ACTA SCIENTIARUM POLONORUM

Acta Sci. Pol. Hortorum Cultus, 17(5) 2018, 157–165

www.acta.media.pl 1644-0692 e-ISSN 2545-1405 DOI: 10.24326/asphc.2018.5.14

ORIGINAL PAPER

Accepted: 27.03.2018

COMPARISION OF THE COMPOSITIONS, ANTIMICROBIAL AND ANTIOXIDANT ACTIVITIES OF ESSENTIAL OILS FROM THE ENDEMIC SPECIES *Thymus malyi* Ronninger AND *Thymus lykae* Degen et Jav.

Marija Marin[1] ✉, Miroslav Novaković [2], Vele Tešević [3], Stoimir Kolarević[1], Branka Vuković-Gačić[1]

[1] Faculty of Biology, University of Belgrade, Studentski trg 16, 11000 Belgrade, Serbia

[2] Institute of Chemistry, Technology and Metallurgy, University of Belgrade, Njegoševa 12, 11000 Belgrade, Serbia

[3] Faculty of Chemistry, University of Belgrade, Studentski trg 16, 11000 Belgrade, Serbia

ABSTRACT

In this study, we compared the compositions, antimicrobial and potential antioxidant activities of essential oils of two *Thymus* endemic, wild-growing species – *T. malyi* Ronninger and *T. lykae* Degen et Jav. No information about its composition and biological activities of oils has been reported to the present day. Forty three compounds were identified from *T. malyi* and major component was α-pinene (26.4%). In the case of *T. lykae*, forty four compounds were identified, with geranyl acetate (35.1%) as the main component. Antioxidant activities of the oils were evaluated using DPPH assay. The antimicrobial effect of essential oils were tested against Gram-negative, Gram-positive bacteria, and two fungi. Results indicated higher antibacterial activity of the essential oils of *T. lykae* in comparison with *T. malyi*, while for antifungal activity, it was reverse. The essential oils from *T. malyi* and *T. lykae* possess antioxidant and antimicrobial activities and could be used as a potential source of natural antioxidants for the food industry.

Key words: antimicrobial activity, antioxidant activity, essential oil, GC-MS, *Thymus lykae*, *Thymus malyi*

INTRODUCTION

The genus *Thymus* L. (Lamiaceae) includes about 215 species of herbaceous perennials and sub-shrubs, and its species are widespread in the arid parts of the Mediterranean region [Horwath et al. 2008]. These species are characterized by high content of essential oils (EOs), considering them as aromatic herbs with potential health benefits [Adelheid et al. 2005].

The composition of their EOs has been studied earlier [Lee et al. 2005, Slavkovska et al. 2006, Rota et al. 2008]. *Thymus* L. species are important due to their wide use in traditional medicine, as an antiseptic agent in many pharmaceutical preparations. It is also popular for antioxidant, digestion-stimulating, hypolipidemic, anti-inflammatory and anticarcinogenic activities and as a flavoring agent for many kinds of food products [Viuda-Martos et al. 2011].

It has significant antibacterial and antifungal properties [Kizil 2006, Saad et al. 2010], which are

✉ majamarin@bio.bg.ac.rs

related to the rich content of monoterpenes, in particular the phenolic compounds, thymol and carvacrol, accompanied by a range of other, more or less biologically active compounds, including eugenol, p-cymene, terpinene, linalool, geraniol, and borneol [Nedorostova et al. 2008].

Thymus malyi Ronninger and *Thymus lykae* Degen et Jav. are endemic species from the central Balkan peninsula, which grow on serpentine hills, spread on dry, rocky, sunny hillsides, from the lowland to the mountains. *Thymus malyi* is low creeping shrub, with ovate leaves and capitate inflorescence with purple corolla, while *Thymus lykae* is small semi-shrub, also with ovate leaves and purple corolla.

Natural antioxidants have been attracted a big attention as food additives in order to provide protection against oxidative degradation of foods by free radicals. In this work, the 2,2-diphenyl-1-picryl hydrazyl (DPPH) radical-scavenging method was used to estimate the antioxidant activity of the oils.

In this work, antimicrobial properties of EOs of two endemic species of genus *Thymus*, *T. malyi* and *T. lykae*, were investigated against Gram-negative, Gram-positive bacteria, and fungi. The antimicrobial activity of *Thymus* EOs was analyzed using Microtitre plate-based resazurin assay for detection of minimum inhibitory concentration (MIC) for bacteria [Sarker et al. 2007] and broth Macrodilution Trypane blue assay to determine the minimum inhibitory concentration (MIC) for fungi.

