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Antibacterial activity of *Thymus vulgaris* essential oil alone and in combination with other essential oils

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Abstract. *Kon K, Rai M. 2012. Antibacterial activity of Thymus vulgaris essential oil alone and in combination with other essential oils. Nusantara Bioscience 4: 50-56.* Essential oils (EOs) from plants represent an alternative approach in combating antibiotic-resistant bacteria. One of the EOs with proven antibacterial properties is *Thymus vulgaris* EO. The purpose of the present work was to investigate *in vitro* antibacterial activity of *T. vulgaris* EO alone and in combination with other EOs. The activity of *T. vulgaris* EO was screened in combination with 34 EOs against *Staphylococcus aureus* and *Escherichia coli* by disk diffusion method; then the most effective combinations were evaluated by broth microdilution method. Against *S. aureus* the synergistic effect was found in combination of *T. vulgaris* and *Cinnamomum zeylanicum* EOs with fractional inhibitory concentration (FIC) index of 0.26; *Juniperus communis* and *Picea abies* EOs showed additive effect (FIC indexes were 0.74 and 0.78, respectively). Combination of *T. vulgaris* EO with *Aniba rosaeodora* and *Melissa officinalis* EOs demonstrated synergistic effect against *E. coli* (FIC indexes were 0.23 and 0.34, respectively); combination of *T. vulgaris* and *Mentha piperita* EOs was additive (FIC index 0.55). Therefore, combining *T. vulgaris* EO with other EOs has potential in further enhancing its antibacterial properties.

Keywords: *Thymus vulgaris*, essential oils, combinations, *Staphylococcus aureus*, *Escherichia coli*.

Abstrak. *Kon K, Rai M. 2012. Aktivitas antibakteri minyak atsiri* Thymus vulgaris *tunggal atau campuran dengan minyak atsiri lain. Bioscience Nusantara 4: 50-56.* Minyak atsiri tumbuhan merupakan senyawa alternatif untuk melawan bakteri resisten antibiotik. Salah satu minyak atsiri yang terbukti bersifat antibakteri adalah minyak atsiri *Thymus vulgaris.* Penelitian ini bertujuan untuk mengetahui aktivitas *in vitro* antibakteri minyak atsiri *T. vulgaris* tunggal atau campuran dengan minyak atsiri lain. Aktivitas antibakteri minyak atsiri *T. vulgaris* dan campurannya dengan 34 minyak atsiri lain terhadap *Staphylococcus aureus* dan *Escherichia coli* ditapis dengan metode cawan difusi, kemudian campuran yang paling efektif diuji dengan metode mikrodilusi kaldu. Efek sinergis terhadap *S. aureus* ditemukan pada campuran antara minyak atsiri *T. vulgaris* dan *Cinnamomum zeylanicum* dengan indeks konsentrasi hambat fraksional (FIC) 0,26; minyak atsiri *Juniperus communis* dan *Picea abies* menunjukkan efek aditif (indeks FIC masing-masing adalah 0,74 dan 0,78). Campuran minyak atsiri *T. vulgaris* dengan *Aniba rosaeodora* dan *Melissa officinalis* menunjukkan efek sinergis terhadap *E. coli* (indeks FIC masing-masing adalah 0,23 dan 0,34); campuran minyak atsiri *T. vulgaris* dengan *Mentha piperita* menunjukkan efek aditif (indeks FIC 0,55). Oleh karena itu, campuran minyak atsiri *T. vulgaris* dengan minyak atsiri lainnya memiliki potensi untuk meningkatkan sifat antibakteri.

Kata kunci: *Timus vulgaris*, minyak atsiri, kombinasi, *Staphylococcus aureus, Escherichia coli*

INTRODUCTION

Widespread of antibiotic resistance remains a serious clinical problem, which stimulates studies for search of new methods for coping with drug resistance or renews interest in traditionally used and forgotten methods, such as treatment with antibacterial plant extracts and essential oils (EOs) (Ríos and Recio 2005; Fisher and Phillips 2009). Combined therapy is traditionally used to increase antimicrobial activity and reduce toxic effects of agents (Houghton 2009).

Thyme plant is used since ancient times to achieve healing, antiseptic fumigator, food preservation and other useful effects (Stahl-Biskup and Sáez 2002). Nowadays, *Thymus vulgaris* EO belongs to EOs with the most pronounced antimicrobial activity (Iten et al. 2009). It was shown to be active against many bacteria, viruses, and fungi. High antimicrobial activity of thyme oil and its components, first of all, thymol and carvacrol, was demonstrated against *Staphylococcus aureus* (Al-Bayati 2008; Soković et al. 2010; Lević et al. 2011), including methicillin-resistant isolates (Tohidpour et al. 2010), *S. epidermidis* (Soković et al. 2010), *Enterococcus faecalis* (Lević et al. 2011), *Bacillus cereus* (Al-Bayati 2008), *Vibrio cholerae* (Rattanachaikunsopon and Phumkhachorn 2010), *Escherichia coli* (Lević et al. 2011), *Proteus mirabilis* (Soković et al. 2010; Lević et al. 2011), *P. vulgaris* (Al-Bayati 2008), *Pseudomonas aeruginosa* (Soković et al. 2010), *Salmonella enteritidis* (Soković et al. 2010), *S. choleraesuis* (Lević et al. 2011), *S. typhimurium*

(Soković et al. 2010), and other microorganisms.

