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Phytochemical composition and antimicrobial activity of *Satureja montana* L. and *Satureja cuneifolia* Ten. essential oils

NADA BEZIĆ*, MIRJANA SKOČIBUŠIĆ, VALERIJA DUNKIĆ

Department of Biology, Faculty of Natural Sciences, Mathematics and Education, University of Split, Teslina12, 21000 Split, Croatia

The phytochemical composition and the antibacterial activity of the essential oils obtained from the aerial parts of two Lamiaceae species, winter savory (*Satureja montana* L.) and wild savory (*Satureja cuneifolia* Ten.) were evaluated. Gas chromatography-mass spectrometry (GC-MS) analysis of the isolated oils resulted in the identification of twenty compounds in the oil of *S. montana* representing 97% of the total oil and 25 compounds of *S. cuneifolia*, representing 80% of the total oil. Carvacrol was the major constituent of the *S. montana* oil (45.7%). Other important compounds were the monoterpenic hydrocarbons *p*-cymene, γ-terpinene and the oxygenated compounds carvacrol methyl ether, borneol and thymol. Conversely, the oil of *S. cuneifolia* contained a low percentage of carvacrol and thymol. The major constituents of wild savory oil were sesquiterpenes β-cubebene (8.7%), spathulenol, β-caryophyllene, followed by the monoterpenic hydrocarbons limonene and α-pinene. The screening of the antimicrobial activities of essential oils were individually evalated against nine microorganisms, using a disc diffusion metod. The oil of *S. montana* exhibited greater antimicrobial activity than the oil of wild savory. Maximum activity of winter savory oil was observed against *Escherichia coli,* the methicillin-resistant *Staphylococcus aureus* and against yeast (*Candida albicans*). The essential oil of *S. cuneifolia* was also found to inhibit the growth of pathogens such as *S. aureus* and *E. coli.* A fungicidal activity against *C. albicans* and *Saccharomyces cerevisiae* was also found in both oils.

Key words: *Satureja montana, Satureja cuneifolia,* essential oil, antimicrobial activity, bacteria, fungi

Introduction

Plants produce an enormous array of secondary metabolites, and it is commonly accepted that a significant part of this chemical diversity serves to protect plants against microbial pathogens (DIXON 2001). Researchers have been interested in biologically active compounds isolated from plant species for the elimination of pathogenic microorganisms because of the resistance that they have developed to antibiotics (HUNTER and REEVES 2002). The antimicrobial properties of essential oils have been well recognized for many

* Corresponding author: e-mail: bezic@pmfst.hr

years (DEANS and RITCHIE 1987, BEZIĆ et al. 1999, HAMMER et al. 1999, COSENTINO et al. 1999, MOUREY and CANILLAC 2002, DAFERERA et al. 2003), and preparations of them have found applications as naturally occurring antimicrobial agents in pharmacology, pharmaceutical botany, phytopathology, medical and clinical microbiology and food preservation. Thus, the discovery of essential oils or components of such oils that possess biological properties has been the subject of many investigations: antiviral (BISHOP 1995), antimycotic (AZZOUZ and BULLERMAN 1982, MARI et al. 2003), antitoxigenic (AKGÜL et al. 1991, ULTEE and SMID 2001, JUGLAL et al. 2002), insecticidal (KONSTANTOPOULOU et al. 1992, KARPOUHTSIS et al. 1998). *Satureja* is a genus of the well-known medicinal plant of Lamiaceae family and comprises numerous species growing wild in the Mediterranean area. Certain species of Satureja, including a well-known species *Satureja montana* L. known as winter savory and *Satureja cuneifolia* Ten. or wild savory, are rich sources of biologically active phytochemicals. The positive effects of savory on human health are attributed to its active constituents such as essential oil, triterpenes ESCUDERO et al. (1985), flavonoids THOMAS-BARBERAN et al. (1987), and rosmarinic acid (RESCHKE 1983). The essential oil of savory contains antioxidative compounds, namely carvacrol, thymol, β-caryophyllene, γ-terpinene, *p*-cymene, together with linalool, which was reported to possess a strong antioxidant activity (RUBERTO and BARATTA 2000). Thus, there is a considerable research interest in the assay of composition and/or biological properties of various *Satureja* essential oils. To our knowledge no data have been published on the biological properties of *S. cuneifolia* and there is a lack of information on the antimicrobial activity of the two species from Croatia. In the present work, a wide range of potentially pathogenic and multidrug resistant bacteria was used to evaluate antibacterial activity and to relate it to the chemical composition of essential oils of the *S. montana* and *S. cuneifolia* growing in similar environmental conditions. The phytochemical composition of the essential oils was evaluated by using gas chromatography-mass spectrometry (GC-MS) analysis and antimicrobial activity was determined by the disc diffusion method.

