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Phytochemical composition and antimicrobial activities of the essential oils from *Satureja subspicata* Vis. growing in Croatia

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Abstract

The present study describes the phytochemical profile and antimicrobial activity of *Satureja subspicata* Vis. essential oils, collected in Dalmatia (Croatia). Three samples of essential oils were obtained from the aerial parts of the plant by hydrodistillation and analyzed by GC–MS. From the 24 compounds representing 97.47% of the oils, carvacrol (16.76%), α-pinene (13.58), *p*-cymene (10.76%), γ-terpinene (9.54%) and thymol methyl ether (8.83%) appear as the main components. The oils also contained smaller percentages of myrcene, linalool, β-caryophyllene, limonene, geranyl acetate, 1-Octen-3-ol, nerol, thymol and borneol. Furthermore, antimicrobial activity of the oil was evaluated using agar diffusion and broth microdilution methods. The antimicrobial test results showed that the oils had a great potential antimicrobial activity against all 13 bacteria and 9 fungal strains. Gram-positive bacteria are more sensitive to the investigated oil, with a range of 0.09 to 6.25 μl/ml than Gram-negative bacteria in the range which is significantly higher from 1.56 to 25.00 μl/ml. Results presented here may suggest that the essential oil of *S. subspicata* possesses antimicrobial properties, and is therefore a potential source of antimicrobial ingredients for the food and pharmaceutical industry.

Keywords: *Satureja subspicata*; Essential oil; Antimicrobial activity; GC-MS

1. Introduction

Food safety is a fundamental concern of both consumers and the food industry, especially as the number of reported cases of food-associated infections continues to increase and is rapidly changing (Alzoreky & Nakahara, 2003). It has been estimated that as many as 30% of people in industrialized countries suffer from a food borne disease each year and in 2000 according to the World Health Organization at least two million people died from diarrhoeal disease worldwide (WHO, 2002). The increasing incidence of food borne diseases, coupled with the resultant social and economic implications, means there is a constant striving to produce safer food and to develop new natural antimicrobial agents. There is therefore still a need for new methods of reducing or eliminating food borne pathogens, possibly in combination with existing methods. Thus, the food industry at present uses chemical preservatives to prevent the growth of food borne and spoiling microbes. It has been suggested that some synthetic preservatives convert some ingested materials into toxic substances or carcinogens by increasing the activity of microsomal enzymes (Farag, Daw, Hewedi, & El-Baroty, 1989). In recent years there has been a considerable pressure from consumers to reduce or eliminate chemically synthesized additives in their foods. Most plants produce antimicrobial secondary metabolites, either as part of their normal program of growth and development or in response to pathogens attack or stress. A novel way to reduce the proliferation of microorganisms is the use of essential oils. The oils are natural products extracted from plant materials, which because of their antibacterial, antifungal,

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antioxidant and anticarcinogenic properties, can be used as natural additives in many foods (Aureli, Costantini, & Zolea, 1992; Lambert, Skandamis, Coote, & Nychas, 2001; Soliman & Badeaa, 2002; Teissedre & Waterhouse, 2000). Essential oils have been proven to be inhibitory against a wide range of food spoiling microbes, dependent upon their concentration, method of testing, and active constituents present (Aureli et al., 1992; Lis-Balchin, Ochoka, Deans, Asztemborska, & Hart, 1999; Paster, Menasherov, Ravid, & Juven, 1995; Smith-Palmer, Stewart, & Fyfe, 2001).

The genus *Satureja* (Lamiaceae, subfamily Nepetoideae and tribe Satureja) constitutes about 200 species of herbs and shrubs, often aromatic, widely distributed in Mediterranean area, Asia and boreal America (Cantino, Harley, & Wagstaff, 1992). The flora of Croatia has nine species of genus *Satureja*, five subspecies and seven varieties (Palić, Šilić, & Gašić, 1983). Savory species grow abundantly on stony slopes and in rocky mountain areas of Dalmatia. Evidently, members of the genus *Satureja* are such natural sources, including a well-known species *Satureja hortensis* L., known as summer savory, *Satureja montana* L., or winter savory and *Satureja cuneifolia* Ten. or wild savory as an aromatic plant which is frequently used in local spices and as a traditional medicinal plant in Dalmatia. Some members of this genus are of economic importance since they have been used as culinary herbs, flavouring agents in perfumery and cosmetics. Because of the strong phenolic character of its essential oil it is reminiscent of the taste and fragrance of commercial oregano and thyme oils. Among these savory is an important plant widely used in South European cuisine and is a very popular herb in the Mediterranean countries. The positive effects of savory on human health have now been attributed to its various biologically active constituents such as essential oil, triterpenes (Escudero, Lopez, Rabanal, & Valverde, 1985) and flavonoids (Thomas-Barberan, Huisain, & Gil, 1987). The essential oil of savory contains antioxidative compounds, namely carvacrol, thymol, β-caryophyllene, γ-terpinene, *p*-cymene, together with linalool, which has been reported to possess strong antioxidant effects (Ruberto & Baratta, 2000). Thymoquinone is the main aglycone (20.7%) of *S. montana*, while the glycosides of thymol, carvacrol, linalool and geraniol were also detected (Radonić & Miloš, 2003). Phenolic aglycones and thymoquinone are to be the antioxidants for certain organic substances and might be involved in plant pathogenic defence mechanisms, like naphthoquinone (Houghton, Zarka, Heras, & Hoult, 1995). Savory is a very versatile plant and a popular remedy; only now is it starting to be recognized for its potential therapeutic role such as carminative, digestive, antispasmodic, expectorant, fungicidal, antidiuretic, sedative and antioxidant activities. The essential oils isolated from various species of *Satureja* have biological properties such as antimicrobial (Ciani et al., 2000; Dorman & Deans, 2000; Gulluce et al., 2003; Panizi, Flamini, Cioni, & Morelli, 1993; Skočibušić, Bezić, & Dunkić, 2004) antiviral (Abad et al., 1999; Yamasaki et al., 1998) antioxidant (Esquived, Ribeiro, & Bernardo-Gil, 1999; Radonić & Miloš, 2003) antispasmodic and antidiarrhoeal (Hajhashemi, Sadraei, Ghannadi, & Mohseni, 2000; Skočibušić & Bezić, 2003).