In spite of many studies devoted to thyme oil, its combinations with other EOs have not been paid much attention. Gutierrez et al. (2009) studied combinations composed of thyme and oregano EOs against *B. cereus*, *E. coli*, *Listeria monocytogenes* and *P. aeruginosa* by checkerboard method and found that thyme-oregano EO combination had additive effect against *B. cereus* and *P. aeruginosa*, and indifferent effect against *E. coli* and *L. monocytogenes*. Furthermore, against *L. monocytogenes* the authors studied five more thyme EO combinations – with basil, lemon balm, marjoram, rosemary, and sage EOs. The results showed that basil, rosemary and sage EOs with thyme oil had additive effect, while lemon balm and marjoram EOs were indifferent.

The analysis of available literature shows that EO combinations, including combinations with thyme EO, represent perspective approach in antimicrobial treatment and prevention, however, in contrast to combinations of traditional antibiotics, this topic is not still well studied and requires further investigations.

The main goal of the present study was to investigate antimicrobial activity of thyme EO in combination with different EOs against *S. aureus* and *E. coli*.

MATERIALS AND METHODS

Essential oils. We used commercial EO of *Thymus vulgaris* (purchased from NPF Zarstvo Aromatov, Sudak, Ukraine) and 34 different EOs (purchased from Aroma Inter, Mykolaiv, Ukraine; Aromatika, Kiyiv, Ukraine; NPF Zarstvo Aromatov, Sudak, Ukraine) (Table 1).

Strains, preparation of inocula. We used reference strains *Staphylococcus aureus* (ATCC 25923) and *Escherichia coli* (ATCC 25922). The cultures of bacteria were maintained in meat peptone agar slants at 4°C throughout the study and used as stock cultures. For preparation of inocula, cultures were grown until logarithmic phase, and then bacterial density was adjusted to approximately 10^8 colony forming units (CFU) per mL for disk diffusion method and 10^5 CFU/mL for microdilution method with sterile saline solution. Bacterial counts were confirmed by plating out on meat-peptone agar, plates were incubated at 37°C for 24 h.

Disk diffusion method. This method was used for the screening of EOs for increase of antibacterial activity in the presence of thyme oil. Bacterial suspension was spread over the plates 85 mm in diameter containing Mueller-Hinton agar using a sterile cotton swab in three directions in order to get a uniform microbial growth. Under aseptic conditions empty sterile disks were impregnated with 5 µl of EO. Disks were left for 5 min at room temperature for better oil absorption and were then placed on inoculated agar surface. A standard disc with ciprofloxacin (10 µg/disc) was used as a reference control. The Petri dishes were left for 30 min at room temperature (20-22°C) for better oil diffusion and were then placed to an incubator at 37°C for 24 h. After an incubation period diameters of inhibition zones around the disks with EOs were measured.

We assessed diameter of inhibition zones around the disks with EOs mixtures. For this purpose, we prepared blends of EOs in sterile Eppendorf tubes by mixing 50 µl of thyme oil with 50 µl of correspondent second oil. Paper disks were then impregnated with 5 µl of appropriate mixture of EOs. Results of disk diffusion assay for study of EO mixture were assessed by comparing the experimental inhibition zone area of oils mixed with theoretical inhibition zone area of indifferent combinatory effect (calculated as ½ of inhibition zone area for thyme oil + ½ of inhibition zone area for the second oil).

Minimal inhibitory concentration (MIC) test. We prepared serial doubling dilutions of each plant EO in 96-well microtiter plates in volume 50 µL of Mueller Hinton Broth to give a range of concentrations from 0.0025% to 5% (volume/volume). After preparations of suspension of tested cultures, 50 µL were added to oil dilutions to produce total volume of 100 µL. The resulting suspensions were then mixed with a micro-pipettor. Two controls were used: positive (50 µL of medium and 50 µL of culture), and negative (100 µL of medium). All microtiter plates with microorganisms were incubated at 37°C for 24 h. Inhibition of bacterial growth in the wells containing test oil was judged by comparison with growth in negative control well. The MICs were determined by measuring optical density at 570 nm and defined as the concentration of oil at which there was a sharp decline in the absorbance value.

MICs determination of mixtures of EOs. Mixture of thyme and different EOs in ratios 1:1 were tested for determinations of MICs by broth microdilution method.

In order to assess results of MICs of EOs in mixtures, we calculated fractional inhibitory concentrations (FIC) with FIC indexes (Houghton 2009). Because mixtures were used in ratio 1:1, individual MIC of EO in blend was calculated as ½ of MIC of blend. According to this, FIC indexes were calculated as the following:

FIC of thyme oil = (1/2 MIC of blend)/ (MIC of thyme oil alone);

FIC of second oils = (1/2 MIC of blend)/ (MIC of second oil alone);

FIC index = (FIC of thyme oil) + (FIC of second oil),

Where, second oil is the EO which was tested in combination with thyme oil.

