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# *In vitro* antibacterial activity of essential oils from plant family *Lamiaceae*

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**LEVIĆ JOVANKA[1], ČABARKAPA IVANA[1], TODOROVIĆ GORAN[2], PAVKOV SAVA[2], SREDANOVIĆ SLAVICA [1], COGHILL-GALONJA TAMARA[3], KOSTADINOVIĆ LJILJANA[3]**
[1]*Institute for Food Technology, Novi Sad, Bulevar cara Lazara 1, Serbia*
*Tel/Fax: 38121 450-781; E-mail: jovanka.levic@fins.uns.ac.rs*
[2]*Institute for Medicinal Plant Research „Dr. Josif Pančić", Belgrade, Tadeuša Košćuška 1, Serbia*
[3]*Faculty of Biofarming, Bačka Topola, M. Tita 39, Serbia*

## Abstract

*Medicinal plants and herbs have been used for many years in treating various animal and human diseases. Secondary metabolites from medicinal plants are widely unexploited in 'conventional' animal production systems. The aim of the investigation was to study antimicrobial activities of three essential oils on five bacterial species (Escherichia coli, Salmonella choleraesuis, Proteus mirabilis, Staphylococcus aureus, Enterococcus faecalis) obtained from the American Type Culture Collection. The plants used in this study were oregano (Origanum vulgare L.), thyme (Thymus vulgaris L.) and wild thyme (Thymus serpyllum L.), all members of Lamiaceae family. The antibacterial activity of essential oils was tested by disc diffusion method and broth micro-dilution susceptibility assay, according to the National Committee for Clinical Laboratory Standards Guidelines. All the tested oils demonstrated antimicrobial activity on used bacterial strains.*

*The analysis of variance revealed that there were highly significant differences (P<1%) between the effects of essential oils, concentrations and interactions of essential oils within all tested groups of bacteria. The strongest antibacterial effect was shown by oregano essential oil, while the oil extracted from wild thyme was least potent. The antibacterial activity of essential oils declined with decreasing concentration, regardless of the tested bacteria. Antibacterial effects of essential oils showed by broth micro-dilution method are in accordance with the results of a preliminary screening performed by the disc diffusion method.*

**Key words:** Bacteria, antimicrobial activity, essential oils.

## Introduction

In recent years there has been growing concern about the greater occurrence of antibiotic resistance among bacteria isolated from animal feeds and the environment **(**PALANIAPPAN & HOLLEY [1]). Antibiotics have been widely used in animal production for decades. Although some of them were used therapeutically, most were given for prophylactic purposes and to increase growth rate and feed conversion efficiency, as antimicrobial growth performance promoters or AGPs (HUYGHEBAERT & *al*. [2]**)**.

The uncontrolled overuse of antibiotics as common feed supplements could lead to increased numbers of antibiotic-resistant bacteria, and could ultimately compromise treatments of bacterial infections in humans (MC DERMOTT & al. [3]). Also, there is the possibility to transfer the antibiotic-resistant microorganisms into humans both directly via food chain and indirectly, by animal waste spreading throughout fields (GHOSH & LA PARA, [4]; HAMMERUM & HEUER, [5])**.** However, the emergence of antibiotic resistance in humans has increased public awareness on use of antibiotics in animal feeds, which finally led to their ban in the European Union (Regulation 1831/2003/EC). For these reasons, the

interest in evaluating natural alternatives for currently used antibiotics is increasing worldwide.

Medicinal plants and herbs have been used for many years in treatments of various animal and human diseases. Nowadays, plant extracts are more popular as animal feed supplements because most of the antimicrobial growth promoters in animal feed have been banned due to their residual effects. They act as antibacterial, antioxidant, anticarcinogenic, antifungal, analgesic, insecticidal, anticoccidial agents as well as growth promoters **(**TIPU & al. [6]). Medicinal plants have become the focus of intense study in terms of validation of their traditional uses through the determination of their actual pharmacological effects (BHASKARWAR & al. [7]).

Among the aromatic plant species from family *Lamiaceae* (*Labiateae*), genera *Origanum* and *Thymus* have a special position. Their essential oils are known to exhibit antimicrobial activities on bacteria (DORMAN & DEANS, [8]; SKANDAMIS & al. [9]). This is basically due to their major components being carvacrol and thymol, which act as preservatives (ULTEE & al. [10], BURT [11]). The biological activity of essential oils depends on their chemical composition, which is determined by genotype and influenced by environmental and agronomic conditions (BURT [11]). Thymol and carvacrol do not exhibit adverse effects on human health, and are proven not to cause either significant or marginal toxic effects at cellular level. Also, the concentrations at which they exhibit antimicrobial activities are not at possible genotoxic level (STAMMATI & *al.* [12]; BURT [11]).