In contrast to numerous studies of the EOs of *Thymus* species [Miguel et al. 2004, Sarikurkcu et al. 2010], there are no data of the composition and activities of the oils of wild *T. malyi* and *T. lykae*, therefore the aim of this study was to determine the chemical composition, potential antioxidant and antimicrobial properties of the EOs of these aromatic herbs, both endemic and both from Serbia.

MATERIAL AND METHODS

Plant material

Aerial parts of wild *T. malyi* and *T. lykae* were collected at the flowering stage in June 2010 in Studenica and Maglič (Serbia), respectively. A voucher specimen (BEOU 16623) of *T. malyi* and (BEOU 16622) of *T. lykae* has been deposited at the herbarium of the Institute of Botany and Botanical Garden "Jevremovac", Faculty of Biology, University of Belgrade, Serbia.

Isolation of essential oils. Essential oils were isolated from fresh plant material collected as detailed above. Air-dried aerial parts of plant material were cut up into small pieces and subjected to hydrodistillation for 2 h using Clevenger apparatus to obtain the EO.

Gas chromatography–mass spectrometry analysis of the Eos. Gas chromatography–mass spectrometry (GC-MS) and gas chromatography–flame ionization detector (GC-FID) analysis (EI) were performed using an Agilent 7890A GC system equipped with a 5975C inert XL EI/CI MSD and a FID detector connected by capillary flow technology (Agilent Technologies, Santa Clara, California, USA). The separation was achieved using an Agilent HP-5MSI fused silica capillary column HP-5, 5% phenyl methyl siloxane (30 m × 0.25 mm i.d., 0.25 μm film thickness). GC oven temperature was programmed from 60°C to 285°C at a rate of 4°C/min. Helium was used as a carrier gas; inlet pressure was 25 kPa; linear velocity: 1 ml/min at 210°C. Injector temperature: 250°C. Injection mode: splitless. MS scan conditions: source temperature 200°C; interface temperature 250°C; EI energy 70 eV; mass scan range 40–550 amu. Identification of the EOs components was carried out on the basis of retention indices and the comparison with reference spectra (Wiley and NIST databases). The quantitative data are obtained from the electronic integration of the GC-FID peak areas.

Antioxidant activity – DPPH assay. The antioxidant activity of the *T. malyi* and *T. lykae* EOs were measured in terms of hydrogen donating or radical scavenging ability, using the stable radical, 2,2-diphenyl-1-picryl hydrazil – DPPH. Concentrations of the EOs, experimentally found to satisfy linear dependence of absorbance from concentration of remaining DPPH, were 15, 50, 100 and 200 μl/ml for *T. malyi*, and 20, 50, 100 and 200 μl/ml for *T. lykae*. Volume of 200 μl was mixed with 1800 μl of 100 μM methanolic solution of DPPH. The decrease in absorbance at 517 nm after 30 min reaction in the dark was determined by UV-VIS spectropho-

tometer Cintra 40 (GBC Scientific Equipment, Melbourne, Australia) for all samples (A_u). The absorbance of the DPPH radical without antioxidant (with 200 µl of pure methanol) was the control (A_c). Special care was taken to minimize the loss of free radical activity of the DPPH radical stock solution (keeping in the dark and cooling). All determinations were performed in triplicate [Marin et al. 2012].

The percentage of inhibition of the DPPH radical by samples was calculated according to the equation:

$$\text{DPPH radical scavenging (\%)} = A_c - A_u / A_c \cdot 100$$

where A_c is the absorbance of the control and A_u is the absorbance of the remaining DPPH radical after reaction with antioxidant for 30 min. EC50 value was obtained from the graph A = f c(EO) and represents concentration of the EO that decreases the absorbance of remaining DPPH for 50%. DPPH scavenging activity (EC_{50} value) was also determined for butylated hydroxy toluene (BHT) that was used as known artificial antioxidant.

Determination of antimicrobial activity. Determination of the minimum inhibitory concentration was performed by micro-dilution assay for bacteria and macro-dilution assay for fungi. The applied procedures were performed following the instructions of [CLSI 2015]. The MIC values were expressed as µl/ml for EOs and µg/ml for antibiotics.

Bacteria and media. Antibacterial activity of the EOs was tested against five strains of Gram-negative bacteria (*Escherichia coli* SY252, *Escherichia coli* ATCC 25922, *Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 27853, *Burkholderia cepacia* ATCC 25416) and five strains of Gram-positive bacteria (*Enterococcus fecalis* ATCC 29212, *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 12228, *Bacillus subtilis* ATCC 6633, *Listeria innocua* ATCC 33090). Selected bacterial strains were inoculated in Mueller-Hinton Broth (MHB, Biomedics, Madrid, Spain) and cultivated 24 h at 37°C.