Materials and methods

Plant material

The aerial part of wild growing populations of *Satureja montana* L. and *S. cuneifolia* Ten. were collected from Biokovo mountain (Croatia), at altitudes from 400 to 1000 m above sea level. The plants used in this study were gathered from September and October 2002 during flowering periods. Air-drying of the plant was performed in a shady place for twenty days at room temperature. The voucher specimen was identified by Dr. Juraj Kamenjarin at the Department of Biology, Faculty of Natural Science and Education of the University of Split and deposited in the Herbarium of the Department of Biology, Faculty of Natural Science and Education of the University of Split (FNSUEST-2002-38).

Extraction of the essential oils

Leaves and flowers of species were used for the analysis of essential oil composition. A hundred grams of dried plant material were subjected to hydro-distillation for 3 hours using a Clevenger-type apparatus to produce oils. The essential oils obtained were dried over anhydrous sodium sulphate and stored in sealed vials at low temperature (2°C).

Gas chromatography-mass spectrometry analysis (GC-MS) condition

The analyses of the volatile compounds were carried out on a Hewlet Packard gas chromatograph (GC 5890 Series II; MSD 5971A). A fused silica HP-20 M polyethylene glycol column (50m x 0.2 mm, 0.2 μm film thickness) was directly coupled to the mass spectrometer. The carrier gas was helium (1 mL min^{-1}). The programme used was 4 min. isothermal at 70°C, then 70–180°C at a rate of 4°C min^{-1} then held isothermal for 10 min. The injection port temperature was 250°C. The ionization of the sample components was performed in the E.I. mode (70eV). The linear retention indices for all the compounds were determined by co-injection of the sample with a solution containing homologous series of C_8–C_{22} *n*-alkanes (VAN DEN DOOL and KARTZ 1963). The individual constituents were identified by their identical retention indices, referring to compounds known from literature data (ADAMS 1995: 68) and also by comparing their mass spectra with either known compounds or with the Wiley mass spectral database.

Antimicrobial activity

Microbial strains

The antimicrobial activity of essential oils was evaluated using a panel which included both clinical pathogens and laboratory control strains, all of them belonging to the Merck Culture Collection: three Gram-positive bacteria *Bacillus subtilis* (MB 964), *Enterococcus faecium* (MB 5571) and *Staphylococcus aureus* (MB 5393); Gram-negative bacteria *Pseudomonas aeruginosa* (MB 979), *Serratia marcescens* (MB 252) and *Escherichia coli*, two yeasts (*Candida albicans,* MY 1055 and *Saccharomyces cerevisiae*, W 303) and one filamentous fungus (*Aspergillus fumigatus,* MF 5668).

All strains used for the tests, except for *B. subtilis and E. coli,* were resistant to at least one known antimicrobial agent: *S. aureus* was methicillin-resistant (MRSA), *E. faecium* was resistant to vancomycin and β-lactam antibiotics, and the two Gram-negative strains were resistant to penicillin, cephalosporins and macrolides. *A. fumigatus,* (MF 5668) and *C. albicans* (MY 1055) are major causes of systematic fungal infection, particularly in immuno-depressed patients, including those with acquired immunodeficiency syndrome.

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 guidelines (NCCLS, 2001) using 100μL of suspension of the tested microorganisms, containing 2.0 x 10^6 colony forming units (cfu mL^{-1}) for bacteria and 2.0x10^5 spore mL^{-1} 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, Difco) were individually impregnated with 10μL and 20μL of the essential oils was dissolved in dimethylsulfoxide (DMSO), which was subsequently placed in the centre of the inoculated Petri dishes. The DMSO concentration was adjusted to 0.1% (v/v). The Petri dishes were kept at 4°C for 2 h. The plates inoculated with bacteria incubated at 37°C for 24 h and at 30 °C for 48 h for the yeasts. The diameters of the inhibition zones were measured in millimetres. Controls were set up with equivalent quantities of DMSO. Studies were performed in triplicate, and the developing

inhibition zones were compared with those of reference disks. In addition, reference antibiotic discs such as vancomycine, tetracycline and nystatin were used for comparison. All the tests were performed in duplicate.