FIC indexes were interpreted as following: synergy, FIC < 0.5; addition, 0.5≤FIC≤1; indifference, 1<FIC≤4; antagonism, FIC>4 (Gutierrez et al. 2009).

Chemical composition. The main components of EOs were identified by mass-spectrometry analysis. The relative amount of individual components of the total oil was expressed as percentage peak area relative to total peak area. Qualitative identification of the constituents was performed by comparison of their relative retention times and mass spectra with those stored in NIST library or with mass spectra from literature (Stein et al. 2002).

Statistical analysis of data. All experiments were repeated in triplicates, and then mean values for diameters of inhibition zones, geometric mean MICs and accordingly to them FICs were calculated. Results were analyzed using statistical software SPSS (version 20.0). The results are expressed as mean value ± standard deviation or as

geometric mean. Comparison of groups was performed by U test Mann-Whitney and Kruskal-Wallis 1-way analysis of variance (ANOVA); differences were considered as statistically significant at $p<0.05$.

RESULTS AND DISCUSSION

Antibacterial activity of essential oils alone

The antibacterial activity of thyme oil and 34 EOs is summarized in Table 1. The results proved that thyme EO had significant activity against *S. aureus* and *E. coli* with diameters of inhibition zones 22.74±1.56 mm and 22.46±5.48 mm, respectively. Furthermore, the majority of EOs possessed antimicrobial activity, but in very wide ranges. In general, activity of EOs was higher against *S. aureus* than against *E. coli*.

Multivariate analysis showed presence of significant differences between activity of EOs from different plant families ($p=0.036$). The highest activity against both tested strains was demonstrated by EOs of plants from Lamiaceae family with the mean inhibition zone 21.7±17.0 mm against *S. aureus* and 13.2±10.3 mm against *E. coli*. Rather high activity was also present in Lauraceae plant EOs against *S. aureus* (13.7±10.0 mm) and Myrtaceae plant EOs against *E. coli* (12.4±6.2 mm). Activity of Pinaceae and Rutaceae plant EOs against both strains was rather low.

S. aureus did not show any sensitivity to two EOs – eucalyptus and lemon. We found weak activity in juniper berry, rosemary, silver fir, grapefruit, pontica wormwood, and camphor white EOs. High antistaphylococcal activity was found in lavender, ylang-ylang, clary sage, clove, cedarwood, geranium, and especially in cinnamon EO.

Against *E. coli* total absence of activity was noticed in eight EOs: calamus, camphor white, cedarwood, juniper berry, patchouli, sandalwood, Satsuma mandarin, and silver fir. Seven more EOs showed very weak antimicrobial activity with diameter of inhibition zone not exceeding 7 mm: thuja, bitter orange, grapefruit, lime, bay laurel, ylang-ylang, and dill. Interestingly, among these EOs without antimicrobial effect against *E. coli* some EOs possessed high activity against *S. aureus*, such as cedarwood, which did not inhibit growth of *E. coli* but had inhibition zone against *S. aureus* 28.4±14.1 mm; ylang-ylang EO had inhibition zones 7.0±0.9 mm against *E. coli* and 21.7±8.0 mm against *S. aureus*; patchouli and sandalwood EOs also did not inhibit growth of *E. coli* but had inhibition zones against *S. aureus* 16.9±2.8 mm and 15.3±5.1 mm, respectively.

Along with high activity of thyme EO against *E. coli*, high sensitivity of this strain was also shown only to two more EOs – clove and cinnamon (diameters of inhibition zones were 22.0±1.8 mm and 37.4±4.0 mm, respectively). Moderate level of activity against *E. coli* was demonstrated by lemon balm, peppermint and tea tree EOs with diameters of inhibition zones 10.4±1.3 mm, 10.8±1.3 mm, and 15.0±1.6 mm, respectively.

Twenty-one of 35 studied EOs had significant differences in antibacterial activity against *S. aureus* and *E. coli*, and 17 of these oils (basil, clary sage, lavender, patchouli, bay laurel, camphor white, cedarwood, silver fir, bitter orange, lime, Satsuma mandarin, calamus, dill, geranium, sandalwood, thuja, and ylang-ylang) had higher activity against *S. aureus*. Interestingly, peppermint, eucalyptus, tea tree, and lemon EOs were more active against *E. coli*. Such differences in spectrum of antibacterial activity may be a good basis for further assessment of combinations between EOs.

Antibacterial activity of essential oils in combination with thyme oil: results of disk diffusion method

EOs exhibited wide range of interaction effects with thyme oil from strong antagonism to strong synergism against both tested strains. In general, enhancing effect with thyme EO was more noticeable against *S. aureus* than against *E. coli*: mean change of inhibition zone areas compared with theoretical area of indifferent interaction was (32.3±60.0)% against *S. aureus*, while against *E. coli* it was (-13.5±42.5)% ($p < 0.001$). Therefore, against *S. aureus*, in general, interactions between thyme and other EOs were synergistic, while against *E. coli* – antagonistic.