Determination of minimum inhibitory concentration for bacteria in micro-dilution assay. Minimum inhibitory concentrations (MICs) are defined as the lowest concentration of antimicrobial substance that will inhibit the visible growth without bactericidal effect. MICs are considered as the 'gold standard' for determining the susceptibility of organisms to antimicrobials. We determined MICs of *Thymus* EOs for tested bacteria using microtitre plate-based antibacterial assay. In sterile 96-well plates, the gradient of *Thymus* EOs concentration was made. In the first row, the mixture of 90 µl of MHB and 10 µl EO was added, and 50 µl of MHB was added to the remaining wells. The serial dilutions were performed by pipetting 50 µl of the EO from row to row in serially decreasing concentrations. To each row, 10 µl of indicator resazurin water solution was added (0.675 mg per ml resazurin sodium salt, TCI Europe nv, Belgium). Finally, 10 µl of bacterial suspension (10^7 CFU/ml) and 30 µl MHB volume was added to each well. Controls included: negative control (MHB and resazurin), bacteria growth control (MHB, resazurin, bacteria) and positive control (serial gradient of antibiotic tetracycline, first concentration was 800 µg/ml). The plates were wrapped with parafilm to prevent dehydration of plates and incubated at 37°C for 24 h. Each experiment was repeated in triplicate. The results were analyzed by observing the changes in resazurin color. The resazurin is blue oxidation-reduction indicator that was reduced to resorufin by oxidoreductases within viable cells and became pink. The color change was then assessed visually. Any color changes from purple to pink or colorless were recorded as positive. The lowest Eos' concentration with no change of resazurin color was taken as the MIC value, which was represented as average of three values. A confirmation that was obtained due to antibacterial activities are caused by bacteriostatic effect, 10 µl from each well with suspected MIC were spread, in triplicate, on MHB agar (MHB whit 1.5% of agar, Biomedics, Madrid, Spain) and incubated for 24 h at 37°C. Appearance of bacterial growth was considered as positive test result.

Fungi and media. Antifungal activity of EOs was tested against *Candida albicans* ATCC 10231 and *Saccharomyces cerevisiae* D7. Selected fungi strains were inoculated in yeast extract peptone dextrose (YPD) Broth (1% Peptone, 0.5% Yeas extract, 2% Glucose, Difco & co, Corpus Christi, TX, USA) and cultivated for 24 h at 30°C. An antimicrobial activity of *Thymus* EOs was analyzed using Microtitre plate-

based resazurin assay for detection of minimum inhibitory concentration (MIC) for bacteria [Sarker et al. 2007] and broth Macrodilution Trypane blue assay to determine the minimum inhibitory concentration (MIC) for fungi.

Determination of minimum inhibitory concentration for fungi in macro-dilution assay. Determination of MIC for fungi was performed in 2 ml-microtubes. In the first tube, the mixture of 1 ml YPD Broth and 0.01ml of EO was added. The volume of 0.5 ml YPD Broth was added to seven test tubes and serial dilutions were performed by pipetting 0.5 ml of the EO from first tube in serially decreasing concentrations. Finally, 0.1 ml of cell suspension *colony-forming unit* (10^6 CFU/ml) and 0.4 ml YPD Broth were added. The sets of controls included negative control (YPD Broth), positive control (serial gradient of antimycotic-cyclopiroxolamine, first concentration was 0.2 μg/ml) and cell control (YPD Broth and cells suspension). The CFU per ml in cell control culture was counted before incubation and these values were MICs (MIC concentrations of EO will inhibit a cell growth indicating that the CFU/ml should not be significantly changed after incubation period). Cultures were incubated in triplicate at 30°C for 24 h. Each experiment was repeated three times. The viability of fungi was analyzed by staining of 10 μl cell suspension with 10 μl 0.25% solution of Trypan blue (Trypan blue; TCI Europe nv, Belgium) in a *hemocytometer*; cells stained blue were considered non-viable. MIC was confirmed by resazurin assay. After incubation period, 90 μl was sampled from each microtube and transferred into wells of 96-well plate, 10 μl of resazurin solution was added into each well and plates were incubated for 3 h at 30°C.

