Arch. Biol. Sci., Belgrade, 65 (2), 639-643, 2013 DOI:10.2298/ABS1302639S

INVESTIGATION ON ANTIBACTERIAL SYNERGISM OF *ORIGANUM VULGARE* AND *THYMUS VULGARIS* ESSENTIAL OILS

D. STOJKOVIĆ[1], JASMINA GLAMOČLIJA[1], ANA ĆIRIĆ[1], M. NIKOLIĆ[1], M. RISTIĆ[2], JOVANA ŠILJEGOVIĆ[1] and MARINA SOKOVIĆ[1,*]

[1] *University of Belgrade, Department of Plant Physiology, Institute for Biological Research "Siniša Stanković",* 11000 Belgrade, Serbia
[2] *Institute for Medicinal Plant Research "Josif Pančić",* 11000 Belgrade, Serbia

Abstract - Essential oils are well known as strong antimicrobial agents of plant origin. In spite of this, the antimicrobial synergism of essential oils isolated from different plant species is poorly investigated. The following study examines the synergism of the essential oils of *Origanum vulgare* L. and *Thymus vulgaris* L against pathogenic bacteria, *Staphylococcus aureus* and *Salmonella typhimurium*. First, the antibacterial effect of the oils was tested, and the minimal inhibitory concentrations (MIC) of both oils were determined using the microdilution method. To test whether the oils act synergistically, every possible combination of essential oil concentrations was used in a dynamic checkerboard method. The results indicated that the oils indeed acted synergistically with fractional inhibitory concentration indexes of 0.45 and 0.50.

Key words: Essential oil, synergism, *Origanum vulgare*, *Thymus vulgaris*, antimicrobial.

INTRODUCTION

Despite progress in medical care and treatment, infectious diseases caused by pathogen microorganisms are still a major threat to public health (WHO, 2002). New drugs are introduced but they are expensive and relatively unavailable in developing countries (Okeke et al., 2005). Emerging resistance to synthetic products also poses a public concern. New strategies are needed, and in the last two decades a growing interest in the use of essential oils as safe alternatives is increasing.

Essential oils (EO) are products of plant secondary metabolites. They are a mixture of compounds, mainly mono- and sesquiterpenes, carbohydrates, alcohols, aldehydes and ketones. The biological activity is in direct dependence of the genetically induced specific chemical composition of an EO.

Origanum vulgare L. (oregano) is a spice herb from the family Lamiaceae, growing from 20-80 cm in height, with opposite leaves and purple flowers produced in erect spikes. It is native to western and southwestern Eurasia and the Mediterranean region. The main components of its EO are phenolic compounds: carvacrol and thymol. Chemical compositions vary depending on geographical region and session of collecting (Faleiro et al., 2002). *Thymus vulgaris* L. (thyme) is a low-growing herbaceous plant that hardly grows above 20 cm, forming a small shrub. A member of the Lamiaceae family, it is native to southern Europe. The main components of its EO are thymol, linalool or carvacrol depends on chemotype. The EOs of oregano and thyme are known to possess a wide range of biological activities (Adam et al., 1998; Sartoratto et al., 2004). Both plants are commonly used in foods, for flavor and aroma, in pharmaceutical and cosmetic industry.

There have been reports of that EOs are more strongly antimicrobial than their major components individually (Lachowicz et al., 1998) which indicates that the minor components play a important role and may have a synergistic effect. The synergistic effect of the major components of EOs has been reported in several studies (Ipek et al., 2005, Ettayebi et al., 2000, Hummelbrunner and Isman, 2001), however, examinations of whole oil are rare.

The concept of synergisms appears to have a great potential and influence on the biological activity of EO. For this reason, we have evaluated the synergistic potential of *O. vulgare* and *T. vulgaris* EO against pathogens.

MATERIALS AND METHODS

Essential oil isolation

Essential oils were isolated by hydrodistillation in a Clevenger type apparatus for 3 h. The essential oils obtained were stored at +4 °C until further tests.

Gas chromatography (GC) and gas chromatography/mass spectrometry (GC/MS)

Qualitative and quantitative analyses of the oils were performed using GC and GC/MS. The GC analysis of the oil was carried out on a GC HP-5890 II apparatus, equipped with a split/splitless injector, attached to an HP-5 column (25 m x 0.32 mm, 0.52 μm film thickness) and fitted to a flame ionization detector (FID). Carrier gas flow rate (H2) was 1 ml/min, split ratio 1:30, injector temperature was 250°C, detector temperature 300°C, while column temperature was linearly programmed from 40-240°C (at rate of 4°/min). The same analytical conditions were employed for GC/MS analysis, where HP G 1800C Series II GCD system equipped with an HP-5MS column (30 m x 0.25 mm, 0.25 μm film thickness) was used. The transfer line was heated at 260°C. Mass spectra were acquired in EI mode (70 eV), in an m/z range of 40-400. Electron impact identification of individual constituents was made by comparison of their retention times with those of analytical standards of available terpenoids, and by computer searching, matching mass spectra with those held in the Wiley 275 library of mass spectra. Confirmation was done using a calibrated AMDIS program for the determination of experimental values for retention indices of recorded constituents and comparing them with those from the literature (Adams, 2001). For quantification purpose, area percent data obtained by FID were used.