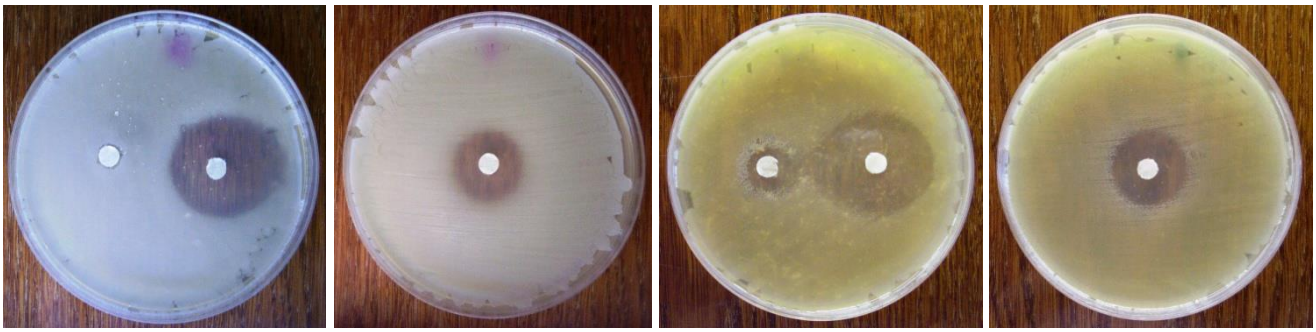
Compared with EOs alone, in combination with thyme oil a smaller number of EOs demonstrated significant differences in activity against tested strains: 14 EOs (basil, clary sage, lemon balm, patchouli, cedarwood, clove, Siberian cedar, neroli, Satsuma mandarin, geranium, pontica wormwood, sandalwood, thuja, and ylang-ylang) were significantly more active against *S. aureus* than against *E. coli*. EOs, which alone were significantly more active against *E. coli* (peppermint, eucalyptus, tea tree, and lemon), in combination with thyme oil demonstrated equal activity against both strains.

Against *S. aureus* the highest level of enhancing effect by using disk diffusion method was detected in Norway spruce EO: diameter of zone inhibition was changed from 8.6±1.5 mm without thyme oil to 32.1±13.7 mm in the mixture with thyme oil. Therefore, area of inhibition zone of mixture of thyme and Norway spruce oils was bigger than theoretical area of indifferent combination by 275.4%. High enhancing effect with thyme oil was also characteristic for juniper berry EO (Figure 1). Interestingly, that with almost absent antibacterial activity alone, in combination with thyme oil inhibition zone area increased by 145.1% compared with theoretical area of indifferent interaction. Significant enhancing effect with thyme oil was also demonstrated by thuja oil (inhibition zone area increased by 95.2%), clove (93.5%), cinnamon (77.0%), and Siberian cedar EOs (76.2%). It is worth to mention that eucalyptus and lemon EOs, which did not show antibacterial activity, in combination with thyme oil demonstrated noticeable increase in inhibition zone areas – by 55.1% and 56.1% respectively. Near 50% increase in inhibition, areas were also found in lavender and lemon balm oils combined with thyme EO. Among 34 studied EOs 9 had antagonistic interactions with thyme oil: bay laurel, bitter orange, peppermint, camphor white, patchouli, silver fir, myrtle, rosemary, and especially calamus EO.

Table 1. Diameters of inhibition zones of essential oils alone and in mixture with thyme oil