The chemical analysis of the oregano (*Origanum vulgare* L.) essential oil revealed the presence of several ingredients, most of them having important antioxidant and antimicrobial properties (OZKAN & al. [20]). Carvacrol and thymol, the two main phenols that constitute about 78–85% of oregano oil, are mainly responsible for the antimicrobial activity of the oil **(**KOKKINI & al. [13]). In addition, other minor constituents such as monoterpene hydrocarbons, γ-terpinene and p-cymene also contribute to antibacterial activity of the oil (BURT [11]).

Essential oil of thyme (*Thymus vulgaris* L.) contains more than 60 ingredients with important antioxidant and antimicrobial properties (BARANAUSKIENE & al. [15]). The most important compounds of thyme essential oil are two phenols: thymol (44–60%) and carvacrol (2.2–4.2%) that are major and more active than monoterpene hydrocarbons p-cymene (18.5–23.5%) and γ-terpinene (16.1–18.9%), (DI BARANAUSKIENE & al. [15]**;** PASQUA & al. [16]; DAFERERA & al. [17]). *In vitro* studies showed that these compounds express their antimicrobial activity on a broad spectrum of Gram-negative and Gram-positive bacteria (BURT& al. [18]; OZCAN & al. [13]).

According to J. Passet, the chemotypes of *T. vulgaris* are thymol, carvacrol, linalol, α-terpineol/terpinyl acetate, geraniol/geranyl acetate, and trans-4-tuyenol. Besides those six main chemotypes, there are also p-cimen, limonene and 1,8 cineole. *T. serpyllum* contains thymol, carvacrol, linalol and geraniol (MARKOVIC, [19]).

This paper presents the results of the antimicrobial activity of essential oils from oregano (*Origanum vulgare* L.), thyme (*Thymus vulgaris* L.), and wild thyme (*Thymus serpillum* L.) on five important pathogenic bacteria. The aim of the study was to compare the effectiveness of different concentrations of the essential oils on pathogenic microorganisms.

## Material and methods

The plants used in this study were: oregano-$E_1$ (*Origanum vulgare* L.), wild thyme-$E_2$ (*Thymus serpyllum* L.) and thyme-$E_3$ (*Thymus vulgaris* L.) from family *Lamiaceae*. All plants

were obtained from the Institute for Medicinal Plant Research "Dr Josif Pančić", Belgrade, Serbia.

The essential oils were isolated from dried plant material by hydro-distillation according to the standard procedure reported in the Sixth European Pharmacopeia, [20]. The distillation was performed using Clevenger type apparatus, for 2 hours.

The antimicrobial activity of essential oil was evaluated using laboratory control strains, *Escherichia coli* ATCC 1053, *Salmonella choleraesuis* ATCC 10708, *Proteus mirabilis* ATCC 12453, *Staphylococcus aureus* ATCC 11632 and *Enterococcus faecalis* ATCC 14506, obtained from the American Type Culture Collection.

Antibacterial activity of essential oils was tested by the disc diffusion method according to the National Committee for Clinical Laboratory Standards Guidelines. This method is presented as a consensus standard by the NCCLS **(**National Committee for Clinical Laboratory Standards, 1999) [21]. Essential oils were diluted in propylene-glycol (2-(2-hydroxypropoxy)-1-propanol) to the test concentration ranging from 500 to 2 µl/ml. Antimicrobial tests were carried out by the disc diffusion method using 100 µl of suspension containing 2.0 x $10^8$ CFU/ml of bacteria spread on Mueller-Hinton agar (MHA, Himedia) in sterilized Petri dishes (90 mm diameter). The discs (6 mm in diameter) were impregnated with 10 µl of the oil dilution in concentration range from 500 to2 µl/ml and placed onto the inoculated agar. Negative controls were prepared using the same solvents to dissolve the essential oil propylene-glycol (2-(2-hydroxypropoxy)-1-propanol). The diameters of the inhibition zones were measured in millimeters. Experiments were performed in triplicate. The effectiveness was classified according to the size of zones of inhibition measured as: strongly inhibitory (more than 20 mm inhibition zone), moderately inhibitory (20-12 mm inhibition zone) and with no inhibitory effect (less than 12 mm zone), according to Rusenova (RUSENOVA & al. [22]).

Broth microdilution method was used to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) according to the National Committee for Clinical Laboratory Standards (NCCLS, [23]). The bacterial inoculates were prepared using overnight cultures and suspensions were adjusted to 0.5 McFarland standard turbidity using (the company's name) turbidimeter.