Microorganisms and culture conditions

For the bioassays, Gram (-) bacterial species *Salmonella typhimurium* (ATCC 13311), and Gram (+) bacterial species *Staphylococcus aureus*, were used. The organisms tested were from the Mycological Laboratory, Department of Plant Physiology, Institute for Biological Research "Siniša Stanković", Belgrade, Serbia. Bacteria were maintained on Mueller-Hinton agar (MHA).

Microdilution method

In order to investigate the antimicrobial activity of the isolated essential oils and the main constituents, the modified microdilution technique was used (Daouk et al., 1995). Bacterial species were cultured overnight at 37°C in Tryptic Soy Broth (TSB) medium The bacterial cell and fungal suspension was adjusted with sterile saline to a concentration of 1.0 x 10^5 units in a final volume of 100 μl per well. The inocula were stored at +4°C for further use. Dilutions of the inocula were cultured on solid MHA for bacteria to verify the absence of contamination and to check the validity of the inoculum.

Minimum inhibitory concentration (MIC) determination was performed by a serial dilution technique using 96-well microtiter plates. The investigated essential oils (0.01-10.00 μl/ml) were added in TSB medium with inoculum. The microplates were incubated for 24 h at 37°C for bacteria. Bacterial growth was detected by the addition of 30 μl of a 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT) water solution (0.5 mg/ml), (Sigma). The bacteria were again incubated at 37°C

for 30 min, and in the wells where bacterial growth occurred, INT changed from yellow to purple. MIC values were defined as the lowest concentration of essential oil to completely inhibit microbial growth.

Checkerboard method

This method was carried out using 96-well microplates containing Tryptic Soy Broth (TSB) with essential oil concentrations ranging from 1/16 - 4 × MIC and combined with each other on the plate in a checkerboard style. The bacterial inoculum was 1.0 x 10^5 colony-forming unit (CFU) per well. The microplates were incubated for 24 h at 37°C (Jacqueline et al., 2005). The fractional inhibitory concentration (FIC) index was calculated as follows: drug A (*O. vulgare* essential oil) FIC (drug A MIC combined/drug A MIC alone) + drug B (*T. vulgaris* essential oil) FIC (drug B MIC combined/drug B MIC alone). Synergism was defined as FIC index ≤ 0.5; additivity FIC index > 0.5-< 2; indifference FIC index ≥ 2-< 4 and antagonism FIC index ≥ 4 (Lorian, 2005). The strains that had MIC > 32 µg/ml and MIC > 1024 µg/ml were considered to be MICs of 64 µg/ml and 2048 µg/ml, respectively.

RESULTS AND DISCUSSION

Results on the chemical composition of the essential oils of *Origanum vulgare* and *Thymus vulgaris* are presented in Table 1. The essential oil of *O. vulgare* was rich in carvacrol (64.50%), while *p*-cymene and γ-terpinene were also dominant constituents with slightly lower percentage (10.90% and 10.80%). *T. vulgaris* essential oil was abundant with thymol (48.92%), followed by *p*-cymene (18.99%). The other constituents were present in amounts less than 5%. Chemical analysis of *O. vulgare* by other authors (Tian and Lai, 2006; Masood et al., 2007) also showed that the main constituents of this oil are phenols: carvacrol and thymol. However, some reports (Bussata et al., 2007) revealed monoterpene hydrocarbons terpinen-4-ol and γ-terpinen as the main components in chemical analysis. The results of chemical analysis of *T. vulgaris* are in accordance with previous studies (Rota et al., 2008, Hudaib et al., 2002) with major constituents being thymol and p-cymene. The high content of phenol compounds from this oil is responsible for its strong antimicrobial and antioxidant activity.

