Arch. Biol. Sci., Belgrade, 63 (4), 1173-1179, 2011 DOI:10.2298/ABS1104173D

EFFECT OF VEGETATION CYCLE ON CHEMICAL CONTENT AND ANTIBACTERIAL ACTIVITY OF *SATUREJA MONTANA* L.

BILJANA DAMJANOVIĆ-VRATNICA[1*], A. PEROVIĆ[2], DANIJELA ŠUKOVIĆ[3] and SVETLANA PEROVIĆ[2*]

[1]*Faculty of Metallurgy and Technology, University of Montenegro,* 81000 Podgorica, Montenegro
[2]*Faculty of Natural Sciences and Mathematics, University of Montenegro,* 81000 Podgorica, Montenegro
[3]*Centre for Ecotoxicological Research of Montenegro,* 81000 Podgorica, Montenegro

Abstract - Effect of vegetation cycle on phytochemical composition of the essential oil obtained from the aerial parts of wild-growing winter savory (*Satureja montana* L.) from Montenegro was analysed by GC-MS and its antibacterial activity tested at different oil concentrations. A total of 36 and 34 constituents were identified in the hydrodistilled oil obtained from herb before flowering and during flowering stage, with major components: thymol (37,36% and 27,68%), carvacrol (15,47% and 4,40%), γ-terpinene (11,75% and 8.66%) and p-cymene (7,86% and 31, 37%), respectively. The gained results revealed that essential oil of *S. montana* has rather significant antibacterial activity against chosen bacteria *Staphylococcus aureus*, *Escherichia coli* and *Bacillus subtilis*. Also, it was found that vegetation cycle affects the chemical composition and antibacterial activity of savory essential oil. Essential oil gained prior to herb flowering period showed stronger antibacterial activity in comparison with the oil gained during herb flowering.

Key words: Satureja montana L., vegetation cycle, essential oil, chemical composition, antibacterial activity

UDC 582.35/.99

INTRODUCTION

Plants produce an enormous array of secondary metabolites, and it is commonly accepted that a significant part of its 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 & Reeves, 2002).

Winter savory (*Satureja montana*) is a perennial herb in the family Lamiaceae, native to warm temperate regions of southern Europe. It is a semi-evergreen, semi-woody subshrub growing to over 20-30 cm tall. The whole herb is mildly antiseptic, aromatic, carminative, digestive, mildly expectorant and stomachic. It is well known as aromatic and medicinal herb while its essential oil is used in the food industry as a flavoring agent, in the making liqueurs and in perfumery. The positive effects of savory on human health are attributed to its active constituents such as essential oil, triterpenes, flavonoids and rosmarinic acid (Bezic et al., 2005).

Essential oils are highly concentrated volatile, aromatic essences of aromatic plants and they have been known since ancient times to possess biological activity. They have a complex composition, containing from a few dozen to several hundred constituents, especially hydrocarbons and oxygenated compounds which are highly odoriferous (Rios & Recio, 2005). The use of essential oils has a potential interest in the pharmacological field and in food preservation technology for its antimicrobial effects (Ciani et al., 2000).

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 & Baratta, 2000).

The objectives of this study were: (i) the extraction of the essential oil from herba of wild-growing winter savory from Montenegro during different vegetation stage, (ii) the identification and quantification of the components of the obtained essential oils and (iii) the determination and comparison of their antibacterial activity against chosen Gram-positive and Gram-negative bacteria by disk diffusion method.

MATERIAL AND METHODS

Plant Material

Many factors can influence the amount of essential oil in aromatic herbs, such as climate and environmental conditions and age of plants. To avoid this influence in the present work fresh leaves of *S. montana* were collected manually from the same collection site in the Podgorica region (central part of Montenegro), in summer 2008, prior the flowering (in May) and during flowering (in August). After flowering (in October), the plant material was not collected, because, in this stage of development, the plant has very small leaves. Voucher specimens were deposited in Herbarium, Department of Biology, Faculty of Natural Sciences and Mathematics, University of Montenegro, voucher numbers: S809/08 and S905/08.