All tests were performed in Mueller Hinton broth. The aliquots of 20 µl of the essential oils were added into each well of the 96-well microtitre plate, in geometric dilutions ranging from 500 to 2 µl/ml. Then, aliquots of 160 µl of MHB were added. As the final step, 20 µl of $2\times10^6$ CFU/ml (according to 0.5 Mc Farland turbidity standards) of standardized microorganisms suspensions were inoculated into each well of the microplate. The test was performed in a volume of 200 µl with final essential oils concentrations of 50 to 0.2 µl/ml. Plates were incubated at 37 °C, for 24 hours. The same tests were performed simultaneously for growth control (MHB + tested microrganism) and sterility control (MHB + tested oil).

The MIC was defined as the lowest concentration of essential oil at which microorganisms show no visible growth. Referring to the results of the MIC assay, the wells showing complete absence of growth were identified and 5 µl solutions from each well was transferred to agar plates (NA-nutrient agar, Torlak) and incubated at 37 °C, for 24 hours. The MBC is defined as the lowest concentration of the essential oil at which inoculated microorganisms were 99.9% killed.

Descriptive and analytical statistics was carried out in the statistical package SPSS 10.0 for Windows, significant difference between the calculated mean values of the studied factors (essential oil and concentration) was tested using analysis of variance (ANOVA) for factorial experiments. Statistical significance was calculated using the F-test and LSD test for significance threshold of 5% and 1%.

## Results and discussion

The analysis of variance revealed very significant differences (P<1%) between the effects of essential oils, concentration of essential oils and interaction of essential oils and their concentration (CxE) for all tested bacteria (table 1).

**Table 1.** Analysis of variance for the tested essential oils (E), applied concentrations (C) and combination of these two factors (C x E)

| Source | Df | Mean Square | | | | |
|---|---|---|---|---|---|---|
| | | *Proteus mirabilis* | *Escherichia coli* | *Salmonella choleraesuis* | *Staphylococcus aureus* | *Enterococcus faecalis* |
| Replication | 2 | 1.57 | 1.81 | 0.53 | 3.42 | 0.59 |
| Essential oil (E) | 2 | 391.35** | 341.44** | 352.09** | 798.86** | 163.85** |
| Concentration (C) | 8 | 797.66** | 853.64** | 785.94** | 1989.60** | 471.40** |
| C x E | 16 | 116.12** | 98.75** | 90.29** | 59.96** | 24.66** |
| Error | 52 | 1.90 | 2.39 | 1.68 | 1.28 | 1.61 |

** P<1%, Df = degrees of freedom

The oregano essential oil showed the highest microbial efficiency while the wild thyme the lowest efficiency. By using disk diffusion method according to the standard conditions (composition and thickness of the substrate, inoculum size, pH of the substrate, incubation time, etc.) the diameter of the inhibition zone is proportional to the logarithm of the concentration of the substance studied. The results obtained with all tested bacteria showed that the inhibition zone diameter was proportional to the logarithm of the concentration of tested oil at a concentration of 500 µl/ml to 31.2 µl/ml.. For the concentrations between 6.25 and 7.8 µl/ml no statistically significant differences were found in antibacterial activities in all bacterial samples (Tables 2 and 3).

The disk diffusion method applied can be used only for preliminary screening of antimicrobial substances, since easily volatile components of essential oils evaporate over a period of incubation together with the solvent, while poorly dissolved components do not pass through the medium (GRIFFIN & al. [24]).

The results of the antibacterial activities of essential oils obtained by broth microdilution method are in accordance with the results of the preliminary screening using the disk diffusion method.

The results of broth microdilution assay showed in table 4 probed that the essential oil of oregano was active against all tested Gram-negative bacteria at lower concentrations: MIC/MBC=0.39/0.78 µl/ml. Gram-positive bacteria were inhibited by this oil at concentrations of MIC/MBC =0.78/1.56 µl/ml.

Thyme oil expressed antibacterial effects on Gram-negative bacteria in the range of MIC/MBC=0.39-1.56/0.78-3.125 µl/ml. Gram-positive bacteria were slightly more resistant on essential oil of thyme MIC/MBC=3.125-6.25/6.25 µl/ml.

All tested strains showed lower susceptibility on the essential oil of wild thyme. Wild thyme oil was efficient in the following range of concentration MIC/MBC=3.125-6.25/6.25-12.5 µl/ml for Gram-positive bacteria used, while it only showed an effect at concentrations MIC/MBC=1.56/3.125 µl/ml for Gram-negative bacterial strains (Table 4).