Investigating the antimicrobial synergism of a combination of thyme and oregano essential oils we obtained the results presented in Table 1. These results indicated that oregano and thyme essential oils indeed expressed antimicrobial synergism. As for the *S. aureus* MIC results, it can be seen from Table 1 that it was more sensitive than *S. typhimurium* to the effect of oregano essential oil. The MIC for oregano essential oil on *S. aureus* was 0.5 µl/ml, and for *S. typhimurium* was 1.0 µl/ml. The essential oil of thyme expressed the same MICs on both bacteria tested (1.0 µl/ml). The combined effect of thyme and oregano essential oils on *S. aureus* was synergistic in a combination of MIC 0.2 µl/ml for oregano oil and 0.25 µl/ml for thyme oil. The FIC index was calculated to be 0.45, which is ≤0.5 and according to the literature (Lorian, 2005) this value indicates compound synergism. A synergistic effect of combined essential oils on *S. typhimurium* was also obtained with combined MICs of 0.5 µl/ml and 0.05 µl/ml, for oregano and thyme oils, respectively. The value for the FIC index was calculated to be 0.505, which is approximately ≤0.5 and the effect is also considered to be synergistic.

Plant essential oils are a potentially useful source of antimicrobial compounds. The results of different studies are difficult to compare, most probably because of the different test methods, bacterial strains and sources of antimicrobial samples used. Natural products demonstrate different modes of action (McAllister et al., 2000), and in order to achieve the desired inhibition effect, products belonging to different plants should be combined as shown in this study.

By mixing different essential oils, their concentration can be significantly reduced, which possibly eliminates their undesired sensorial impact on antimicrobial preparation. The antibacterial properties of terpenic compounds are due to the presence of de-

Table 1. Chemical composition of essential oils

Components	O. vulgare **%**	T. vulgaris **%**	RI
α-thujene	1.9	1.17	931
α-pinene	-	1.21	939
camphene	-	0.83	948
sabinene	2.20	0.58	973
β-pinene	-	0.41	980
β-myrcene	-	1.06	991
δ-3-carene	2.20	-	1011
α-terpinene	-	0.65	1018
p-cymene	10.90	18.99	1026
limonene	-	0.46	1030
1,8-cineole	-	0.76	1031
trans-ocimene	-	1.30	1050
γ-terpinene	10.80	4.08	1068
linalool	-	0.74	1098
camphor	-	0.17	1143
borneol	-	1.72	1165
terpin-4-ol	-	1.78	1177
thymol-methyl-ether	-	0.16	1235
carvacrol-methyl-ether	-	1.73	1244
thymol	3.50	48.92	1290
carvacrol	64.50	3.45	1298
β-caryophyllene	2.50	3.45	1418
α-humulene	-	0.30	1454
germacrene D	-	0.33	1480
α-cadinene	-	2.23	1538
Total	98.50	96.48	

Table 2. Synergistic property of *O. vulgare* and *T. vulgaris* combined essential oils (μL/mL).

	MIC oregano	MIC thyme	MIC (O^1 + T^2)	FIC index
S. aureus	0.5	1.0	0.2 + 0.25	0.45
S. typhimurium	1.0	1.0	0.5 + 0.05	0.50

[1]Oregano, [2]Thyme

localized electrons in the molecule, which according to Ultee (2002) allows for easier deprotonation, playing an important role in their antibacterial activity. It is reported that terpenoid compounds have a greater inhibiting effect on Gram-positive than on Gram-negative bacteria (Nascimento et al., 2000) and our results confirm these previous findings.

In conclusion, *O. vulgare* and *T. vulgaris* are valuable sources of secondary metabolites with biological activities. This study demonstrates the synergistic effect of a natural mixture of two popular plant essential oils, *O. vulgare* and *T. vulgaris*. This potential should be further evaluated for application in modern medicine and industry against pathogen microorganisms.

Acknowledgments - The authors are grateful to the Ministry of Education and Science of Serbia for financial support (Grant № 173032).

REFERENCES

Adam K., Sivropoulou A., Kokkini S., Lanares T., and *M. Arsenakis* (1998). Antifungal activities of *Origanum vulgare* subsp. *hirtum, Mentha spicata, Lavandula angustifolia*, and *Salvia fruticosa* essential oils against human pathogenic fungi. *J. Agric. Food Chem.* **46**, 1739-1745.

Adams R.P. (2001) Identification of Essential Oil Component by Gas Chromatography/ Quadrupole Mass Spectrometry. Allured Publ. Corp., Carol Stream, IL.

Bussata, C., Mossi A.J., Rodrigues, M.R.A., Cansian, R.L. and *J. V. de Oliveira* (2007). Evaluation of *Origanum vulgare* essential oil as antimicrobial agent in sausage. *Braz. J. Microb*. **38**, 610-616.

Douk K.D., and *Dagher M.S.* (1995). Sattout J.E. Antifungal activity of the essential oil of *Origanum syriacum* L. *J. Food Prot*. **58**, 1147-1149.