Essential oils		Diameter of inhibition zone alone (Mean±SD)			Diameter of inhibition zone in combination with thyme oil (Mean±SD)			Fold increase (%) of inhibition area comparing with theoretical area of indifference	
English name	**Latin name**	***S. aureus***	***E. coli***	***p***	***S. aureus***	***E. coli***	***p***	***S. aureus***	***E. coli***
Lamiaceae		21.7±17.0	13.2±10.3	0.15	26.7±16.0	16.7±6.3	0.07		
Basil	*Ocimum basilicum*	15.8±3.0	8.9±0.6	0.05	20.3±5.6	9.4±1.0	0.05	2.3%	-54.9%
Cinnamon	*Cinnamomum zeylanicum*	64.2±2.3	37.4±4.0	0.08	64.4±6.6	29.9±6.9	0.08	77.0%	-9.7%
Clary sage	*Salvia sclarea*	23.3±6.7	8.4±0.4	0.05	26.4±1.0	16.1±0.9	0.05	27.0%	36.4%
Lavender	*Lavandula angustifolia*	21.5±19.5	7.2±0.1	0.05	27.7±15.6	12.3±0.8	0.13	50.1%	-60.6%
Lemon balm	*Melissa officinalis*	16.4±8.2	10.4±1.3	0.13	25.0±4.9	18.6±1.8	0.05	50.7%	65.9%
Patchouli	*Pogostemon patchouli*	16.9±2.8	-	0.04	18.3±1.8	12.4±1.2	0.05	-20.8%	-38.8%*
Peppermint	*Mentha piperita*	7.7±0.5	10.8±1.3	0.05	16.5±6.4	18.9±1.0	0.51	-12.0%	67.5%
Rosemary	*Rosmarinus officinalis*	7.0±0.3	7.4±0.5	0.28	14.6±3.5	15.7±2.3	0.83	-29.8%	-35.5%
Thyme	*Thymus vulgaris*	22.7±1.6	22.5±5.5	0.85					
Lauraceae		13.7±10.0	7.1±1.6	0.08	21.0±6.6	17.8±5.3	0.25		
Bay laurel	*Laurus nobilis*	10.9±1.6	6.9±0.5	0.05	17.6±3.5	16.2±3.1	0.83	-8.5%	1.3%
Camphor white	*Cinnamomum camphora*	7.4±0.4	-	0.03	16.2±4.2	17.4±5.9	0.83	-14.8%	-19.1%*
Cedarwood	*Juniperus virginiana*	28.4±14.1	-	0.03	30.6±8.8	12.6±1.0	0.05	36.9%	-37.2%*
Rosewood	*Aniba rosaeodora*	7.9±0.8	9.5±3.4	0.51	19.4±3.4	25.2±5.2	0.28	21.5%	128.6%
Myrtaceae		10.8±7.7	12.4±6.2	0.35	21.0±6.6	16.3±1.8	0.12		
Cajuput	*Melaleuca cajuputi*	7.7±0.9	8.1±0.6	0.28	17.4±2.9	14.7±1.2	0.13	-2.0%	-44.3%
Clove	*Eugenia caryophyllata*	24.6±7.2	22.0±1.8	0.83	32.3±6.4	16.7±2.9	0.05	93.5%	-47.3%
Eucalyptus	*Eucalyptus globulus*	6.0±0.0	9.5±0.7	0.04	19.9±4.6	17.9±0.2	0.51	55.1%*	-4.5%
Myrtle	*Myrtus communis*	7.8±2.1	7.3±0.4	0.83	15.4±5.7	14.1±1.9	0.83	-23.4%	-23.9%
Tea tree	*Melaleuca alternifolia*	8±1.0	15.0±1.6	0.05	20.0±4.3	17.9±0.2	0.51	47.6%	-20.2%
Pinaceae		8.2±1.0	6.8±0.7	0.13	23.7±8.4	15.0±1.2	0.13		
Norway spruce	*Picea abies*	8.6±1.5	7.5±1.1	0.48	32.1±13.7	16.1±0.5	0.13	275.4%	-17.2%
Siberian cedar	*Pinus sibirica*	8.9±0.9	7.4±1.4	0.28	23.7±4.9	15.1±1.5	0.05	76.2%	26.1%
Silver fir	*Abies sibirica*	7.1±0.7	-	0.04	15.3±2.1	13.7±1.6	0.51	-22.9%	-49.4%*
Rutaceae		8.3±1.8	7.4±1.5	0.30	19.0±1.3	15.7±1.8	0.01		
Bitter orange	*Citrus aurantium* (fruits)	8.2±0.1	6.6±1.0	0.05	16.9±1.0	16.4±0.3	0.83	-8.7%	5.8%
Grapefruit	*Citrus paradisi*	7.2±0.2	6.6±1.0	0.51	18.0±4.9	12.7±2.8	0.28	5.4%	-36.5%
Lemon	*Citrus limon*	6.0±0.0	8.8±0.5	0.04	20.0±5.3	18.3±0.5	0.51	56.1%*	2.3%
Lime	*Citrus aurantifolia*	10.0±1.2	6.8±0.7	0.05	18.9±3.0	15.8±2.3	0.28	8.5%	-3.0%
Neroli	*C. aurantium* (flowers)	10.6±2.9	9.8±1.0	0.83	20.1±1.5	15.8±2.2	0.05	20.3%	-11.4%
Satsuma mandarin	*Citrus unshiu*	7.7±0.5	-	0.04	19.9±0.3	15.2±4.2	0.05	28.3%	-8.2%*
Other									
Calamus	*Acorus calamus* (Araceae)	13.1±3.3	-	0.04	13.1±2.7	9.3±2.5	0.28	-53.4%	-76.8%*
Dill	*Anethum graveolens* (Apiaceae)	9.1±0.7	7.0±0.8	0.05	18.4±5.1	16.8±3.6	0.83	5.3%	9.6%
Geranium	*Pelargonium roseum* (Geraniaceae)	29.2±5.6	8.3±0.5	0.05	25.7±2.5	14.1±1.2	0.05	-1.0%	-48.9%
Juniper berry	*Juniperus communis* (Cupressaceae)	6.7±0.6	-	0.12	25.3±4.6	20.4±5.9	0.28	145.1%	12.0%*
Pontica wormwood	*Artemisia pontica* (Asteraceae)	7.3±0.6	7.9±0.6	0.27	21.2±3.0	13.5±0.8	0.05	46.2%	-2.0%
Sandalwood	*Santalum album* (Santalaceae)	15.3±5.1	-	0.04	21.6±8.1	10.5±0.6	0.05	31.6%	-64.4%*
Thuja	*Thuja occidentalis* (Cupressaceae)	9.7±1.6	6.5±0.8	0.05	25.2±1.2	13.7±1.1	0.05	95.2%	-50.2%
Ylang-ylang	*Cananga odorata* (Annonaceae)	21.7±8.0	7.0±0.9	0.05	26.7±6.9	11.5±1.6	0.05	38.4%	-48.9%
Control									
Ciprofloxacin		28.8±1.7	38.7±0.2						