Satureja subspicata Vis. is a rare species narrowly distributed on the Adriatic coast on open rocky Dinaride of Croatia. This plant is a perennial shrub sprouting every spring with new twigs full of linear and leathery leaves and purple flowering during October. To our knowledge, there are no published reports on the phytochemical composition and antimicrobial activity of the *S. subspicata* essential oil. Therefore, we focused our study on the phytochemical composition by GC–MS analysis and antimicrobial activity was determined by using agar disc diffusion and broth microdilution methods.

2. Materials and methods

2.1. Plant material

The aerial parts (tops) of wild plant materials (*S. subspicata* Vis.) were collected three times during October 2002 (1, 15 and 30), in the Kozjak Mountain (Croatia, near the city of Split) at altitudes of 500 m. Voucher specimens (No. FNSUEST-2002-98) were deposited in the herbarium at the Faculty of Natural Science, Mathematics and Education of the University of Split.

2.2. Extraction of the essential oils

Air-drying of the plant was performed in a shady place at room temperature for 10 days. Plant tops (during and after flowering) were used for the analysis of essential oil composition. A portion (100 g) of the aerial parts of *S. subspicata* was submitted for 3 h to water-distillation, using a Clevenger-type apparatus. The obtained essential oil (EO) was dried over anhydrous sodium sulphate and 2 μl was used for GC–MS measurements.

2.3. GC–MS analysis conditions

The analyses of the volatile compounds were carried out on a Hewlett–Packard GC–MS system (GC 5890 Series II; MSD 5971A). The fused-silica HP-20 M polyethylene glycol column (50 m × 0.2 mm i.d., 0.2 μm film thickness) was directly coupled to the mass spectrometer. The carrier gas was helium (1 ml/min) and the program used was 4 min isothermal at 70 °C, followed by 70–180 ° C at a rate of 4 °C/min, then held at 180 °C

for 10 min; the injection port temperature was 250 °C. Ionization of the sample components was performed in the E.I. mode (70 eV). The linear retention indices for all the compounds were determined by co-injection of the sample with a solution containing the homologous series of C_8–C_{22} *n*-alkanes (Van Den Dool & Kratz, 1963). Individual constituents were identified by referring to compounds known in the literature data (Adams, 1995), and also by comparing their mass spectra with either known compounds or with the Wiley mass spectral database.

2.4. Antimicrobial activity

2.4.1. Microbial strains

The antimicrobial activity of *S. subspicata* Vis. essential oil was evaluated using a panel which included laboratory control strains obtained from the American Type Culture Collection (Rockville, MD, USA): Gram-positive bacteria *Bacillus cereus* (ATCC 11778), *Bacillus subtilis* (ATCC 26633), *Enterococcus faecium* (ATCC 12755) *Enterococcus faecalis* (ATCC 29212), *Listeria monocytogenes* (ATCC15313), *Staphylococcus aureus* (ATCC 25923), Gram-negative bacteria: *Aeromonas hydrophila* (ATCC 7965), *Escherichia coli* (ATCC 25922), *E. coli* O157:H7 (ATCC 43895), *Klebsiella pneumoniae* (ATCC 13883), *Pseudomonas aeruginosa* (ATCC 27853), *Proteus mirabilis* (ATCC 25933), *Salmonella typhimurium* (ATCC 19430), and fungal microorganisms *Aspergillus niger* (ATCC 6275), *Aspergillus fumigatus* (ATCC 9142), *Aspergillus flavus* (ATCC 9643), *Candida albicans* (ATCC 10231), *Candida rugosa* (ATCC 10571), *Cladosporium cladosporioides* (ATCC 13276), and *Saccharomyces cerevisiae* (ATCC 561). Stock cultures were maintained at 4 °C on slopes of Tryptic soy broth (BBL, Cockeysville, MD) amended with 5 g/l Yeast extract (Oxoid, Nepean, ON) and 15 g/l Agar agar (BDH, Toronto, ON). Active cultures for experiments were prepared by transferring a loopful of cells from the stock cultures to flasks of Mueller–Hinton broth (MHB) (Oxoid) for bacteria and Sabouraud dextrose broth (SDB) for fungi that were incubated without agitation for 24 h at 37 and 25 °C. The cultures were diluted with fresh Mueller–Hinton and Sabouraud dextrose broth to achieve optical densities corresponding to 2.0×10^6 colony forming units (CFU/ml) for bacteria and 2.0×10^5 spore/ml for fungal strains.

2.4.2. Antimicrobial screening

Two different methods were employed for the determination of in vitro antimicrobial activities of the *S. subspicata* Vis. essential oil: an agar disc diffusion method and broth microdilution method. The minimum inhibitory concentration (MIC) and minimum bactericidal or fungicidal concentration (MBC/MFC) of the essential oils against the test microorganisms were determined by the broth microdilution method. The MIC and MBC of levofloxacin were also determined in parallel experiments in order to control the sensitivity of the test microorganisms. All tests were performed in duplicate.