Results

The oils isolated by hydro-distillation from the aerial part of *S. montana* and *S. cuneifolia* were found to be yellow liquids and were obtained in yields of 1.7% (v/w) for

Tab. 1. Phytochemical analysis of *Satureja montana* L. and *Satureja cuneifolia* Ten. essential oils (% v/w, volume/dried weight). 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 – trace < 0.1% v/w

Component	RI	*Satureja montana*	*Satureja cuneifolia*	Mode of Identification
α-Thujene	1031	1.8	–	GC, MS
α-Pinene	1038	1.0	6.9	GC, MS
Myrcene	1149	0.8	3.9	GC, MS
α-Terpinene	1161	1.5	–	GC, MS
Limonene	1183	–	8.3	GC, MS
cis-β-Ocimene	1218	–	4.4	GC, MS
γ-Terpinene	1231	8.1	–	GC, MS
p-Cymene	1247	12.6	3.8	GC, MS, RC
allo-Ocimene	1351	–	2.5	GC, MS
1-Octen-3-ol	1411	0.7	–	GC, MS, RC
Camphor	1472	–	1.3	GC, MS
β-Bourbonene	1496	–	1.0	GC, MS
Linalool	1507	0.5	1.6	GC, MS, RC
Terpinen-4-ol	1559	–	2.4	GC, MS
Thymol methyl ether	1563	2.3	–	GC, MS
Carvacrol methyl ether	1576	11.0	–	GC, MS
β-Caryophyllene	1578	–	5.2	GC, MS
Aromadendrene	1583	–	0.8	GC, MS
Neral	1633	–	3.5	GC, MS
α-Terpineol	1646	0.5	1.2	GC, MS
Borneol	1653	4.8	3.8	GC, MS, RC
β-Cubebene	1680	0.5	8.7	GC, MS
Geranial	1680	–	2.1	GC, MS, RC
δ-Cadinene	1729	0.4	1.5	GC, MS
Nerol	1752	–	0.9	GC, MS
Geraniol	1796	–	0.6	GC, MS
Thymyl acetate	1809	0.2	–	GC, MS
Caryophyllene oxide	1927	0.4	1.0	GC, MS
Viridiflorol	2023	–	1.6	GC, MS
Spathulenol	2061	0.3	5.3	GC, MS
Thymol	2115	3.9	3.6	GC, MS, RC
Carvacrol	2140	45.7	4.1	GC, MS, RC

winter savory and 0.3% [(v/w) volume/dried weight] for wild savory. Twenty compounds were identified in the oil of *S. montana* representing 97% of the total oil, and 25 compounds were identified in the oil of *S. cuneifolia*, representing 80% of the total oil (Tab. 1).

The oil of *S. montana* is characterized by a high content of the phenolic monoterpene carvacrol (45.7%). Other important compounds were the monoterpenic hydrocarbons *p*-cymene (12.6%), γ-terpinene (8.1%) and the oxygen-containing compounds carvacrol methyl ether (11.0%), borneol (4.8%) thymol (3.9%) and thymol methyl ether (2.3%). The essential oil also contained smaller percentages of *a*-thujene, *a*-terpinene, *a*-pinene and myrcene.

The GC-MS analysis of *S. cuneifolia* oil demonstrated an abundance of the sesquiterpenes β-cubebene (8.7%), spathulenol (5.3%), β-caryophyllene (5.2%) while other components are limonene (8.3%) and α-pinene (6.9%). The oil of *S. cuneifolia* contained a low percentage of carvacrol and thymol compared with the *S. montana* oil investigated (Fig. 1). Chemical analysis revealed that there are significant qualitative and quantitative differences among the oils tested.

The antimicrobial activity of *S. montana* and *S. cuneifolia* essential oils was evaluated by the disc diffusion method against Gram-positive, Gram-negative bacteria and fungal strains. All strains used for the tests, except for *Bacillus subtilis* and *Esherichia coli,* were resistant to at least one known antimicrobial agent. Essential oils exhibited antimicrobial activity of various degrees against the tested strains (Tab. 2). It is evident from the table that the antimicrobial activities greatly increase with increase of the oil concentrations from 10µL to 20µL/disc. The data indicated that *E. coli* was the most sensitive strain tested to the oil of *S. montana,* with the strongest inhibition zones (23–32 mm), followed *Candida albicans* with inhibition zones (21–28 mm). The methicillin-resistant *Staphylococcus aureus* (MRSA), which causes many public health problems, was susceptible to winter savory oil, with an inhibition zone of 19–25 mm. The tested strains *B. subtilis, Serratia*