**Table 4.** Minimal inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) of essential oils (µl/ml)

| Test microorganism | *Oregano oil* | | *Thyme oil* | | *Wild thyme oil* | |
|---|---|---|---|---|---|---|
| | MIC | MBC | MIC | MBC | MIC | MBC |
| *P. mirabillis* | 0.39 | 0.78 | 0.39 | 0.78 | 1.56 | 3.125 |
| *E. coli* | 0.39 | 0.78 | 1.56 | 3.125 | 1.56 | 3.125 |
| *S. choleraesuis* | 0.39 | 0.78 | 1.56 | 3.125 | 1.56 | 3.125 |
| *S. aureus* | 0.78 | 1.56 | 3.125 | 6.25 | 3.125 | 6.25 |
| *E. faecalis* | 0.78 | 1.56 | 6.25 | 6.25 | 6.25 | 12.5 |

Many studies have shown that the essential oils of the herbs oregano and thyme were effective against strains of *E. coli, Salmonella enteritidis*, *Salmonella choleraesuis* and *Salmonella typhimurium* and that is based upon their high contents of thymol and carvacrol, p-cimen and γ-terpinen (SANTOYO & *al.*[25]; BENNIS & *al.* [26]; NOSTRO & *al.* [27]; BEN ARFA & *al.* [28]; KRIST & *al.*[29]). Carvacrol and thymol are able to disintegrate the outer membrane of Gram-negative bacteria, releasing lipopolysaccharides and increasing the permeability of the cytoplasmic membrane to ATP and depolarize the cytoplasmic membrane (XU & al. [30]).

In the previous studies, the essential oils of oregano were active against *E. coli, Salmonella typhimurium,* and *S. aureus* at the concentration of 0.5-1.2 µl/ml (BURT, [11]). Essential oil of thyme was active against *E.coli, Salmonella typhimurium,* and *S. aureus* in the following range of concentrations 0.45-1.25, 0.45->20, 0.2-2.5 (µl/ml), respectively (BURT, [11]). It appears that the difference in antibacterial activities may be related to the concentration and nature of contents, the functional groups, the structural configuration of the components and their possible synergistic interaction. These differences found between essential oils are due to ecological and plant growth factors.

**Table 2.** The effect of essential oils (E), their concentrations (C) and combination of those two parameters (ExC) on the bacterial strains tested *Proteus mirabilis* ATCC 12453, *Escherichia coli* ATCC 1053 and *Salmonella choleraesuis* ATCC 10708.

| Concentration (µl/ml) | *Proteus mirabilis* | | | Average | *Escherichia coli* | | | Average | *Salmonella choleraesuis* | | | Average |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $E_1$ | $E_2$ | $E_3$ | | $E_1$ | $E_2$ | $E_3$ | | $E_1$ | $E_2$ | $E_3$ | |
| 500 | 49.7 | 24.0 | 34.7 | 36.1 | 46.7 | 24.3 | 39.0 | 36.7 | 42.7 | 20.0 | 42.3 | 35.0 |
| 250 | 44.3 | 21.0 | 31.7 | 32.3 | 43.7 | 20.3 | 34.3 | 32.8 | 39.3 | 19.7 | 42.0 | 33.7 |
| 125 | 38.7 | 18.7 | 21.7 | 26.3 | 37.0 | 18.3 | 30.0 | 28.4 | 28.0 | 18.0 | 27.7 | 24.6 |
| 62.5 | 20.7 | 18.3 | 20.3 | 19.8 | 22.3 | 17.0 | 20.0 | 19.8 | 19.0 | 18.0 | 20.0 | 19.0 |
| 31.2 | 18.0 | 18.0 | 12.7 | 16.2 | 19.3 | 16.3 | 14.7 | 16.8 | 18.0 | 16.7 | 14.7 | 16.4 |
| 15.6 | 13.3 | 15.7 | 11.7 | 13.6 | 13.3 | 15.7 | 13.0 | 14.0 | 17.0 | 15.7 | 13.3 | 15.3 |
| 7.8 | 11.7 | 14.0 | 11.0 | 12.2 | 12.7 | 14.7 | 12.0 | 13.1 | 12.7 | 11.7 | 12.3 | 12.2 |
| 3.9 | 11.3 | 13.7 | 10.7 | 11.9 | 11.7 | 12.7 | 10.3 | 11.6 | 12.0 | 10.3 | 11.7 | 11.3 |
| 2.0 | 11.3 | 12.3 | 10.3 | 11.3 | 9.7 | 13.0 | 10.0 | 10.9 | 10.0 | 10.3 | 10.3 | 10.2 |
| Average | 24.3 | 17.3 | 18.3 | | 24.0 | 16.9 | 20.4 | | 22.1 | 15.6 | 21.6 | |
| $Lsd_{0.05}$ (E) | | | 0.75 | | | | 0.85 | | | | 0.71 | |
| $Lsd_{0.01}$ (E) | | | 1.01 | | | | 1.12 | | | | 0.94 | |
| $Lsd_{0.05}$ (C) | | | 1.30 | | | | 1.46 | | | | 1.23 | |
| $Lsd_{0.01}$ (C) | | | 1.74 | | | | 1.95 | | | | 1.64 | |
| $Lsd_{0.05}$ (ExC) | | | 2.26 | | | | 2.53 | | | | 2.13 | |
| $Lsd_{0.01}$ (ExC) | | | 3.01 | | | | 3.38 | | | | 2.83 | |