2.4.3. Determination of antibacterial activity by the disc diffusion method

The essential oil was tested for antibacterial activity by the disc diffusion method according to the National Committee for Clinical Laboratory Standards (NCCLS, 2001) using 100 μl of suspension of the tested microorganisms, containing 2.0×10^6 CFU/ml for bacteria and 2.0×10^5 cfu/ml spore for fungal strains. Mueller–Hinton agar (MHA) (Oxoid) and Sabouraud dextrose agar (SDA) sterilized in a flask and cooled to 45–50 °C were distributed to sterilized Petri dishes with a diameter of 9 cm (15 ml). The filter paper discs (6 mm in diameter) were individually impregnated with 10 and 20 μl of the oil and then placed onto the agar plates which had previously been inoculated with the tested microorganisms. The Petri dishes were kept at 4 °C for 2 h. The plates were inoculated with bacteria incubated at 37 °C for 24 h and at 30 °C for 48 h for the yeasts and fungal strains. The diameters of the inhibition zones were measured in millimetres. All the tests were performed in duplicate. Vancomycin (30 μg), tetracycline (30 μg) and nystatin (30 μg) served as positive controls.

2.4.4. Determinations of the minimum inhibitory concentration (MIC) and minimum bactericidal or fungicidal concentration (MBC/MFC)

A broth microdilution method was used to determine the minimum inhibitory concentration (MIC) and minimum bactericidal or fungicidal concentration (MBC/MFC) according to the National Committee for Clinical Laboratory Standards (NCCLS, 2001). All tests were performed in Mueller Hinton broth (MHB), with the exception of the yeasts and fungal strains (Sabouraud dextrose broth; SDB). The investigated oil was dissolved in 1% dimethylsulphoxide (DMSO) and then diluted to the highest concentration. A serial doubling dilution of the oil was prepared in a 96-well microtiter plate over the range of 0.02–50.00 μl/ml. 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 bacteria and 2.0×10^5 CFU/ml spore for fungal strains. Petri dishes were kept at 4 °C for 2 h, placques injected with yeasts were incubated at 25 °C for 48 h, and the bacteria were incubated at 37 °C for 24 h. The MIC is defined as the lowest concentration of the essential oil at which the microorganism does not demonstrate visible growth. The microbial growth was determined by absorbance at 600 nm using the universal microplate reader (Biotek Instrument Inc, Highland Park, VT, USA). To determine MBC, broth was taken from each well 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 25 °C for the fungi. The MBC is defined as the lowest concentration of the essential oil at which inoculated microorganisms were completely killed. All determinations were performed in duplicate and two growth controls consisting of MHB and SDB medium with 1.0% (v/v) DMSO was included. Levofloxacin served as a positive control.

3. Results and discussion

3.1. Chemical composition of the essential oil

Air-dried herbal parts of the plant were subjected to hydrodistillation using a Clevenger apparatus and the whitish-coloured essential oil. The yields of oils ranged from 1.72% to 1.79% w/w (SD = 0.04). The percentage compositions and modes of identification of the oil components are listed in Table 1. GC–MS analysis resulted in the identification of 24 compounds representing 97.47–92.03% of the oils. Monoterpene hydrocarbons and their derivates dominated the chemical composition of the investigated oils. The sesquiterpenes are present in smaller quantities. The major compound was phenolic monoterpene carvacrol (16.76%, SD = 0.27). Other important compounds were the monoterpenic hydrocarbons α-pinene (13.58, SD = 0.01), *p*-cymene (10.76%, SD = 0.21), γ-terpinene (9.54%, SD = 0.26), and the oxygen-containing compounds thymol methyl ether (8.83%, SD = 0.08). The essential oil also contained smaller percentages of myrcene (4.82%, SD = 0.03), linalool (3.94%, SD = 0.14), β-caryophyllene (3.76%, SD = 0.21), limonene (3.45%, SD = 0.02), geranyl acetate (2.81%, SD = 0.02), 1-Octen-3-ol (2.21%, SD = 0.01), nerol (2.13%, SD = 0.05), thymol (2.12%, SD = 0.32) and borneol (2.11%, SD = 0.05).

Table 1
Phytochemical composition of *Satureja subspicata* Vis. essential oils (%)

No.	Phytochemicals	RI	%	SD	Mode of identification
1.	α-Pinene	1038	13.58	0.01	GC, MS
2.	Myrcene	1149	4.82	0.03	GC, MS
3.	Limonene	1183	3.45	0.02	GC, MS
4.	*cis*-β-Ocimene	1218	1.76	0.01	GC, MS
5.	γ-Terpinene	1231	9.54	0.26	GC, MS
6.	*p*-Cymene	1247	10.76	0.21	GC, MS, RC
7.	Alloocimene	1351	1.27	0.07	GC, MS
8.	1-Octen-3-ol	1411	2.21	0.01	GC, MS, RC
9.	Sabinene hydrate	1423	1.01	0.08	GC, MS
10.	β-Bourbonene	1496	1.13	0.04	GC, MS
11.	Linalool	1507	3.94	0.14	GC, MS, RC
12.	Terpinen-4-ol	1559	0.24	0.05	GC, MS
13.	Thymol methyl ether	1563	8.83	0.08	GC, MS
14.	β-Caryophyllene	1578	3.76	0.21	GC, MS
15.	Borneol	1653	2.11	0.05	GC, MS, RC
16.	Geranial	1680	1.20	0.01	GC, MS, RC
17.	Geranyl acetate	1729	2.81	0.02	GC, MS
18.	Myrtenol	1733	0.75	0.05	GC, MS
19.	Muurolene	1735	0.19	0.07	GC, MS
20.	Nerol	1752	2.13	0.05	GC, MS
21.	Caryophyllene oxide	1927	1.80	0.31	GC, MS
22.	Spathulenol	2061	1.31	0.03	GC, MS
23.	Thymol	2115	2.12	0.32	GC, MS, RC
24.	Carvacrol	2140	16.76	0.27	GC, MS, RC
	Oil yield (%)		1.75	0.04	

RI: retention indices (Kovats index) on HP-20M column.
GC: identification by comparison of retention indices.
MS: identification on the basis of the mass spectra Wiley (MS) only.
RC: identification by comparison of their mass spectra of reference compounds.
SD: standard deviation.