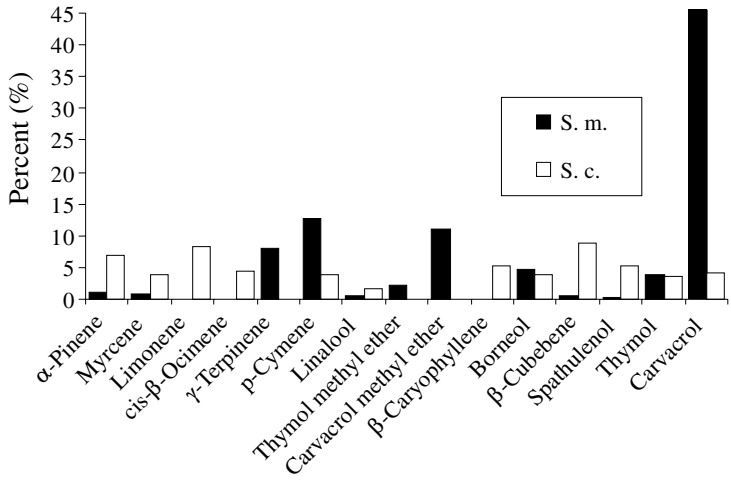

Fig. 1. Comparison of the composition principal monoterpenes of the *Satureja montana* L. and *Satureja cuneifolia* Ten. essential oils analyzed by gas chromatography.

Tab. 2. Antimicrobial activity of the *Satureja montana* L. and *Satureja cuneifolia* Ten. essential oils. Includes diameter of disc (6 mm). nt – non tested; na – non active. Inactive (–); moderately active (7–13mm); highly active (>14mm). VA: vancomycin 30 μg; TE: tetracycline 30 μg; NY: nystatin 30 μg.

Microorganisms	Inhibition zone (mm)[a]						
	Satureja montana		*Satureja cuneifolia*		Standard antibiotics		
					VA	TE	NY
	10 μL	20 μL	10 μL	20 μL	30 μg	30 μg	30 μg
Gram-positive bacteria							
Bacillus subtilis	22	23	14	16	15	24	nt
Enterococcus faecium	14	21	_	_	7	11	nt
Staphylococcus aureus	19	25	13	17	10	16	nt
Gram -negative bacteria							
Escherichia coli	23	32	7	11	24	17	nt
Pseudomonas aeruginosa	4	7	_	_	8	12	nt
Serratia marcescens	12	16	9	11	15	na	nt
Fungi							
Aspergillus fumigatus	10	16	11	15	nt	nt	9
Candida albicans	21	28	14	17	nt	nt	14
Saccharomyces cerevisiae	16	19	11	14	nt	nt	11

marcescens and *Enterococcus faecium* were more sensitive to the oil of *S. montana*. *Pseudomonas aeruginosa,* known to be very resistant even to synthetic drugs, exhibited weak inhibition zones (4–7 mm).

The essential oil of *S. cuneifolia* was also found to inhibit the growth of important pathogens such as *B. subtilis*, with an inhibition zone of 14–16 mm. The oil was found to possess moderate activity against methicillin-resistant *S. aureus* (MRSA), with a 13–17 mm inhibition zone. Gram-negative bacteria *E. coli* exhibited a weak inhibition zone (7–11 mm). This oil had poor activity against *E. faecium,* which is resistant to vancomycin and β-lactam antibiotics. Among the microorganisms tested, *E. faecium* and *P. aeruginosa* were resistant to the oil investigated. This oil was observed to have its highest antifungal activity against *C. albicans* (14–17 mm)*, Aspergillus fumigatus* (11–15 mm) and *Saccharomyces cerevisiae* (11–14 mm). Significant differences were evident between *S. montana* and *S. cuneifolia* essential oils in terms of the antimicrobial spectrum.

Discussion

The genus *Satureja* presents great variability in the concentration of the major components in its essential oils composition due to the existence of different species and subspecies, but also to a numerous of parameters, mainly the environmental and climatic conditions (GULLUCE et al. 2003; KUŠTRAK et al. 1996; SKOČIBUŠIĆ and BEZIĆ 2003). With reference to previous studies, thymol and carvacrol, in particular, were found to be principal constituents of the oils isolated from several Croatian *Satureja* species (MILOŠ et al. 2001;

SKOČIBUŠIĆ and BEZIĆ 2004). It is interesting that various isolates of winter savory from Croatia and Bosnia and Herzegovina have carvacrol (up to 84.19%) as main constituent (KUŠTRAK et al. 1996). A review of the published literature CAZIN et al. (1985) reveals that the oil composition of winter savory shows large variations in the relative concentration of major components: carvacrol (5–69%), linalool (1–62%), γ-terpinene (1–31%) and *p*-cymene (3–27%), arising from the existence of different chemotypes. The main constituents of the *S. hortensis* (summer savory) essential oil in are the phenols, thymol (29.0%), carvacrol (26.5%), *γ*-terpinene (22.6%), *p*-cymene (9.3%) and other terpenoids (GULLUCE et al. 2003).