$E_1$ – oregano oil (*Origanum vulgare* L.), $E_2$ - thyme oil (*Thymus vulgaris* L.) and $E_3$ – wild thyme oil (*Thymus serpyllum* L.)
Least significant difference test ($Lsd_{0.05}$ and $Lsd_{0.01}$)

**Table 3**. The effect of essential oils (E), their concentrations (C) and combination of those two parameters (ExC) on the *Staphylococcus aureus* ATCC 11632 and *Enterococcus faecalis* ATCC14506

| Concentration (µl/ml) | *Staphylococcus aureus* | | | Average | *Enterococcus faecalis* | | | Average |
|---|---|---|---|---|---|---|---|---|
| | $E_1$ | $E_2$ | $E_3$ | | $E_1$ | $E_2$ | $E_3$ | |
| 500 | 56.0 | 33.7 | 44.3 | 44.7 | 37.0 | 21.0 | 28.7 | 28.9 |
| 250 | 54.0 | 30.3 | 40.3 | 41.6 | 34.3 | 19.3 | 25.7 | 26.4 |
| 125 | 43.3 | 28.3 | 30.3 | 34.0 | 27.0 | 16.3 | 17.7 | 20.3 |
| 62.5 | 30.0 | 16.7 | 24.3 | 23.7 | 18.0 | 14.3 | 16.3 | 16.2 |
| 31.2 | 19.7 | 13.7 | 13.3 | 15.6 | 13.3 | 12.3 | 15.3 | 13.7 |
| 15.6 | 13.3 | 11.7 | 12.0 | 12.3 | 11.3 | 11.7 | 12.0 | 11.7 |
| 7.8 | 12.3 | 10.3 | 11.3 | 11.3 | 10.7 | 10.7 | 10.7 | 10.7 |
| 3.9 | 12.0 | 10.0 | 10.0 | 10.7 | 10.3 | 0.0 | 10.0 | 6.8 |
| 2.0 | 10.0 | 0.0 | 0.0 | 3.3 | 0.0 | 0.0 | 0.0 | 0.0 |
| Average | 27.8 | 17.2 | 20.7 | | 18.0 | 11.7 | 15.1 | |
| $Lsd_{0.05}$ (E) | | | 0.62 | | | | 0.69 | |
| $Lsd_{0.01}$ (E) | | | 0.82 | | | | 0.93 | |
| $Lsd_{0.05}$ (C) | | | 1.07 | | | | 1.20 | |
| $Lsd_{0.01}$ (C) | | | 1.42 | | | | 1.60 | |
| $Lsd_{0.05}$ (ExC) | | | 1.85 | | | | 2.08 | |
| $Lsd_{0.01}$ (ExC) | | | 2.45 | | | | 2.78 | |

$E_1$ – oregano oil (*Origanum vulgare* L.), $E_2$ -wild thyme oil (*Thymus serpyllum* L.) and $E_3$ – thyme oil (*Thymus vulgaris* L.)
Least significant difference test ($Lsd_{0.05}$ and $Lsd_{0.01}$)

## Conclusions

The analysis of variance revealed very significant differences (P<1%) between the effects of essential oils used at various concentrations on all tested bacteria. Antimicrobial efficiency of oregano essential oils was the highest while for the wild thyme it was the lowest regardless of the bacterial strains tested.

Reduction of essential oils concentration significantly reduces the diameter of inhibition zone for all bacterial strains tested, except for the concentrations at 6.25 and 7.8 µl/ml where no statistically significant difference in diameter of inhibition was observed.

The results of the study on antibacterial effects of essential oils obtained by broth microdilution method are in accordance with the results of preliminary screening performed by using disk diffusion method. Gram-negative strains tested were more resistant to the antimicrobial activity essential oils.

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