Literature review showed variation between the chemical compositions of different *Satureja* species oils (Azaz, Demirci, Satíla, Kurcuoglu, & Baser, 2002; Baydar, Sağdic, Özkan, & Karadoğan, 2004; Gulluce et al., 2003; Kuštrak, Kuftinec, Blažević, & Maffei, 1996; Miloš, Radonić, Bezić, & Dunkić, 2001; Tumen, Baser, Demirci, & Ermin, 1998). Generally, the savory oils were characterized by high percentage of the monoterpene phenols that is specific for the carvacrol chemotype growing in Croatia. It is interesting that oils extracted from *S. montana* collected from central parts of Dalmatia have carvacrol (84%) as their main constituent, other important components were *p*-cymene and sometimes thymol (Kuštrak et al., 1996). Several studies of other *Satureja* species such as *Satureja hortensis* (summer savory) *Satureja icarica*, *Satureja pilosa* and *Satureja boissieri* also showed that the oils have carvacrol, thymol, γ-terpinene, *p*-cymene and other terpenoids as the main components (Azaz et al., 2002; Gulluce et al., 2003). The oil of *S. cuneifolia* from Dalmatia contained carvacrol and was relatively rich in linalool, γ-terpinene, *p*-cymene, limonene and α-pinene (Miloš et al., 2001; Skočibušić et al., 2004). The studied essential oils displayed different chemical profiles from those observed *Satureja* plants of other geographical origins (Sefidkon, Jamzad, & Mirza, 2004). Compared to other *Satureja* species collected from this flora, carvacrol content was lower than those of *S. montana* and *S. cuneifolia* oils, but more sesquiterpenes were present. The chemical composition of essential oils of *Satureja* spp. shows a large interspecies variability and, within the same species, it seems to depend on the genetic characteristics of the plant and on the conditions under which it has grown. The biosynthetic relationships between these components in savory explain clearly their regular and correspondent variation within the plant life cycle (Skočibušić & Bezić, 2004).

The in vitro antimicrobial activity of *S. subspicata* essential oil against the microorganisms employed and its activity potentials were qualitatively and quantitatively assessed by the presence or absence of inhibition zones, zone diameters and MIC values. According to

the results given in Tables 2 and 3, the essential oil of the investigated species had great in vitro potential of antimicrobial activities against all 13 bacteria, 9 moulds and a yeast species tested. In this study, the antimicrobial activities of essential oil having two different concentrations of 10 and 20 μl/discs are compared with standard antibiotics such as vancomicin, tetracycline and nystatin used as positive controls. Results from the antimicrobial disc diffusion assay are summarized in Table 2. The data obtained from the disc diffusion method indicated that the essential oil displayed a variable degree of antimicrobial activity on different tested strains. The inhibitory effect increased with increase of the oils concentration from 10 to 20 μl. The data indicated that Gram-positive *S. aureus* was the most sensitive strain tested to the oil of *S. subspicata* with the strongest inhibition zones (28–34 mm). The *Enterococcus* group D was, in general, found to be more sensitive among Gram-positive bacteria with *E. faecalis* being the most sensitive (19–25 mm) and *E. faecium*, with inhibition zones of 17–21 mm. The oil also exhibited high antimicrobial activity against *B. subtilis* and *B. cereus*. Modest activities were observed against important food pathogens such as *L. monocytogenes*, with inhibition zones of 16–19 mm. Among these, Gram-negative strains also displayed variable degree of susceptibility against investigated oil. Maximum activity was observed against *P. mirabilis* (16–19 mm), followed by *E. coli* (16–19 mm) and *A. hydrophila* (14–21 mm). Gram-negative bacteria, *P. aeruginosa* exhibited weak inhibition zones (8–11 mm), since it is known to have high level of intrinsic resistance to virtually all known antimicrobials and antibiotics due to a combination of a very restrictive outer membrane barrier, highly resistant even to synthetic drugs. The highest antifungal activity of this oil was observed against *C. albicans* (24–36 mm), *C. rugosa* (21–32 mm), followed by *A. fumigatus* (15–28 mm) and *S. cerevisiae* (14–19 mm).

The in vitro activity of *S. subspicata* essential oil was evaluated by a broth microdilution method using a panel of microorganisms, which included laboratory control strains. Antimicrobial activity was expressed as minimum inhibitory concentration (MIC) and minimum bactericidal or fungicidal concentration (MBC/MFC) of the oil. The results of the MIC and MBC or MFC are shown in Table 3. The data indicate that the oil exhibited varying levels of antimicrobial activity against the investigated food pathogens. The inhibitory properties of the oil were observed within a range of concentrations from 0.09 to 25.00 μl/ml. In liquid medium the essential

Table 2
Antimicrobial activity of the *Satureja subspicata* Vis. essential oil, by the disc diffusion method

Microorganisms	Source No.	Essential oil zone inhibition (mm)		Antimicrobial agents zone inhibition (mm)		
		10 μl/disc	20 μl/disc	Vancomicin 30 μg/disc	Tetracycline 30 μg/disc	Nystatin 30 μg/disc
Gram-positive						
Bacillus cereus	ATCC 11778	14	23	12	28	nd
B. subtilis	ATCC 26633	17	21	15	24	nd
Enterococcus faecium	ATCC 12755	19	23	24	–	nd
E. faecalis	ATCC 29212	19	25	28	–	nd
Listeria monocytogenes	ATCC 15313	16	19	24	28	nd
Staphylococcus aureus	ATCC 25923	28	34	16	26	nd
Gram-negative						
Aeromonas hydrophila	ATCC 7965	14	21	18	31	nd
Escherichia coli	ATCC 25922	18	21	22	28	nd
E. coli O157:H7	ATCC 43895	17	19	18	26	nd
Klebsiella pneumoniae	ATCC 13883	14	17	11	26	nd
Pseudomonas aeruginosa	ATCC 27853	8	11	8	11	nd
Proteus mirabilis	ATCC 25933	21	24	20	24	nd
Salmonella typhimurium	ATCC 19430	16	19	19	22	nd
Fungi						
Aspergillus niger	ATCC 6275	19	25	nd	nd	13
Aspergillus fumigatus	ATCC 9142	15	27	nd	nd	10
Aspergillus flavus	ATCC 9643	19	28	nd	nd	12
Candida albicans	ATCC 10231	24	36	nd	nd	25
C. rugosa	ATCC 10571	21	32	nd	nd	18
Cladosporium cladosporioides	ATCC 13276	26	28	nd	nd	16
Saccharomyces cerevisiae	ATCC 561	14	19	nd	nd	18