The initial water inherent in the herb leaves found to be 10.7 % (w/w) using a Dean and Stark apparatus with n-heptane as the reflux solvent. Herb material was milled in a coffee mill and, after sieving, sample with a mean particle diameter size of 0.8 mm was obtained. A prepared batch was kept in an airtight resalable polypropylene bag and stored at 8°C for maximum 2 days before use, in order to avoid losses of volatile compounds.

Herb material (40g) was submitted to hydrodistillation in a Clevenger-type apparatus for 2 hours according to Yugoslav Pharmacopoeia IV. The obtained oil was dried over anhydrous sodium sulphate, measured, poured in hermetically sealed dark-glass containers and stored in a freezer at -4°C until analyzed by GC-MS.

Gas Chromatography-Mass Spectrometry

The GC-MS analyses were carried out using a Shimadzu 2010+ gas chromatograph-mass spectrometer equipped with a ZB-5ms (30m x 0,25mm x 0,25μm) capillary column. The column temperature was programmed from 35°C (5 min) to 300°C at 5°C/min. The injection port temperature was 260°C while the interface temperature was 305°C. The samples of oil were injected by splitting and the split ratio was adjusted to 1:100. Helium was used as the carrier gas at a flow rate of 1.2 ml/min and 61.8 kPa inlet pressure. The MS conditions were: the ionisation voltage 70 eV, scanning interval 1.5 s, detector voltage 1.0 kV and *m/z* range 40 - 500. The components were identified by comparing their mass spectral data with those in the WILEY229 and the NIST107 mass spectra libraries, as well as by comparison of the fragmentation patterns of the mass spectra with those reported in the literature and whenever possible, by co-injection with authentic standards (Fluka, Great Britain).

Disc diffusion method

The antimicrobial activity of the oil was evaluated by the disc diffusion method, using Mueller–Hinton agar for bacteria, by determination of inhibition zones. Two Gram-positive (*Staphylococcus aureus* and *Bacillus subtilis*) and Gram-negative (*Escherichia coli*) bacteria were used. Test microorganisms were obtained from Clinical Department of National Institute in Montenegro. Ceftalexine, erythromycin and nalidixine acid were used as positive standards in order to control the sensitivity of the microorganisms. A 100 µl suspension of any tested bacteria containing about 10^8cells/ml spreaded on Mueller Hinton Agar (MHA) mediums using sterile swabs. Sterile blank disk 6.0 mm in diameter were impregnated with 1, 3 and 5 µl essential oil/disk and finally placed on the agar surface. Plates were incubated at 37°C for 24 hours and then the inhibition zones were measured in diameters. Disks soaked in the solvent (5% DMSO) were used as a negative control.

All tests of inhibitory activity were carried out in duplicate and the developing inhibition zones were compared with those of reference disks.