Ettayebi K., El Yamani J. and *B.D Rossi-Hassani* (2000). Synergistic effects of nisin and thymol on antimicrobial activities in *Listeria monocytogenes* and *Bacillus subtilis*. *Fems. Microbiol. Lett.* **183**, 191-195.

Faleiro M.L., Miguel M.G., Ladeiro F., Venancio F., Tavares R., Brito J.C., Figueiredo A.C., Barroso J.G. and *L.G. Pedro* (2003). Antimicrobial activity of essential oils isolated from Portuguese endemic species of *Thymus*. *Lett. Appl. Microbiol.* **36**, 35-40.

Hudaib, M., Speroni, E., Di Pietra, A.M. and *V. Cavrini* (2002). GC/MS evaluation of thyme (*Thymus vulgaris L.*) oil composition and variations during the vegetative cycle. *J. Pharm. Biomed. Anal.* **29**, 691-700.

Hummelbrunner L.A. and *M.B. Isman* (2001). Acute, sublethal, antifeedant, and synergistic effects of monoterpenoid essential oil compounds on the tobacco cutworm, *Spodoptera litura* (Lep., Noctuidae). *J. Agric. Food Chem.* **49**, 715-720.

Ipek E., Zeytinoglu H., Okay S., Tuylu B.A., Kurkcuoglu M., and *K. Husnu Can Baser* (2005). Genotoxicity and antigenotoxicity of *Origanum* oil and carvacrol evaluated by Ames *Salmonella*/microsomal test. *Food Chem.* **93**, 551-556.

Jacqueline C., Navas D., Batard E., Miegeville A.F., Le Macbecque V., Kergueris M.F., Bugnon D., Potel G. and J. *Caillon* (2005). *In vitro* and *in vivo* synergistic activities of linezolid combined with subinhibitory concentrations of imipenem against methicillin-resistant *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* **49**, 45-51.

Lorian V. (2005). Antibiotics in laboratory medicine, 5th ed., Lippincot Williams and Wilkins, Philadelphia, p 889.

Lachowicz K.J., Jones G.P., Briggs D.R., Bienvenu F.E., Wan J., Wilcock A. and *M.J. Coventry* (1998). The synergistic preservative effects of the essential oils of sweet basil (*Ocimum basilicum* L.) against acid-tolerant food microflora. *Letters Appl. Microbiol.* **26**, 209-214.

Masood, N., Chaudhry, A., Saeed, S. and *P. Tariq* (2007). Antibacterial effect of Oregano (Oreganum vulgare) against Gram negative bacilli. *Pak. J. Bot*., **39**, 609-613.

Mcallister J.C. and *M.F. Adams* (2010). Mode of Action for Natural Products Isolated From Essential Oils of Two Trees Is Different From Available Mosquito Adulticides. *J. Medl. Entomol.* **47**, 1123-1126.

Nascimento G.G.F., Locatelli J., Freitas P.C. and *G.L. Silv*a (2000). Antibacterial activity of plant extracts and phytochemicals on antibiotic-resistant bacteria. *Brazilian J. Microbiol.* **31**, 247-256.

Okeke I.N., Laximinarayan R., Bhutta Z.A., Dusa A.G., Jenkins P., O'Brien T.F., Pablos-Mendez A. and *K.P. Klugman* (2005). Antimicrobial resistance in developing countries. Part I: recent trends and current status. *Lancet Infect. Dis,* **5**, 481-493.

Rota, M.C., Herrera, A., Martinez, R.M., Sotomayor, J.A. and *M.J. Jordan* (2008). Antimicrobial activity and chemical composition of *Thymus vulgaris, Thymus zygis* and *Thymus hyemalis* essential oils. *Food Control*. **19**(7), 681-687.

Sartoratto A., Machado A.L.M., Delarmelina C., Figueira G.M., Duarte M.C.T. and V.L.G. Rehder (2004). Composition and antimicrobial activity of essential oils from aromatic plants used in Brazil. *Brazilian J. Microbiol.* **35**, 275-280.

Tian, H., and *D.M. Lai* (2006). Analysis on the volatile oil in *Origanum vulgare*. *Zhong Yao Cai*., **29**(9): 920-921.

Ultee A., Bennik M.H.J. and *R. Moezelaar* (2002). The phenolic hydroxyl group of carvacrol is essential for action against the food-borne pathogen *Bacillus cereus*. *Appl Environ. Microbiol*.; **68**, 1561-1568.

WHO. (2002). Food safety and foodborne illness. World Health Organization Fact sheet 237. Geneva.