Inactive (–); moderately active (713 mm); highly active (14 mm).
VA: vancomycin 30 μg/disc; TE: tetracycline 30 μg/disc; NY: nystatin 30 μg/disc.
nd, not detected; –, not active.

Table 3
Antimicrobial activity expressed as minimum inhibitory concentration (MIC) and minimum bactericidal or fungicidal concentration (MBC/MFC) of the *Satureja subspicata* Vis. essential oil, by the broth microdilution method

Microorganisms	Source No.	Essential oil (μl/ml)		Antimicrobial agents	
		MIC (μl/ml)	MBC/MFC (μl/ml)	MIC (μg/ml)	MBC (μg/ml)
Gram-positive				Levofloxacin	
Bacillus cereus	ATCC 11778	3.12	6.25	1.00	2.00
B. subtilis	ATCC 26633	3.12	3.12	0.50	2.00
Enterococcus faecium	ATCC 12755	1.56	3.12	0.50	8.00
E. faecalis	ATCC 29212	0.78	1.56	1.00	8.00
Listeria monocytogenes	ATCC 15313	1.56	3.12	0.50	2.00
Staphylococcus aureus	ATCC 25923	0.09	0.19	0.12	4.00
Gram-negative					
Aeromonas hydrophila	ATCC 7965	1.56	3.12	0.050	1.00
Escherichia coli	ATCC 25922	0.78	1.56	0.016	0.62
E. coli O157:H7	ATCC 43895	1.56	3.12	0.016	2.00
Klebsiella pneumoniae	ATCC 13883	6.25	12.50	0.062	1.00
Pseudomonas aeruginosa	ATCC 27853	12.50	25.00	0.050	8.00
Proteus mirabilis	ATCC 25933	1.56	3.12	0.062	1.00
Salmonella typhimurium	ATCC 19430	6.25	12.50	0.50	0.50
Fungi				Amphotericin	
Aspergillus niger	ATCC 6275	0.78	0.78	0.25	1.00
Aspergillus fumigatus	ATCC 9142	0.78	1.56	0.38	2.00
Aspergillus flavus	ATCC 9643	0.19	3.12	0.50	2.00
Candida albicans	ATCC 10231	0.09	0.19	0.25	2.00
Candida rugosa	ATCC 10571	0.19	0.39	0.50	2.00
Cladosporium cladosporioides	ATCC 13276	0.09	0.19	0.12	8.00
Saccharomyces cerevisiae	ATCC 561	1.56	3.12	0.25	4.00

oil was active against all the test strains. The Gram-negative *P. aeruginosa* seemed to be resistant to the investigated oil with a range of 12.50 to 25.00 μl/ml. Maximum activity was observed against the yeast such as *C. albicans*, fungal strain *C. cladosporioides* and *S. aureus* with MIC of 0.09 and MFC/MBC of 0.19 μl/ml to the oil. *E. faecium*, *L. monocytogenes*, *A. hydrophila* and *P. mirabilis* showed similar susceptibility to the investigated oil, ranging from 1.56 to 3.12 μl/ml. The oil exhibited the highest inhibitory effect against Gram-negative bacteria *K. pneumoniae* and *S. thyphmurium* in a range between 6.25 and 12.50 μl/ml. The oil of *S. subspicata* was effective against all fungal strains tested in the study, with a range of 0.09 to 3.12 μl/ml.

Plant essential oil is a potentially useful source of antimicrobial compounds. By inhibiting the growth of all human and plant pathogenic and/or food spoilage bacteria, moulds and the yeast tested, *S. subspicata* essential oil exerted a broad antimicrobial spectrum. Numerous studies demonstrated that the essential oils of other *Satureja* species are among the most potent essential oils with regard to antimicrobial properties (Ciani et al., 2000; Dorman & Deans, 2000; Gulluce et al., 2003; Müller-Riebau, Berger, & Yegen, 1995; Panizi et al., 1993; Sahin et al., 2003; Skočibušić & Bezić, 2004). Some researchers reported that there is a relationship between the chemical structures of the most abundant compounds in the tested oil and the antimicrobial activity. The essential oils rich in phenolic compounds are widely reported to possess high levels of antimicrobial activity (Baydar et al., 2004; Cosentino et al., 1999; Dorman & Deans, 2000; Lambert et al., 2001; Panizi et al., 1993; Sivropoulou, Papanikolaou, Nikolaou, & Kokkini, 1996), which has been confirmed and extended in the present studies.The antimicrobial nature of the essential oil studied is apparently related to its phenolic components, such as carvacrol, oxygenated derivatives (thymol methyl ether) and its precursors *p*-cymene and γ-terpinene (Table 1). The bacteriostatic and fungistatic properties of the oil are suspected to be associated with the carvacrol content, which has been tested previously and was found to have a significant antibiotic activity (Cosentino et al., 1999; Dorman & Deans, 2000; Juliano, Mattana, & Usai, 2000; Lambert et al., 2001). Carvacrol has been reported to have significant bactericidal effects towards *Salmonella* in pieces of fish stored at 4 °C (Hulin, Mathot, Mafart, & Dufosse, 1998). Aside from the inhibition of the growth of vegetative bacterial cells, the inhibition of toxin production is also of interest to food microbiologists. Carvacrol is able to inhibit the production of diarrhoeal toxin by *B. cereus* in broth and in soup. Two theories are offered for the mode of action of toxin limitation: If toxin excretion is an active process, there may be insufficient ATP to export it from the cell. Alternatively, the lower specific growth rate may mean that the cells use all the available energy to sustain viability, leaving little over for toxin production (Ultee, Kets, Alberda, Hoekstra, & Smid, 2000a). Carvacrol showed very high antifungal