According to AZAZ et al. (2002) carvacrol (42.1–59.2%) was the main component in the oils of *Satureja icarica*, *S. pilosa* and *S. boissieri* and other major components were identified as *p*-cymene (8.1–35.5%) and borneol (4.5–6.3%). Chemical analysis revealed that there are significant qualitative and quantitative differences among the oils tested (Fig. 1).

Conversely, the oil of *S. cuneifolia* contained a low percentage of carvacrol and thymol compared with the oil of *S. montana* investigated. However, TÜMEN et al. (1998) revealed the occurrence of carvacrol-rich (26–72%) and thymol-rich (22–58%) oils in *Satureja cuneifolia.* It is interesting that Croatian *S. cuneifolia* is relatively rich in the sesquiterpenes *β*-cubebene, spathulenol, *β*-caryophyllene, and perhaps exists in different chemotypes during ontogenesis (SKOČIBUŠIĆ et al. 2004). The other components are monoterpene hydrocarbons such as limonene and *α*-pinene (Tab.1). Among other things the species from Croatia (which have relatively low percentages of both thymol and carvacrol) appeared to be very different from those collected in the Mediterranean parts of Turkey (AKGÜL et al. 1999).

Populations of *S. montana* and *S. cuneifolia* growing wild on Biokovo mountain in similar environmental conditions, exhibited significant differences in essential oil yield and composition, this difference being mainly attributed to genetic diversity. The essential oils of both investigated plants show antimicrobial activity against a wide range of multidrug resistant bacteria including resistant fungal strains. Antibacterial activities have been attributed to the presence of active volatile components. Essential oils rich in phenolic compounds are widely reported to possess high levels of antimicrobial activity (PANIZI et al., 1993; SIVROPOULOU et al., 1996). The essential oil of *S. montana* showed greater antimicrobial activity against all the tested strains than the oil of *S. cuneifolia* (Tab. 2). Taking into account the chemical composition of the oil, it is clear that there is a relationship between the high activity of the carvacrol-type oil and the presence of phenolic components, such as carvacrol and its precursors, *γ*-terpinene and *p*-cymene which has been confirmed and extended in the present studies (SKOČIBUŠIĆ and BEZIĆ 2003, SKOČIBUŠIĆ and BEZIĆ 2004). Carvacrol is an isomer of thymol and has been shown to cause damage in *B. cereus* cells, finally leading to its death ULTEE and SMID (2001). Thymol disintegrated the outer membrane and increased the permeability of the cytoplasmic membrane to ATP of *E. coli* and *S. typhimurium* cells (HELANDER et al. 1998). However, the presence of the hydroxyl group seems to be more important for the antimicrobial activities of these compounds than the ability to expand and consequently to destabilize the bacterial membrane. Thymol and *p*-cymene have almost the same structure, although cymene lacks the hydroxyl group present in thymol that results in an increase of the antibacterial activity. However, it has to be considered that minor components, such as thymol and *α*-terpineol, contribute to the

antimicrobial activity of the oil (COSENTINO et al. 1999). It has frequently been reported that Gram-positive bacteria are more susceptible to essential oils than Gram-negative bacteria (SMITH-PALMER et al. 1998). Confirming previous reports, it was found that the strength and spectrum of activity varied between investigated *Satureja* species and Gram-positive bacteria were generally more sensitive to the effects of the oils. The tolerance of Gram-negative bacteria to essential oils has been ascribed to the presence of a hydrophilic outer membrane that blocked the penetration of hydrophobic components of oil to the target cell membrane (MANN et al. 2000). The oil of *S. cuneifolia* contained a higher percentage of sesquiterpenes then the monoterpenes and showed moderately antimicrobial activities (Tabs. 1, 2). In addition, it is evident that these antimicrobial activities do not result only from monoterpenic hydrocarbons such as carvacrol and thymol.

In conclusion, the findings of the present study indicate that savory oils, in addition to other properties, have a potential as a topical antibacterial agent against important human pathogens, particularly against methicillin-resistant *Staphylococcus aureus* (MRSA). Further studies are needed to evaluate the in vivo potential of both oils in animal models.

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