RESULTS AND DISCUSSION

This is the first report on the analysis of EOs chemical composition of *T. malyi* and *T. lykae*, two endemic species growing wild in Serbia. Chemical composition of *T. malyi* and *T. lykae* EOs were investigated using GC-MS analysis. The EO of *T. malyi* was obtained in the yield of 0.10%. The oil was yellow in color with density of 0.888 g cm^{-3}. The EO of *T. lykae* was also yellow with density of 0.896 g cm^{-3} and the yield amounted to 0.40%.

Oils showed great differences in composition of the main components, not only between each other, but also to other Thymus species (Tab. 1). Forty-three compounds of *T. malyi* were identified, accounting for 98.6% of the total oil content. The major constituents were α-pinene (26.4%), myrcene (13.4%) and germacrene D (11.9%). For *T. lykae*, forty-four compounds were identified representing 99.4% of the oil. The main components were geranyl acetate (35.1%), followed by geraniol (24.2%) and α-terpinyl acetate (6.2%).

Table 1. Chemical composition of the essential oils of *Thymus malyi* and *Thymus lykae*

No.	Compound[a]	RI[b]	RI[c]	*T. malyi* (%)	*T. lykae* (%)
1	2	3	4	5	6
1	Tricyclene	908	904	0.1	0.1
2	α-Thujene	910	909	0.2	0.2
3	α-Pinene	919	939	26.4	0.6
4	Camphene	937	953	3.5	1.4
5	Sabinene	968	966	0.4	0.1
6	β-Pinene	972	980	2.8	–
7	1-Octen-3-ol	973	974	–	0.9
8	3-Octanone	981	972	0.1	0.1
9	Myrcene	990	988	13.4	0.5
10	3-Octanol	997	990	0.3	0.1
11	α-Phellandrene	1006	1002	0.3	–

1	2	3	4	5	6
12	α-Terpinene	1017	1016	0.1	0.3
13	*p*-Cymene	1021	1023	0.2	5.8
14	Limonene	1025	1026	6.8	0.3
15	1,8-Cineole	1028	1028	3.3	0.1
16	(*Z*)-*β*-Ocimene	1033	1033	0.4	–
17	(*E*)-*β*-Ocimene	1042	1044	0.4	–
18	γ-Terpinene	1054	1054	0.6	1.2
19	*cis*-Sabinene hydrate	1062	1062	0.1	0.1
20	Terpinolene	1081	1084	0.2	0.1
21	Linalol	1094	1093	0.4	0.9
22	1-Octen-2-yl-acetate	1112	1110	–	0.6
23	Camphor	1140	1140	6.4	0.9
24	Borneol	1164	1162	3.6	4.9
25	Terpinen- 4-ol	1177	1177	0.5	0.6
26	α-Terpineol	1189	1190	0.1	0.9
27	*cis*-Dihidrocarvone	1192	1191	0.2	–
28	*trans*-Dihidrocarvone	1195	1200	0.1	–
29	Nerol	1229	1227	–	2.1
30	Thymol, methyl ether	1234	1231	0.1	0.8
31	Neral	1240	1235	–	0.7
32	Carvacrol, metyl ether	1243	1240	0.2	0.4
33	Geraniol	1256	1249	–	24.2
34	Geranial	1272	1264	–	0.9
35	Bornyl acetate	1287	1282	1.1	1.6
36	Thymol	1299	1289	–	1.2
37	Carvacrol	1307	1305	0.5	0.2
38	α-Terpinyl acetate	1352	1346	–	6.2
39	Neryl acetate	1366	1359	–	0.5
40	*β*-Bourbonene	1384	1387	0.5	–
41	*β*-Cubebene	1386	1387	0.1	–
42	β-Elemene	1388	1389	0.1	–
43	Geranyl acetate	1392	1379	–	35.1
44	(*E*)-Caryophyllene	1422	1420	6.4	2.7
45	β-Copaene	1425	1430	0.1	–
46	α-Humulene	1450	1452	0.5	0.1
47	β-(*E*)-Farnestene	1459	1455	4.6	0.1
48	Germacrene D	1480	1480	11.9	0.6
49	Bicyclogermacrene	1494	1494	0.5	0.2
50	β-Bisabolene	1510	1512	0.2	0.1
51	γ-Cadinene	1518	1513	0.1	–
52	δ- Cadinene	1527	1522	0.2	–
53	Geranyl butanoate	1563	1562	–	0.2
54	Spathulenol	1577	1578	–	0.1
55	Caryophyllene oxide	1586	1585	0.6	0.6
56	Geranyl isovalerate	1589	1606	–	0.1
	Total identified (%)			98.6	99.4