potential with much lower MIC (0.1–0.2 μl/ml) and MFC (0.1–0.5 μl/ml) values than miconazole (Sokovic, Tzakou, Pitarokili, & Couladis, 2002). Synergism between carvacrol and its biological precursor *p*-cymene has been noted when acting on *B. cereus* vegetative cells. It appears that *p*-cymene, a very weak antibacterial, swells bacterial cell membranes to a greater extent than carvacrol does. By this mechanism *p*-cymene probably enables carvacrol to be more easily transported into the cell so that a synergistic effect is achieved when the two are used together (Ultee, Bennink, & Moezelaar, 2002). Thus, *p*-cymene, which was found to be the major constituent of the investigated oil, is not an effective antibacterial when used alone (Dorman & Deans, 2000; Juliano et al., 2000; Juven, Kanner, Schved, & Weisslowicz, 1994; Ultee et al., 2000a), but when combined with carvacrol, synergism has been observed against *B. cereus* in vitro and in rice (Ultee, Slump, Steging, & Smid, 2000b). Furthermore, Mourey and Canillac (2002) found that α-pinene, the main compound of *S. subspicata* oil, was the most active component with an average minimal inhibitory concentration (MIC) of 0.19 μl/ml against *L. monocytogenes* 4b. Although the antibacterial activity of essential oils from many plant species has been extensively surveyed, their antimicrobial mechanism has not been reported in great detail. 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 and in some instances structure, causing swelling and increasing its permeability. A consistent observation is an increase in K^+ and often cytoplasmic content effluxes from cells in response to antimicrobial challenge. These effects may develop as a result of membrane depolarization by altered ion transport or through changes in membrane structure, inhibition of energy (ATP) generation by interference with glucose uptake or inhibition of enzymes involved in oxidative or substrate level phosphorylation. 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.

Some studies have concluded that whole essential oils have a greater antibacterial activity than the major components mixed (Gill, Delaquis, Russo, & Holley, 2002; Mourey & Canillac, 2002), which suggests that the minor components are critical to the activity and may have a synergistic effect or potential influence to the essential oil. However, Pattnaik, Subramanyam, Bapaji, and Kole (1997) found that the linalool, as the main of this oil demonstrated strong inhibitory effect against 17 bacteria and 10 fungi. In fact, long chain (C_6–C_{10}) alcohols were particularly active against Gram-positive bacteria (Delaquis, Stanich, Girard, & Mazza, 2002). The antimicrobial properties of alcohols were known to increase with molecular weight. It is also evident that terpene alcohols such as linalool exhibit strong antimicrobial activity, especially pronounced on whole cells, while hydrocarbon derivatives possess lower antifungal properties, as their low water solubility limits their diffusion through the medium (Knobloch, Pauli, Iberl, Weigand, & Weis, 1989). In addition, other minor components such as borneol have been also reported to have antimicrobial potential (Knobloch et al., 1989). Indeed, Mourey and Canillac (2002) also found that the borneol show bacteriostatic activity against *L. monocytogenes* at concentration of less than 0.62 μl/ml. The sesquiterpene β-caryophyllene is known to possess a critical part in plant defense (Ulubelen, Topcu, & Eris, 1994). Most studies investigating the action of essential oils against food spoilage organisms and food borne pathogens agree that, generally, essential oils are slightly more active against Gram-positive than Gram-negative bacteria (Delaquis et al., 2002; Juliano et al., 2000; Lambert et al., 2001; Smith-Palmer et al., 2001). That Gram-negative organisms are less susceptible to the action of antibacterials is perhaps to be expected, since they possess an outer membrane surrounding the cell wall, which restricts diffusion of hydrophobic compounds through its lipopolysaccharide covering. However, our results show that Gram-positive bacteria are more sensitive to the investigated oil, with a range of 0.09 to 6.25 μl/ml than Gram-negative bacteria in the range of 1.56 to 25.00 μl/ml, which has been confirmed and extended in the present studies.

To the best of our knowledge, the essential oil composition and antimicrobial activity of *S. subspicata* Vis. has not been reported before and therefore our results can be evaluated as the first report about the antimicrobial properties in respect to the chemical composition. The results of this study suggest the possibility of using the essential oil or some of their components as natural food preservatives, because the oils possess strong antibacterial activity. Further research is needed in order to obtain information regarding the practical effectiveness of essential oil to prevent the growth of food borne and spoiling microbes under the specific application conditions.

References

Abad, M. J., Bermejo, P., Gonzales, E., Iglesias, I., Irurzun, A., & Carrasco, L. (1999). Antiviral activity of Bolivian plant extracts. *General Pharmacology, 32*, 499–503.

Adams, R. P. (1995). *Identifications of essential oil components by gas chromatography/mass spectroscopy*. Carol Stream, IL: Allured Publishers.

Alzoreky, N. S., & Nakahara, K. (2003). Antimicrobial activity of extracts from some edible plants commonly consumed in Asia. *International Journal of Food Microbiology, 80*, 223–230.

Aureli, P., Costantini, A., & Zolea, S. (1992). Antimicrobial activity of some plant essential oils against *Listeria monocytogenes*. *Journal of Food Protection, 55*, 344–348.