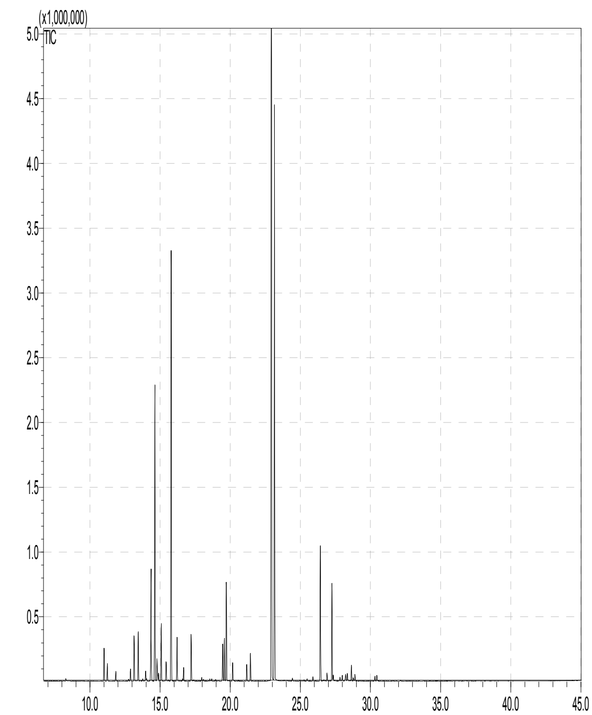
RESULTS AND DISCUSSION

Chemical composition of the essential oil

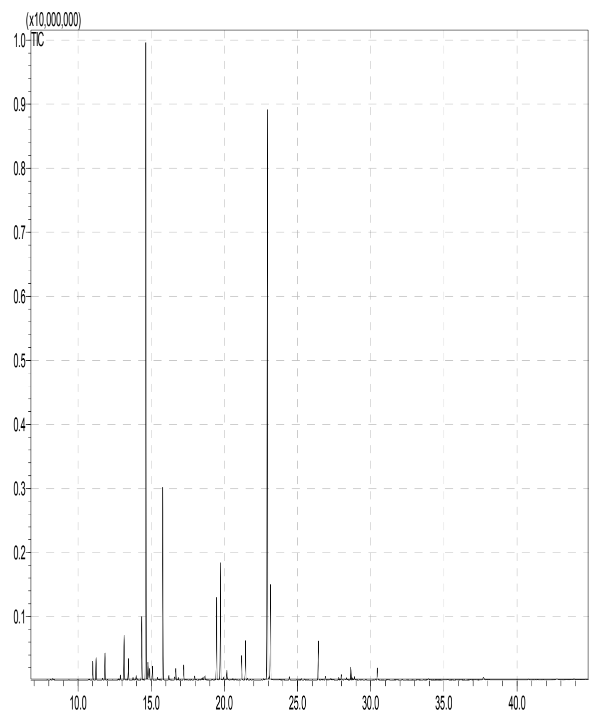
The yield of savory essential oil was higher in May (1,9%, w/w), while with the growing of the plant yield decreased in August (1,1%, w/w). The chemical composition of the hydrodistilled essential oil is shown in Table 1. A total of 36 and 34 constituents were identified in the hydrodistilled oil obtained from herb before flowering (in May) and during flowering stage (in August), with major components: thymol (37,36% and 27,68%), carvacrol (15,47% and 4,40%), γ-terpinene (11,75% and 8.66%) and p-cymene (7,86% and 31, 37%), respectively.

The amount of several minor compounds in the essential oil was also significant: α-terpinene (3.00% and 2.79%), caryophyllene (3.96% and 1.83%) and borneol (1.04% and 3.79%), respectively.

The essential oil of winter savory before and during flowering stage consisted chiefly of oxygenated monoterpenes (60.93% and 45.36%), monoterpene



1(a)



1(b)

Fig. 1. Chromatograms of the hydrodistillated S. montana essential oil (a) prior to flowering, (b) in flowering

Table 1. Relative percentage composition of *Satureja montana* L. essential oil depending on the stage of plant development

Compound	Retention time prior flowering	Prior flowering	Retention time in flowering	In flowering
		% Composition		% Composition
2-heksenal	n.d.	n.d.	8.265	0.08
α-tujene	11.002	0.90	10.999	0.85
α-pinene	11.236	0.48	11.232	1.02
Camphene	11.842	0.27	11.838	1.28
Sabinene	12.754	0.04	12.751	0.05
β-pinene	12.887	0.35	12.884	0.24
1-oktan-3-ol	13.144	1.19	13.142	1.90
β-myrcene	13.439	1.31	13.436	0.94
α-phellandrene	13.969	0.27	13.966	0.24
α-terpinene	14.349	3.00	14.348	2.79
p-cymene	14.625	7.86	14.629	31.37
cis-ocimene	15.080	1.51	15.077	0.60
β-trans-ocimene	15.428	0.51	15.425	0.12
γ-terpinene	15.786	11.75	15.785	8.66
trans-sabinen-hidrate	16.206	1.20	16.204	0.21
α-terpinolene	16.678	0.36	16.675	0.53
Linalool	17.211	1.31	17.209	0.71
Borneol	19.461	1.04	19.459	3.79
Pinocamphone	19.581	1.16	n.d.	n.d.
terpinen-4-ol	19.720	2.75	19.717	5.24
α-terpineole	20.172	0.51	20.169	0.48
thymol-methyl-ethar	21.175	0.43	21.171	1.14
carvacrol-methyl-ethar	21.439	0.72	21.435	1.75
Thymol	22.933	37.36	22.927	27.68
Carvacrole	23.152	15.47	23.143	4.40
timol-acetate	24.433	0.07	24.429	0.17
α-copaene	25.258	0.03	25.256	0.05
eugenol-methyl-ethar	25.895	0.11	n.d.	n.d.
Caryophyllene	26.423	3.96	26.418	1.83
Aromadendrene	26.900	0.23	26.896	0.18
β-farnesene	27.252	2.60	27.250	0.05
germacrene D	27.986	0.15	27.984	0.25
Ledene	28.225	0.18	n.d.	n.d.
β-bisabolene	28.642	0.43	28.641	0.59
δ-cadinene	28.894	0.19	28.891	0.15
Spatulenol	30.318	0.13	30.316	0.04
caryophyllene-oxide	30.453	0.17	30.454	0.62