[a] Compounds are listed in order of their elution from a HP-5 column
[b] Retention index on an HP-5column, experimentally determined using a homologous series of n-alkanes
[c] Relative retention index taken from Adams and/or NIST 05 for HP-5 capillary column

Table 2. Minimum inhibitory concentration of EO of *Thymus lykae* and *Thymus malyi*

	EO of *T. lykae*	EO of *T. malyi*	Tetracycline (μg/ml)[b] Cyclopiroxolamine (μg/ml)
Gram-negative$^-$ bacteria (μl/ml)			
Escherichia coli SY252	5.-0	nd[a]	10.-0
Escherichia coli ATCC 25922	5.-0	5.-0	40.-0
Escherichia coli ATCC 8739	5.-0	5.-0	40.-0
Pseudomonas aeruginosa ATCC 27853	1.-3	nd[a]	40.-0
Burkholderia cepacia ATCC 25416	1.-3	nd[a]	40.-0
Gram-positive$^+$ bacteria (μl/ml)			
Bacillus subtilis ATCC 6633	2.-5	nd[a]	40.-0
Enterococcus fecalis ATCC 29212	5.-0	nd[a]	40.-0
Staphylococcus aureus ATCC 25923	3	6	40.-0
Staphylococcus epidermidis ATCC 12228	6	2	20.-0
Listeria innocua ATCC 33090	5.-0	nd[a]	1.-3
Fungi (μl/ml)			
Candida albicans ATCC 10231	5.0	1.3	0.1
Saccharomyces cerevisiae D7	5.0	2.5	0.2

[a] Not detected. The EO concentration higher than 50 μl/ml were not tested
[b] Positive control for bacteria Tetracycline and for fungi Cyclopiroxolamin
Abbreviations: ATCC – American type culture collection, BHT – butylated hydroxy toluene, CFU – *colony-forming unit*, DPPH – 2,2-diphenyl--1-picryl hydrazyl, FID – flame ionization detector, GC – gas chromatography, MIC – minimal inhibitory concentration, MHB – Mueller-Hinton broth, YPD – yeast extract peptone dextrose.

Thymus species from the Mediterranean region mainly possesses thymol and carvacrol as major compounds. EO of *T. boveii* contained carvacrol (41.34%), *p*-cymene (19.80%), thymol (8.92%) and borneol (5.04%), while the major constituents of EO of *T. hyemalis* were carvacrol (30.25%), thymol (18.32%) and *p*-cymene (13.21%) [Tepe et al. 2011]. The paper of [Russo et al. 2013] reported that the essential oils isolated by hydro-distillation from the aerial parts of *T. capitatus* (L.) Hoffmanns and Link, Lamiaceae from Southern Italy, were characterized by high level of carvacrol, which was the main component (81.52%–78.40%). Similar results were found in the study of [Küçükbay et al. 2014], in which carvacrol was a dominant compound with a percentage 66.1% of the essential oil of *T pubescens* var. *pubescens.* The essential oil from *T. pulegioides* from Sicily were represented by thymol (21.8%), *p*-cymene (17.6%), *β*-caryophyllene (5.9%), while the essential oil of *T. pulegioides* from Campania was mainly constituted by phenolic compound, where thymol (26.3%) was the main one. In the case of *T. longicaulis* from Sicily [Martino et al. 2009], the composition was: thymol acetate (12.8%), τ-cadinol (9.2%), *p*-cymene (9.0%), γ-terpinene (5.5%) and linalool (3.5%), in the EO of *T. longicaulis* from Campania, the predominant compound was geraniol, which was absent in the oil of *T. longicaulis* from Sicily and in the oil of *T. pulegioides.* Major compounds of the EO of *T. numidicus* were thymol (54.1%), *p*-cymene (15,3%), linalool (5.4%) and carvacrol (3.8%) [Benayache et al. 2014].

Comparing our results with the literature ones, significant differences have been noticed. In general, Thyme EOs are characterized by a high amount of monoterpens; carvacrol occurs more frequently [Napoli et al. 2010, Economou et al. 2011]. EOs obtained

from *T. malyi* and *T. lykae* possess low concentrations of carvacrol and in EO of *T. malyi* thymol has not been found. Major components, which were found in *T. malyi* and *T. lykae*, were also recorded in some other species of *Thymus* genus, but at lower amounts [Ruiz-Navajas et al. 2012].