Azaz, D., Demirci, F., Satíla, F., Kurcuoglu, M., & Baser, K. H. (2002). Antimicrobial activity of some *Satureja* essential oils. *Zeitschrift für Naturforschung, 57c*, 817–821.

Baydar, H., Sağdic, O., Özkan, G., & Karadoğan, T. (2004). Antibacterial activity and composition of essential oils from *Origanum*, *Thymbra* and *Satureja* species with commercial importance in Turkey. *Food Control, 15*, 169–172.

Cantino, P. D., Harley, R. M., & Wagstaff, S. J. (1992). Genera of Labiatae: Status and Classification. In R. M. Harley & T. Reynolds (Eds.), *Avances in Labiatae Science* (pp. 511–522). Kew: Royal Botanical Gardens.

Ciani, M., Menghini, L., Mariani, F., Pagiotti, R., Menghini, A., & Fatichenti, F. (2000). Antimicrobial properties of essential oil of *Satureja montana* L. on pathogenic and spoilage yeasts. *Biotechnology Letters, 22*, 1007–1010.

Cosentino, S., Tuberoso, C. I. G., Pisano, B., Satta, M., Mascia, V., Arzedi, E., et al. (1999). In vitro antimicrobial activity and chemical composition of Sardinian *Thymus* essential oils. *Letters in Applied Microbiology, 29*, 130–135.

Delaquis, P. J., Stanich, K., Girard, B., & Mazza, G. (2002). Antimicrobial activity of individual and mixed fractions of dill, cilantro, coriander and eucalyptus essential oils. *International Journal of Food Microbiology, 74*, 101–109.

Dorman, H. J. D., & Deans, S. G. (2000). Antimicrobial agents from plants: antibacterial activity of plant volatile oils. *Journal of Applied Microbiology, 88*, 308–316.

Escudero, J., Lopez, J. C., Rabanal, R. M., & Valverde, S. (1985). Secondary metabolites form Satureja species – new triterpenoid from *Satureja acinos*. *Journal of Natural Products, 48*, 128–132.

Esquived, M. M., Ribeiro, M. A., & Bernardo-Gil, M. G. (1999). Supercritical extraction of savory oils: study of antioxidant activity und extract characterisation. *Journal Supercritical Fluids, 14*, 129–138.

Farag, R. S., Daw, Z. Y., Hewedi, F. M., & El-Baroty, G. S. A. (1989). Antimicrobial activity of some Egyptian spice essential oils. *Journal of Food Protection, 52*, 665–667.

Gill, A. O., Delaquis, P., Russo, P., & Holley, R. A. (2002). Evaluation of antilisterial action of cilantro oil on vacuum packed ham. *International Journal of Food Microbiology, 73*, 83–92.

Gulluce, M., Sokmen, M., Daferera, D., Agar, G., Ozkan, H., & Kartal, N. (2003). In vitro antibacterial, antifungal, and antioxidant activities of the essential oil and methanol extracts of herbal parts and callus cultures of *Satureja hortensis* L. *Journal of Agricultural and Food Chemistry, 51*, 3958–3965.

Hajhashemi, V., Sadraei, H., Ghannadi, A. R., & Mohseni, M. (2000). Antispasmodic and anti-diarrhoeal effect of *Satureja hortensis* L. essential oil. *Journal Ethnopharmacology, 71*, 187–192.

Houghton, P. J., Zarka, R., Heras, B., & Hoult, J. R. S. (1995). Fixed oil of *Nigella sativa* and derived thymoquinone inhibit eicosanoid generation in leucocytes and membrane lipid peroxidation. *Planta Medica, 61*, 33–36.

Hulin, V., Mathot, A., Mafart, P., & Dufosse, L. (1998). Les proprieties anti-microbiennes des huiles essentielles et composes d'aromes. *Sciences of Aliments, 18*, 563–582.

Juliano, C., Mattana, A., & Usai, M. (2000). Composition and in vitro antimicrobial activity of the essential oil of *Thymus* herba-barona Loisel growing wild in Sardinia. *Journal of Essential Oil Research, 12*, 516–522.

Juven, B. J., Kanner, J., Schved, F., & Weisslowicz, H. (1994). Factors that interact with the antibacterial action of thyme essential oil and its active constituents. *Journal of Applied Bacteriology, 76*, 626–631.

Kuštrak, D., Kuftinec, J., Blažević, N., & Maffei, M. (1996). Comparison of the essential oil composition of two subspecies of *Satureja montana*. *Journal of Essential Oil Research, 8*, 7–13.

Knobloch, K., Pauli, A., Iberl, B., Weigand, H., & Weis, N. (1989). Antibacterial and antifungal properties of essential oil components. *Journal of Essential Oil Research, 1*, 119–128.

Lambert, R. J. W., Skandamis, P. N., Coote, P., & Nychas, G. J. E. (2001). A study of the minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol. *Journal of Applied Microbiology, 91*, 453–462.

Lis-Balchin, M., Ochoka, R. J., Deans, S. G., Asztemborska, M., & Hart, S. (1999). Differences in bioactivity between the enantiomers of α-pinene. *Journal of Essential Oil Research, 11*, 393–397.

Miloš, M., Radonić, A., Bezić, N., & Dunkić, V. (2001). Localities and seasonal variations in the chemical composition of essential oils of *Satureja montana* L. and *S. cuneifolia* Ten. *Flavour and Fragrance Journal, 16*, 157–160.

Mourey, A., & Canillac, N. (2002). Anti-*Listeria monocytogenes* activity of essential oils components of conifers. *Food Control, 13*, 289–292.

Müller-Riebau, F., Berger, B., & Yegen, O. (1995). Chemical composition and fungitoxic properties to phytopathogenic fungi of essential oils of selected aromatic plants growing wild in Turkey. *Journal of Agricultural and Food Chemistry, 43*, 2262–2266.