*n.d.- not detected

hydrocarbons (31.00% and 50.8%), while sesquiterpene hydrocarbons and oxygenated sesquiterpenes were only present in a relatively small percentage (8.07% and 3.76%), respectively.

Characteristic chromatographs of the hydrodistillated *S. montana* essential oil at isolated from the herb at different vegetation stage, prior to flowering and during flowering, are shown in Figure 1.

The higher content of thymol was identified in young plant (May) as well as carvacrol, γ-terpinene, α-terpinene, caryophyllene, linalool and β-pharnesene, which content decrease with plant maturation (Figure 2).

The content of p-cymene increased four times with the maturation of the plant (August) as well as terpinen-4-ol and borneol. The content of thymol methyl ether and carvacrol methyl ether increase with maturation of the plant, while content of eugenol-methyl-ether identified only in young plant. The content of thymol and carvacrol in savory is variable and depends on the origin and vegetative stage of the plant (Slavkovska et al., 2001). 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 (Skocibusic & Bezic, 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 (Kustrak et. al., 1996). A review of the published literature reveals that the essential oil composition of *Satureja montana* shows large variations in the relative concentration of major components: carvacrol (5-96%), linalool (1-62%), γ-terpinene (1-31%) and p-cymene (3-27%), arising from the existence of different chemotypes.

Similar results of essential oil analysis for this plant was reported by (Mastelic & Jerkovic, 2003) for winter-savory from Croatia (near Sinj) with 30,88% of thymol and 3,81 of carvacrol in August while (Bezic et al., 2005) in contrary, reported that

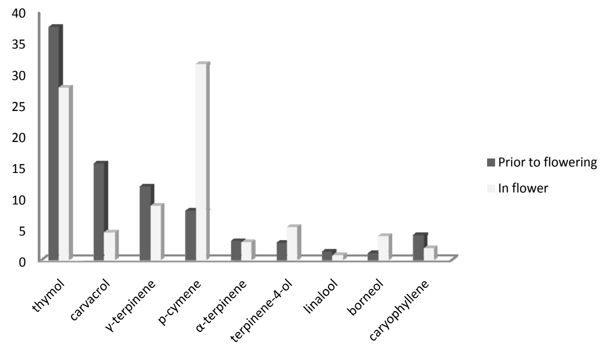


Fig. 2. Comparison of the main compounds identified in essential oil of *Satureja montana* from different vegetation stage

essential oil from Croatia (from Biokovo) has high content of carvacrol (45,7%) and small content of thymol (3,9%). Similar results of essential oil analysis for this plant, but from different locality (also in Croatia) were reported by (Milos et al., 2001) who identified carvacrol as main components of the oil. It is well known that environmental conditions, stage of development and especially chemotype of plant can affect composition and content of essential oil.