Note: * In the absence of bacterial growth inhibition zones, the disks' diameters (6 mm) were used to calculate fold increase, %



1.A **1.B** **2.A** **2.B**

Figure 1. Inhibition zones around the disk with juniper berry essential oil alone (left) and mixture of juniper berry and thyme essential oils (right) (A); inhibition zone around the disk with thyme essential oil alone (B) against *Staphylococcus aureus*

Figure 2. Inhibition zones around the disk with rosewood essential oil alone (left) and mixture of rosewood and thyme essential oils (right) (A); inhibition zone around the disk with thyme essential oil alone (B) against *Escherichia coli*

Against *E. coli* rosewood EO showed significant enhancing effect in combination with thyme oil (Figure 2) – inhibition zone area increased by 128.6% compared with theoretical area of indifferent interaction. High enhancing effect with thyme oil was also demonstrated by peppermint and lemon balm EOs: zones of inhibition increased by 67.5% and 65.9%, respectively. Several more EOs (clary sage, Siberian cedar, juniper berry, dill, and bitter orange) had some enhancing effect in ranges from 36.4% for clary sage to 5.8% for bitter orange EO. Eucalyptus, lime, pontica wormwood, bay laurel, and lemon EOs were indifferent to the presence of thyme oil, while majority of EOs (21 of 34) exhibited antagonistic interactions with thyme oil from mild (decrease of inhibition zone by 9.7% for cinnamon oil) to strong antagonism in lavender, sandalwood and calamus EOs (zones of inhibition decreased by 60.6%, 64.4%, and 76.8%, respectively). Interestingly, that calamus EO showed significant antagonistic effect with thyme oil against both tested strains, furthermore, antagonism was more noticeable against *E. coli*: decrease of inhibition zone area was 76.8% against *E. coli* and 53.4% against *S. aureus*.

Antibacterial activity of essential oils in combination with thyme oil: results of microdilution method

For several EOs which showed high synergistic effect with thyme oil in disk diffusion method, we determined MICs alone and in mixture with thyme oil (Tables 2 and 3).

Table 2. Susceptibility of *Staphylococcus aureus* to essential oils alone and in blends

EOs	Geometric mean minimal inhibitory concentrations, % (mg/mL)		Fractional inhibitory concentration index
	Alone	In blend with thyme oil (1:1)	
Thyme	0.4 (4.0)	-	-
Norway spruce	1.3 (11.2)	0.5 (4.5)	0.78
Juniper berry	10.0 (86.7)	0.6 (5.5)	0.74
Cinnamon	0.02 (0.2)	0.01 (0.1)	0.26

Table 3. Susceptibility of *Escherichia coli* to essential oils alone and in blends

EOs	Geometric mean minimal inhibitory concentrations, % (mg/mL)		Fractional inhibitory concentration index
	Alone	In blend with thyme oil (1:1)	
Thyme	0.3 (2.8)	-	-
Peppermint	3.2 (28.5)	0.3 (2.7)	0.55
Rosewood	0.4 (3.3)	0.1 (0.7)	0.23
Lemon balm	10.0 (91.4)	0.2 (1.8)	0.34

The microdilution method demonstrated general agreement with disk diffusion method. Thyme EO showed high activity against both tested strains: MIC was 4.0 mg/mL against *S. aureus* and 2.8 mg/mL against *E. coli* ($p = 0.884$, so differences between susceptibility of *S. aureus* and *E. coli* are not statistically significant). Among activity of three studied EO combinations against *S. aureus* the most active was cinnamon EO alone with MIC 0.2 mg/mL and cinnamon-thyme EO combination with MIC 0.1 mg/mL. This combination also demonstrated the highest synergistic effect with FIC index of 0.26. Norway spruce EO alone was less active than cinnamon oil; juniper berry EO alone inhibited *S. aureus* only at high concentration: MICs were 11.2 mg/mL for Norway spruce and 86.7 mg/mL for juniper berry EOs. However in combination with thyme oil activity was higher and MICs of these oils achieved 4.5 and 5.5 mg/mL, respectively. But, in general, interactions with thyme oil were additive: FIC indexes were 0.8 for Norway spruce and 0.7 for juniper berry EOs.