Since ancient times, spices were added to different types of food to improve its flavor. In most cases, it has been showed that these spices possess antioxidant activity as well, since they acted as food preservatives. EOs of *Thymus* species are well known because of their biological and pharmacological properties. The antioxidant activity of thyme EOs has been evaluated in previous study [Bounatirou et al. 2007].

The action of antioxidants on DPPH radical scavenging is due to their hydrogen donating ability [Kulisica et al. 2004]. EOs exhibited DPPH radical scavenging activity. EC50 values were 140 mg/ml for *T. malyi* and 123 mg/ml for *T. lykae*. EC_{50} value for BHT, a well known artificial antioxidant, obtained in the same conditions of DPPH assay, was 328 μg/ml. These results confirm that EOs of these species possess potential for antioxidant activity.

Burkholderia cepacia, an important human pathogen which most often causes pneumonia, was used in this investigation for the first time.

All tested bacteria and fungi that were screened, could be found in food and some of them are agents of food poisoning [Nostro et al. 2009, Mihajlov-Krstev et al. 2010]. *L. innocua* is a non-pathogenic species and may be used as model for prediction of the activity of EOs in food-borne pathogen *L. monocytogenes* because of its similar response to physical, chemical or thermal treatments [Staszewski et al. 2011].

Essential oils of two *Thymus* species showed different antimicrobial activity against tested strains. In all tested bacteria, *T. lykae* EO showed antibacterial activity, especially against Gram-positive bacteria. The most sensitive to *T. lykae* EO was *S. aureus* ATCC 25923 (3 μl/ml). Antibacterial effects of the *T. malyi* EO were detected only in *E. coli* SY252, *E. coli* ATCC 25922, *S. aureus* ATCC 25923 and *S. epidermidis* ATCC 12228, which was the most sensitive (2 μl/ml).

In the case of fungi, *T. malyi* EO has stronger effect than the EO of *T. lykae*. *C. albicans* ATCC 10231 was found to be more sensitive to *T. malyi* EO than *S. cerevisiae* D7 (Tab. 2).

Contrary to our previous research [Marin et al. 2012], in which a high concentration of carvacrol of EO of *S. montana* exerted significant antimicrobial activity, in present research, investigation of volatile compositions of EOs showed low concentrations of carvacrol. Thymol that has been proved to possess strong antimicrobial activity [Mathela Chandra et al. 2010], was detected in 1.21% in EO of *T. lykae* and absented in EO of *T. malyi*. This difference could be responsible for higher antimicrobial activity of *T. lykae* EO than *T. malyi*. According to [Leite et al. 2007], results have shown significant antibacterial effect of α-pinene on the growth of Gram-positive bacteria, especially on *S. epidermidis*, while [Jirovetz et al. 2006] indicated high antibacterial effect of monoterpenic target-compound myrcene against *E. coli*. Also [Mishra et al. 2011] showed that high concentration of some single compound such as germacrene D of the EO of *Senecio rufinervis* (Asteraceae) has significant antimicrobial activity. These results are in agreement with our findings; high concentrations of α-pinene, myrcene and germacrene D were observed in *T. malyi*, while EO of *T. lykae* was characterized by a high amount of geranyl acetate and geraniol. Geraniol exhibited higher antimicrobial effect on *S. aureus*, than thymol [Gallucci et al. 2010]. Previous investigations confirm that carvacrol had the most potent activity on fungi [Chami et al. 2005], which was not presented at high concentration in tested oils, but its concentration was about three times higher in *T. malyi* EO, which could explained stronger antifungal effect of *T. malyi* EO than *T. lykae* EO.

CONCLUSION

Composition of EOs of *T. malyi* and *T. lykae* showed strong differences comparing to other *Thymus* species that mostly possess thymol and carvacrol as the main constituents in EOs. They are characterized by low concentration of carvacrol and in the EO of *T. malyi*, thymol has not been found. Both oils

exhibited potential for antioxidant activity. Comparing the results of antimicrobial investigation of essential oils of two tested endemic *Thymus* species, higher antibacterial activity was shown by the oil of *T. lykae*, while *T. malyi* was found to have higher antifungal effects.

Based on antimicrobial tests, it is concluded that the essential oils from *T. malyi* and *T. lykae* possess significant antimicrobial activities against medically important pathogens.

ACKNOWLEDGMENT

This study was supported by Ministry of Science and Technologies of Republic of Serbia, Projects No. 172053 and 172058. We are grateful to prof. Petar Marin for providing plant materials.

REFERENCES

Adelheid, H.B., Hartwig, W.P., Ozren, E., Zeljan, M. Misko, P. (2005). Essential oil composition and antioxidant activity of *Thymus bracteosus* Vis. ex Benth. Flav. Frag. J., 20, 596–600.

