMSO, were dissolved in the corresponding medium to the required working concentrations (400, 200, 100, 50 and 25 μg/mL). The final concentration of DMSO never exceeded 0.5%, which was non-toxic to the cells. Target neoplastic HeLa cells (2000 cells per well), MDA-MB-453 cells (3000 cells per well), MDA-MB-361 (7000 cells per well), and normal human fetal lung fibroblast MRC-5 cells (5000 cells per well) were seeded into 96-well microtiter plates and 24 h later, after cell adherence, 5 different, double diluted, concentrations of investigated compounds, were added to the wells except for the control cells to which a

nutrient medium only was added. The cultures were incubated for 72 h.

Determination of cell survival: The effects of essential oils on cancer cell survival were determined by the MTT test, according to Mosmann [26] with modification by Ohno and Abe [27], 72 h after addition of the compounds. The detailed procedure was described Stanojković *et al*. [28].

Acknowledgments - The authors are grateful to the Ministry of Science (Grant Nos. 173021 and 175011) for financial support.

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