In combination with thyme oil against *E. coli* the best synergistic effect was demonstrated by rosewood EO: FIC index was 0.2 and final MIC of combination was 0.7 mg/mL. Lemon balm EO also showed synergistic effect with thyme oil and high activity against *E. coli*: FIC index was 0.3 and MIC of combination achieved 1.8 mg/mL. Peppermint oil interacted with thyme oil in an additive manner with FIC index of 0.6. Activity of peppermint-thyme EO combination was also rather high against *E. coli* with MIC 2.7 mg/mL. Therefore, all studied combinations can be used in order to inhibit growth of *S. aureus* and *E. coli*.

Chemical composition of thyme essential oil

The major components of thyme EO were carvacrol, γ-terpinene, and para-cymene (62.3%, 15.8% and 6.0%, respectively), therefore, the present thyme oil belongs to carvacrol chemotype. Thymol and α- terpinene were present in small amount (2.5% and 1.7%, respectively). Minor components were α- pinene (0.8%), α- terpineol (0.4%), camphene (0.4%) and camphor (0.2%).

Discussion

High prevalence of antibiotic resistance among bacteria causing infectious processes of different location has lead to revitalization of interest in EOs. Combined use of EOs has obvious advantages such as increasing activity of both agents, reduction of toxicity and minimizing adverse sensory effect of EOs in case of application of them as food preservatives. In many studies, EO of *T. vulgaris* demonstrated good antimicrobial properties; however, activity of thyme oil in combinations with other EOs is not well investigated. In the present study, we investigated activity of combinations of thyme oil with different EOs against representatives of two major bacterial groups – gram-positive *S. aureus* and gram-negative *E. coli*.

The results proved high antimicrobial activity of thyme EO and also demonstrated general higher susceptibility of *S. aureus* to EOs than *E. coli* in disk diffusion method. Based on these preliminary results of enhancing activity in disk diffusion method, we chose several EOs for more detailed evaluation in micro-broth dilution method – Norway spruce, juniper berry, and cinnamon EOs. For all these EOs, combinations with thyme oil were either synergistic or additive which demonstrated general agreement between disk diffusion and microdilution

methods. However, some differences were present as the best synergistic effect was seen in thyme-cinnamon combination, while two other combinations were additive.

Against *E. coli*, according to disk diffusion method, the most noticeable increase in antibacterial activity was present in combinations of thyme EO with rosewood, peppermint and lemon balm EOs. These three EOs were then studied by microdilution method which proved that the presence of beneficial effect between these EOs: synergism was detected in the combinations between thyme and rosewood, and between thyme and lemon balm EOs, while thyme-peppermint EOs combination was additive.

Effect of interactions between EOs depends on interactions of their components. Polymorphic variations in monoterpene production, characteristic for *T. vulgaris* (Thompson et al. 2003), make it important to determine the phenotype of studied thyme oil. In the present study, according to the major component, thyme oil belonging to carvacrol chemotype. Carvacrol is the substance with phenolic structure in which hydroxyl group plays an important role.

EO components with phenolic structure, such as thymol, carvacrol, and eugenol, possess high antimicrobial activity demonstrated in many studies (Soković et al. 2010; Bassolé et al. 2010). Several mechanisms have been proposed to explain their mechanism of action. Hydroxyl group on eugenol may react with proteins and inhibit action of enzymes; hydrophobic thymol and carvacrol may damage the outer membrane of gram-negative bacterial cell wall releasing lipopolysaccharides (Gómez-Estaca et al. 2010).

Bassolé et al. (2010) demonstrated synergistic interactions against *E. coli* between carvacrol and eugenol, carvacrol and thymol, carvacrol and linalool, carvacrol and menthol, menthol and eugenol, eugenol and thymol, and eugenol and linalool. Synergy between carvacrol of thyme oil and menthol of peppermint oil may be responsible for the additive effect between these EOs against *E. coli* demonstrated in our study. The main component of rosewood and lemon balm EOs, according to manufactures instructions, is linalool. Although linalool mechanism of action is not well understood, its documented synergistic interactions with carvacrol may play a key role in synergy between thyme and rosewood EOs and between thyme and lemon balm EOs against *E. coli*.

Against *S. aureus*, the present study has demonstrated synergistic effect between thyme and cinnamon EOs, the main component of which is cinnamaldehyde. Its mechanism of action includes inhibition of energy metabolism and interaction with bacterial cell membrane leading to its disruption and dispersion of the proton motive force by small ions leakage (Gill and Holley 2004).

Interactions of EO components against *S. aureus*, in general, are less studied. Synergism between thyme and cinnamon EOs may be caused either by not well-understood interactions between cinnamaldehyde and thyme EO components, or by already documented synergistic interactions against other gram-positive bacterium *L. monocytogenes* between carvacrol of thyme oil and eugenol of cinnamon oil, between thymol of thyme oil and eugenol, and between thymol and linalool of cinnamon oil (Bassolé et al. 2010).