Benayache, F., Chalard, P., Figueredo, G., Benayache, F., Benayache, S. (2014). Chemical composition of the essential of *Thymus numidicus* Poiret. Der Pharm. Lett., 6, 182–185.

Bounatirou, S., Smiti, S., Miguel, M.G., Faleiro, L., Rejeb, M.N., Neffati, M., Costa, M., Figueiredo, A.C., Barroso, J.G., Pedro, L.G. (2007). Chemical composition, antioxidant and antibacterial activities of the essential oils isolated from Tunisian *Thymus capitatus* Hoff. et Link. Food Chem., 105, 146–155.

CLSI (2015). Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; Approved standard – Tenth Edition. CLSI document M07-A1. Clinical and Laboratory Standards Institute, Wayne.

Chami, N., Bennis, S., Chami, F., Aboussekhra, A. Remmal, A. (2005). Study of anticandidal activity of carvacrol and eugenol *in vitro* and *in vivo*. J. Antimicrob. Chemother., 20, 106–111.

Economou, G., Panagopoulos, G., Tarantilis, P., Kalivas, D., Kotoulas, V., Travlos, I.S., Polysiou, M., Karamanos, A. (2011). Variability in essential oil content and composition of *Origanum hirtum* L., *Origanum onites* L., *Coridothymus capitatus* (L.) and *Satureja thymbra* L. population from the Greek island Ikaria. Ind. Crop. Prod., 33, 236–241.

Gallucci, N., Oliva, M., Carezzano, E., Zygadlo, J., Demo, M. (2010). Terpenes antimicrobial activity against slime producing and non producing staphylococci. Mol. Med. Chem., 21, 132–136.

Horwath, A.B., Grayer, R.J., Keith-Lucas, D.M., Simmonds, S.J. (2008). Chemical characterisation of wild populations of Thymus from different climatic regions in southeast Spain. Biochem. System. Ecol., 36, 117–133.

Jirovetz, L., Bail, S., Buchbauer, G., Denkova, Z., Slavichev, A., Stoyanova, A., Schmidt, E., Geissler, M. (2006). Antimicrobial testings, gas chromatographic analysis and olfactory evauation of an essential oil of hop cones *Humulus lupulus* L. from Bavaria and some of its main compounds. Sci. Pharm., 74, 189–201.

Kizil, S. Uyart, F. (2006). Antimicrobial activities of some Thyme (*Thymus*, *Satureja*, *Origanum* and *Thymbra*) species against important plant pathogens. Asian J. Chem., 18, 1455–1461.

Kulisica, T., Radonić, A., Katalinić, V., Miloša. M. (2004). Use of different methods for testing antioxidative activity of oregano essential oil. Food Chem., 85, 633– 640.

Küçükbay, F.Z., Kuyumcu, E., Çelen, S., Azaz, A.D., Arabaci, T. (2014). Chemical composition of the essential oils of three Thymus taxa from Turkey with antimicrobial and antioxidant activities. Rec. Nat. Prod., 8, 110–120.

Lee, S.J., Umano, K., Shibamoto, T., Lee, K.G. (2005). Identification of volatile components in basil (*Ocimim basilicum* L.) and thyme leaves (*Thymus vulgaris* L.) and their antioxidant properties. Food Chem., 91, 131–137.

Leite, A.M., Lima, E.O., Souza, E.L., Diniz, M.F.F.M., Trajano, V.N., Medeiros, I.A. (2007). Inhibitory effect of α-pinene, β-pinene and eugenol on the growth of potential infectious endocarditis causing Gram-positive bacteria. Braz. J. Pharm. Sci., 43, 121–126.

Marin, M., Novaković, M., Tešević, V., Vučković, I., Milojević, N., Vuković-Gačić, B., Marin, P.D. (2012). Antioxidative, antibacterial and antifungal activity of the essential oil of wild-growing *Satureja montana* L. from Dalmatia, Croatia. Flav. Frag. J., 27, 216–223.

Martino, L., Bruno, M., Formisano, C., Feo, V., Napolitano, F., Rosselli, S., Senatore, F. (2009). Chemical composition and antimicrobial activity of the essential oils from two species of *Thymus* growing wild in southern Italy. Molecules, 14, 4614–4624.

Mathela Chandra, S.K., Singh Krishna, K., Gupta, V.K. (2010). Synthesis and *in vitro* antibacterial avtivity of

thymol and carvacrol derivatives. Acta Pol. Pharm. Drug Res., 4, 375–380.