NCCLS – National Committee for Clinical Laboratory Standards. (2001). Performance standards for anti-microbial susceptibility testing: eleventh informational supplement. Document M100-S11. National Committee for Clinical Laboratory Standard, Wayne, PA, USA.

Palić, R., Šilić, Č, & Gašić, M. J. (1983). Chemical composition of volatile oil from *Satureja montana* subsp. *montana*. *Acta Pharmceutica Jugoslavica, 33*, 227–231.

Panizi, L., Flamini, G., Cioni, P. L., & Morelli, I. (1993). Composition and antimicrobial properties of essential oils of four Mediterranean Lamiaceae. *Journal of Ethnopharmacology, 39*, 167–170.

Paster, N., Menasherov, M., Ravid, U., & Juven, B. (1995). Antifungal activity of oregano and thyme essential oils applied as fumigants against fungi attacking stored grain. *Journal of Food Protection, 58*(1), 81–85.

Pattnaik, S., Subramanyam, V. R., Bapaji, M., & Kole, C. R. (1997). Antibacterial and antifungal activity of aromatic constituents of essential oils. *Microbios, 89*, 39–46.

Radonić, A., & Miloš, M. (2003). Chemical composition and in vitro evaluation of antioxidant effect of free volatile compounds from *Satureja montana* L. *Free Radical Research, 37*, 673–679.

Ruberto, G., & Baratta, M. T. (2000). Antioxidant activity of selected essential oil components in two lipid model systems. *Food Chemistry, 69*, 167–174.

Sahin, F., Karaman, I., Gulluce, M., Ogutcu, H., Sengul, M., Adiguzel, A., et al. (2003). Evaluation of antimicrobial activities of *Satureja hortensis* L. *Journal Ethnopharmacology, 87*, 61–615.

Sefidkon, F., Jamzad, Z., & Mirza, M. (2004). Chemical variation in the essential oil of *Satureja sahendica* from Iran. *Food Chemistry, 88*, 325–328.

Sivropoulou, A., Papanikolaou, E., Nikolaou, C., & Kokkini, S. (1996). Antimicrobial and cytotoxic activities of *Origanum* essential oils. *Journal of Agricultural and Food Chemistry, 44*, 1202–1205.

Skočibušić, M., & Bezić, N. (2003). Chemical composition and antidiarrhoeal activities of winter savory (*Satureja montana*) essential oil. *Pharmaceutical Biology, 41*, 622–626.

Skočibušić, M., & Bezić, N. (2004). Chemical composition and antimicrobial variability of *Satureja montana* L. essential oils produced during ontogenesis. *Journal of Essential Oil Research, 16*, 387–391.

Skočibušić, M., Bezić, N., & Dunkić, V. (2004). Variability of *Satureja cuneifolia* Ten. essential oils and their antimicrobial activity depending on the stage of development. *European Food Research and Technology, 218*, 367–371.

Smith-Palmer, A., Stewart, J., & Fyfe, L. (2001). The potential application of plant essential oils as natural food preservatives in soft cheese. *Food Microbiology, 18*, 463–470.

Sokovic, M., Tzakou, O., Pitarokili, D., & Couladis, M. (2002). Antifungal activities of selected aromatic plants growing wild in Greece. *Nahrung Food, 46*, 317–320.

Soliman, K. M., & Badeaa, R. I. (2002). Effect of oil extracted from some medicinal plants on different mycotoxigenic fungi. *Food and Chemical Toxicology, 40*, 1669–1675.

Teissedre, P. L., & Waterhouse, A. L. (2000). Inhibition of oxidation of human low-density lipoproteins by phenolic substances in different essential oils varieties. *Journal of Agricultural and Food Chemistry, 48*, 3605–3801.

Thomas-Barberan, F. A., Huisain, S. Z., & Gil, M. J. (1987). The distribution of methylated flavones in the *Lamiaceae*. *Biochemical Systematics and Ecology, 16*, 43–46.

Tumen, G., Baser, K. H. C., Demirci, B., & Ermin, N. (1998). The essential oils of *Satureja coerulea* Janka and *Thymus aznavourii* Velen. *Flavour and Fragrance Journal, 13*, 65–67.

Ultee, A., Kets, E. P. W., Alberda, M., Hoekstra, F. A., & Smid, E. J. (2000a). Adaptation of the food-borne pathogen *Bacillus cereus* to carvacrol. *Archives of Microbiology, 174*, 233–238.

Ultee, A., Slump, R. A., Steging, G., & Smid, E. J. (2000b). Antimicrobial activity of carvacrol toward *Bacillus cereus* on rice. *Journal of Food Protection, 63*, 620–624.

Ultee, A., Bennink, M. H. J., & Moezelaar, R. (2002). The phenolic hydroxyl group of carvacrol is essential for action against the food-borne pathogen *Bacillus cereus*. *Applied and Environmental Microbiology, 68*, 1561–1568.

Ulubelen, A., Topcu, G., & Eris, C. (1994). Terpenoids from *Salvia sclarea*. *Phytochemistry, 36*, 971–974.

Van Den Dool, H., & Kratz, P. D. (1963). A generalization of the retention index system including linear temperature programmed gas-liquid partition chromatography. *Journal of Chromatography, 11*, 463–471.

Yamasaki, K., Nakano, M., Kawahata, T., Mori, H., Otake, T., Ueba, N., et al. (1998). Anti-HIV-1 activity of herbs in *Labiatae*. *Biological & Pharmaceutical Bulletin, 2*, 829–833.

WHO – World Health Organization. (2002). *World health reports 2002: reducing risks, promoting healthy life* (p. 248). Geneva: World Health Organization, 30 October 2002. ISBN 92 4 156207 2 ISSN 1020-3311.