Delgado et al. (2004) showed synergistic effect between thymol and cymene, present in different EOs, on *B. cereus* and proposed an explanation for it. Thymol and cymene have similar structure but, in contrast to thymol, cymene lacks the hydroxyl group. Both compounds are hydrophobic and accumulate preferentially in the cell membranes; after this the action of one compound may facilitate uptake of another into the lipid bilayer of cytoplasmic membrane, causing the observed synergistic effect. Cymene, which is present in juniper berry and cinnamon EOs, may be responsible for beneficial interactions with carvacrol or thymol of thyme oil.

CONCLUSION

Combinations of EOs provide an effective and economically feasible approach in combating antibiotic-resistant bacteria. However, unlike studies on antibiotic-antibiotic combinations, combinations of EOs are not so widely investigated and future studies should be devoted to evaluation of EO combinations against clinical isolates of multidrug-resistant bacteria, and to study combined effect of different EO components including also oil components present in small proportions.

REFERENCES

Al-Bayati FA. 2008. Synergistic antibacterial activity between *Thymus vulgaris* and *Pimpinella anisum* essential oils and methanol extracts. J Ethnopharmacol 116 (3): 403-406.

Bassolé IH, Lamien-Meda A, Bayala B, Tirogo S, Franz C, Novak J, Nebié RC, Dicko MH. 2010. Composition and antimicrobial activities of *Lippia multiflora* Moldenke, *Mentha* x *piperita* L. and *Ocimum basilicum* L. essential oils and their major monoterpene alcohols alone and in combination. Molecules 15 (11): 7825-7839.

Delgado B, Fernández PS, Palop A, Periago PM. 2004. Effect of thymol and cymene on *Bacillus cereus* vegetative cells evaluated through the use of frequency distributions. Food Microbiol 21 (3): 327-334.

Fisher K, Phillips C. 2009. *In vitro* inhibition of vancomycin-susceptible and vancomycin-resistant *Enterococcus faecium* and *E. faecalis* in the presence of citrus essential oils. Br J Biomed Sci 66 (4): 180-185.

Gill AO, Holley RA. 2004. Mechanisms of bactericidal action of cinnamaldehyde against *Listeria monocytogenes* and of eugenol against *L. monocytogenes* and *Lactobacillus sakei*. Appl Environ Microbiol 70 (10): 5750-5755.

Gómez-Estaca J, López de Lacey A, López-Caballero ME, Gómez-Guillén MC, Montero P. 2010. Biodegradable gelatin-chitosan films incorporated with essential oils as antimicrobial agents for fish preservation. Food Microbiol 27 (7): 889-896.

Gutierrez J, Barry-Ryan C, Bourke P. 2009. Antimicrobial activity of plant essential oils using food model media: efficacy, synergistic potential and interactions with food components. Food Microbiol 26 (2): 142-150.

Houghton P. 2009. Synergy and polyvalence: paradigms to explain the activity of herbal products. In: Houghton P, Mukherjee PK (eds) Evaluation of Herbal Medicinal Products: Perspectives on quality, safety and efficacy. Pharmaceutical Press.

Iten F, Saller R, Abel G, Reichling J. 2009. Additive antimicrobial [corrected] effects of the active components of the essential oil of *Thymus vulgaris*-chemotype carvacrol. Planta Med 75 (11): 1231-1236.

Lević J, Čabarkapa I, Todorović G, Pavkov S, Sredanović S, Coghill-Galonja T, Kostadinović L. 2011. *In vitro* antibacterial activity of essential oils from plant family Lamiaceae. Roman Biotechnol Let 16 (2): 6034-6041.

Rattanachaikunsopon P, Phumkhachorn P. 2010. Assessment of factors influencing antimicrobial activity of carvacrol and cymene against *Vibrio cholerae* in food. J Biosci Bioeng 110 (5): 614-619.

Ríos JL, Recio MC. 2005. Medicinal plants and antimicrobial activity. J Ethnopharmacology 100: 80–84.

Soković M, Glamočlija J, Marin PD, Brkić D, van Griensven LJLD. 2010. Antibacterial effects of the essential oils of commonly consumed medicinal herbs using an in vitro model. Molecules 15 (11): 7532-7546.

Stahl-Biskup E, Sáez F. 2002. Thyme: The genus Thymus. Taylor and Francis.

Stein S, Mirokhin D, Tchekhovskoi D, Mallard G. 2002. The NIST Mass Spectral Search Program for the NIST/EPA/NIH Mass Spectra Library; Standard Reference Data Program of the National Institute of Standards and Technology. Gaithersburg, MD, USA.

Thompson JD, Chalchat JC, Michet A, Linhart YB, Ehlers B. 2003. Qualitative and quantitative variation in monoterpene co-occurrence and composition in the essential oil of *Thymus vulgaris* chemotypes. J Chem Ecol 29 (4): 859-880.

Tohidpour A, Sattari M, Omidbaigi R, Yadegar A, Nazemi J. 2010. Antibacterial effect of essential oils from two medicinal plants against Methicillin-resistant *Staphylococcus aureus* (MRSA). Phytomedicine 17 (2): 142-145.