Miguel, G., Simoes, M., Figueiredo, A.C., Barroso, J.G., Pedro, L.G., Carvalho, L. (2004). Composition and antioxidant activities of the essential oils of *Thymus caespititius*, *Thymus camphoratus* and *Thymus mastichina*. Food Chem., 86, 183–188.

Mihajlov-Krstev, T., Radnović, D., Kitić, D., Stojanović-Radić, Z., Zlatković, B. (2010). Antimicrobial activity of *Satureja hortensis* L. essential oils against microbial strains. Arch. Biol. Sci., 62, 159–166.

Mishra, D., Joshi, S., Sah, S.P., Dev, A., Bisht, G. (2011). Chemical composition and antimicrobial activity of the essential oils of *Senecio rufinervis* DC. (Asteraceae). Indian J. Nat. Prod. Res., 2, 44–47.

Napoli, E.M., Curcuruto, G., Ruberto, G. (2010). Screening of the essential oil composition of wild Sicilian thyme. Biochem. Syst. Ecol., 38, 816–822.

Nedorostova, L., Kloucek, P., Kokoska, L., Stolcova, M., Pulkrabek, J. (2008). Antimicrobial properties of selected essential oils in vapour phase against foodborne bacteria. Food Control., 20, 157–160.

Nostro, A., Marino, A., Rita Blanco, A., Cellini, L., Di Giulio, M., Pizzimenti, F., Sudano Roccaro, A., Bisignano, G. (2009). In vitro activity of carvacrol against staphylococcal preformed biofilm by liquid and vapour contact. J. Med. Microbiol., 58, 791–797.

Rota, M.C., Herrera, A., Martinez, R.M., Sotomayor, J.A., Jordan, M.J. (2008). Antimicrobial activity and chemical composition of *Thymus vulgaris*, *Thymus zygis* and *Thymus hyemalis* essential oils. Food Control, 19, 681–687.

Ruiz-Navajas, Y., Viuda-Martos, M., Sendra, E., Perez--Alvarez, J.A., Fernández- López, J. (2012). Chemical characterization and antibacterial activity of *Thymus moroderi* and *Thymus piperella* essential oils, two Thymus endemic species from southeast of Spain. Food Control., 27, 294–299.

Russo, M., Suraci, F., Postorino, S., Serra, D., Roccotelli, A., Agosteo, G.E. (2013). Essential oil chemical composition and antifungal effects on *Sclerotium cepiyorum* of *Thymus capitatus* wild populations from Calabria, southem Italy. Rev. Bras. Farmacogn., 23, 239–248.

Saad, A., Fadli, M., Bouaziz, M., Benharref, A., Mezrioui, N.E., Hassani, L. (2010). Anticandidal activity of the essential oils of *Thymus maroccanus* and *Thymus broussonetii* and their synergism with amphotericin B and fluconazol. Phytomedicine, 17, 1057–1060.

Sarikurkcu, C., Ozer, M.S., Eskici, M., Tepe, B., Can, S., Mete, E. (2010). Essential oil composition and antioxidant activity of *Thymus longicaulis* C. Presl subsp. *longicaulis* var. *longicaulis*. Food Chem. Toxicol., 48, 1801–1805.

Sarker, S., Nahar, L., Kumarasamy, Y. (2007). Microtitre plate-based antibacterial assay incorporating resasurin as an indicator of cell growth, and its application in the *in vitro* antibacterial screening of phytochemicals. Methods, 42, 321–324.

Slavkovska, V.N., Lakušić, B.S., Jančić, R.B., Mimica-Dukić, N.A., Vujičić, Đ.S. (2006). Chemical composition of the essential oil of *Thymus bracteosus* Vis. ex. Bentham (Lamiaceae). J. Essent. Oil Res., 18, 310–314.

Staszewski, M., Pilosof, A.M.R., Jagus, R.J. (2011). Antioxidant and antimicrobial performance of different Argentinean green tea varieties as affected by whey proteins. Food Chem., 125, 186–192.

Tepe, B., Sarikurkcu, C., Berk, S., Alim, A., Akpulat, H.A. (2011). Chemical composition, radical scavenging and antimicrobial activity of the essential oils of *Thymus boveii* and *Thymus hyemalis*. Rec. Nat. Prod., 5, 208–220.

Viuda-Martos, M., Ruiz-Navajas, Y., Fernández-López, J., Pérez-Álvarez, J.A. (2011). Spices as functional foods. Food Sci. Nutr., 51, 13–28.