Antibacterial activity

The antibacterial activity assay for *S. montana* essential oil, as summarized in Table 2, showed that different microorganisms tested had different susceptivity to the same essential oil. Savory oil, isolated from the herb before flowering period, was very potent against all chosen microorganisms at all concentration while essential oil isolated from the herb during flowering period showed activity at all concentration only against medically important pathogen *Staphylococcus aureus*. This oil had significant effect on another medically important pathogen *E. coli* at lower oil concentration (1μL and 3μL) while the activity was rather high at concentration of 5μL (29.5 mm). Additionally this oil showed no effect on *B. subtilis* at lowest oil concentration (1μL) while the activity was rather high at concentration of 3 μL and 5μL (27 mm and 39 mm).

Table 2. Antimicrobial activity of the Satureja montana L. essential oil and some standard antibiotics

	S. montana essential oil (µl)						Standard antibiotics (µg)		
	Before flowering			During flowering			NK	CFK	ERM
	1	3	5	1	3	5	30	30	15
	Inhibition zone (mm)								
Staphylococcus aureus	37	45	55	24	39	41	-	25	33
Escherichia coli	25	45	54	-	12	30	30	19	26
Bacillus subtilis	28	43	53	-	27	39	22	27	34

*(-) not active; NK: nalidixine acid; CFK: ceftalexine; ERM: erythromycin.

The essential oil activities against tested microorganisms were increased with increased amount of investigated essential oil.

High antimicrobial activity of savory essential oil is due to the presence of phenolic components, such as thymol and its isomer carvacrol as well as its precursors, γ-terpinene and p-cymene which activities has been confirmed (Slavkovska et al., 2001). Thymol disintegrates the outer membrane and increase the permeability of the cytoplasmic membrane to ATP of E. coli (Helander et al., 1998). Carvacrol is an isomer of thymol and has been shown to cause damage in *B. subtilis* cells (Ultee & Smid, 2001). 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. Thus, thymol and p-cymene have almost same structure, although cymene lacks the hydroxyl group present in thymol that results in an increase of the antibacterial activity.

The antimicrobial activity of the savory essential oil could, in part, be associated with borneol which antimicrobial activity was previously reported (Tabanca et al., 2001; Knoblach et al., 1989). Also, a number of researchers had shown the components present in lower amount in savory essential oil such as α-terpineol, terpinen-4-ol and p-cymene could also contribute to the antimicrobial activity of the oil. Terpinen-4-ol was concluded to be responsible for bacteriostatic activity against several microorganisms, especially against bacteria that infect the urinary tract (Barel et al., 1991); p-cymene and α-terpineol had been also reported to be antibacterial (Cosentino et al., 1999). In fact, it was also possible that the components in lower percentage might be involved in some type of synergism with the other active compounds.

Although the antibacterial activity of essential oils from many herb species has been extensively surveyed (Rios & Recio, 2005) their antimicrobial mechanism has not been reported in great details. Since the active antimicrobial compounds of essential oils are terpenes and phenolics in nature, it seems reasonable that their mode of action might be similar to that of other phenolic compounds (Shunying et al., 2005). Individual essential oil contains complex mixtures of such compounds; however, little is known about the effect of interaction between individual constituents on antimicrobial activity. Interactions between constituents may lead to additive, synergistic or antagonistic effects (Delaquis et al., 2002).

CONCLUSIONS

The gained results revealed that essential oil of *S. montana* has very significant antibacterial activity against chosen bacteria. These results confirm the potential use of *S. montana* essential oil in the pharmaceutical industry, and may be useful as an alternative antibacterial agent in the folk medicine. Also, it was found that herb vegetation cycle notably affects the yield, chemical composition and antibacterial activity of *S. montana* essential oil. Essential oil gained before flowering period showed stronger antibacterial activity, probably due to the higher content of phenolic monoterpenes, thymol and carvacrol.

Acknowledgments - The authors would like to thank Mr. Vladimir Živković (Center for Ecotoxicological Research, Podgorica, Montenegro) for GC-MS analyses. This work was supported by funds from the Natural Research Project of Ministry of Science and Education, Government of Montenegro (Project No. 05/1-3-3368, 2008) and we gratefully acknowledged this fact.

REFERENCES

Barel, S., Segal, R., and J. Yashphe (1991). The antimicrobial activity of the essential oil from Achillea fragrantissima. J. *Ethnopharmacology* **33 (1/2)**, 187-191.

Bezic, N., Skocibusic, M., and V. Dunkic (2005). Phytochemical composition and antimicrobial activity of *Satureja montana* L. and *Satureja cuneifolia* Ten. essential oils. *Acta Bot. Croatica* **64** (2), 313–322.

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

Cosentino, S., Tuberoso, C.I.G., Pisano, B., Satta, M., Mascia, V., Arzedi, E., and F. Palmas (1999). In-vitro antimicrobial activity and chemical composition of Sardinian *Thymus* essential oils. *Lett. Appl. Microbiology* **29**, 130–135.

Delaquis, P.J., Stanich, K, Girard, B., and G. Mazza (2002). Antimicrobial activity of individual and mixed fractions of dill, cilanto, coriander and eucalyptus essential oils. *Int. J. Food Microbiology* **74 (1),** 101-109.

Dixon, R. A (2001). Natural products and plant disease resistance. *Nature* **411**, 843-847

Helander, I.M., Alakomi, H.L., Latva-Kala, K., Mattil-Sandho, M., Smid, I., Gorris, E.J., L.G.M., and A.V. Wright (1998). Characterization of the action of selected essential oil components on Gram-negative bacteria. *J. Agric. Food Chemistry* **46 (9)**, 3590-3595.

Hunter, P.A., and *D.S. Reeves,* (2002). The current status of surveillance of resistance tp antimicrobial agents: reports on a meeting. *J. Antimicrob. Chemotherapy* **49**, 17-23.

Knoblach, K., Pauli, A., Iberi, B., Wegand, H., and *N. Weis* (1989). Antibacterial and antifungal properties of essential oil components. *J. Essent Oil Research* **1,** 119-128.

Kustrak, D., Kuftinec, J., Blazevic, N., and M. Maffei (1996). Comparison of the essential oil composition of two subspecies of *Satureja montana. J. Essent. Oil Research* **11**, 7-13.

Mastelic, J., and *I. Jerkovic* (2003). Gas chromatography-mass spectrometry analysis of free and glycoconjugated aroma compounds of seasonally collected *Satureja montana* L. *Food Chemistry* **80**, 135-140.

Milos, M., Radonic, A., Bezic.N., and *V. Dunkic* (2001). Localities and seasonal variations in the chemical composition of essential oils of *Satureja montana* L. and S. Cuneifolis Ten. *Flavour & Fragrance Journal* **16**, 157-160.

Rios, J.L., and *M.C. Recio,* (2005). Medicinal plants and antimicrobial activity. *Journal of Ethnopharmacology* **100 (1-2)**, 80–84.

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

Shunying, Z., Yang, Y., Huaidong, Y., Yue, Y., and *Z. Guolin* (2005). Chemical composition and antimicrobial activity of the essential oils of *Chrysanthemum indicum.* J *Ethnopharmacology* **96 (1-2)**, 151-158.

Skocibusic, M., and *N. Bezic,* (2004). Chemical composition and antimicrobial variability of *Satureja montana* L. essential oils in the ontogenesis. *J. Essent. Oil Research* **16**, 387-391.

Slavkovska, V., Jancic, R., Bojovic, S., Milosavljavic., S., and *D. Djokovic* (2001). Variability of essential oils of *Satureja montana* L. and *Satureja kitaibelii* Wierzb. Ex Heuff. from the central part of Balkan Peninsula. *Phytochemistry* **57**, 71-76.

Tabanca, N., Kirimer, N., Demirci, B., Demirci, F., and *K.H.C. Baser* (2001). Composition and antimicrobial activity of the essential oils of *Micromeria cristata* subsp. *phrygia* and the enantiomeric distribution of borneol. *J. Agric. Food Chemistry* **49 (9)**, 4300-4303.

Ultee, A., and *E.J. Smid* (2001). Influence of carvacrol on growth and toxin production by Bacillus cereus. *Int. J. Food Microbiology* **564**, 373-378.