**ABSTRACT:** The modern trends in nutrition suggest the limitation of synthetic food additives or the substitution with natural ones. Aromatic herbs are probably the most important source of natural antimicrobial agents. The aim of this study was to investigate antibacterial effects of various concentrations of *Origanum heracleoticum* essential oil on the food-borne bacteria. The antibacterial activity of *Origanum heracleoticum* essential oil was evaluated using laboratory control strains *Escherichia coli* ATCC/10536, *Salmonella choleraesuis* ATCC/110708, *Salmonella enteritidis* ATCC/13076, *Proteus mirabilis* ATCC/12435, *Pseudomonas aeruginosa* ATCC/10145, *Staphylococcus aureus* ATCC/11632, *Bacillus cereus* ATCC/10876 and *Enterococcus faecalis* ATCC/14506, obtained from the American Type Culture Collection. The antibacterial activity was determined using disk diffusion method and more precise broth microdilution method. Using the broth microdilution method essential oil of *Origanum heracleoticum* L. showed antibacterial activity against all tested strains of microorganisms with exception of the test strain of *Pseudomonas aeruginosa*. The tested oil had antibacterial effect on gram-positive bacteria in the range of MIC/MBC=0.2-0.39/0.78 μl/ml. The essential oil was active in the range from MIC/MBC=0.39 to 50/0.78 to 50 μl/ml against the tested gram-negative bacteria. *P. aeruginosa* ATCC/10145 showed the lowest sensitivity of MIC/MBC=50/50 μl/ml.

**MATERIALS AND METHODS**

**Plant material**

Aerial parts of *Origanum heracleoticum* L. were collected during blooming stage (August 2009) from the locality Kamendol near Smederevo, Serbia. The plant material was dried under laboratory conditions (20-25°C). Institute of Medicinal Plant Research Dr. Josif Pančić identified the plants and voucher specimens were stored in the herbarium of the Institute of Medicinal Plant Research Dr. Josif Pančić.

**Isolation of the essential oil**

The essential oil was isolated from dried plant material by hydro-distillation according to the standard procedure reported in the Sixth European Pharmacopoeia (18). Distillation was performed using Clevenger type apparatus, for 2.5 hours. The resulting essential oil was dried over anhydrous sodium sulfate and stored at 4°C.
Antimicrobial activity assessment

The antimicrobial activity of essential oil was evaluated using laboratory control strains, Escherichia coli ATCC /10536/, Salmonella cholerasuis ATCC /10708/, Salmonella enteritidis ATCC /13076/, Proteus mirabilis ATCC /12453/, Pseudomonas aeruginosa ATCC /10145/, Staphylococcus aureus ATCC /11632/, Bacillus cereus ATCC /10876/ and Enterococcus faecalis ATCC /14506/, obtained from the American Type Culture Collection.

Disc diffusion method

Antibacterial activity of essential oil was tested using disc diffusion method according to the National Committee for Clinical Laboratory Standards Guidelines (NCCLS). This method is presented as a consensus standard by the NCCLS (19). Essential oil was diluted in dimethylsulphoxide (DMSO) to the test concentration ranging from 500 to 0.25 µl/ml. Antimicrobial tests were carried out by the disc diffusion method using 100 µl of suspension containing 2.0 x 10⁶ CFU/ml of bacteria spread on Mueller-Hinton agar (MHA, Himedia) in sterile Petri dishes (90 mm diameter). The discs (6 mm in diameter) were impregnated with 10 µl of the oil dilution in the concentration range of 500-0.25 µl/ml and placed on the inoculated agar. Negative controls were prepared using dimethylsulphoxide (DMSO) which was the same solvent used to dissolve the essential oil. The diameters of the inhibition zones were measured in millimetres. All analysis were performed in triplicate, and the mean values are reported.

Broth microdilution assay

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 (19). The bacterial inoculates were prepared using overnight cultures and suspensions were adjusted to 0.5 McFarland standard turbidity.

All tests were performed in Mueller Hinton broth (MHB, Himedia). Dimethylsulphoxide (DMSO) was used to dissolve the essential oil and then diluted to the concentration (500 - 0.25 µl/ml). The concentration of DMSO used in the broth dilution method was same in each microtiter plate wells and amounted 5 percent. Twenty microliters aliquots of the essential oil were added to 96-well microtitre plates, in geometric dilutions ranging from 500-0.25 µl/ml. After that, aliquots of 160 µl of MHB, were added into each microplate well.

As the final step, 20 µl of 2.10⁶ cfu/ml (according to 0.5 Mc Farland turbidity standards) of standardized microorganisms suspensions were inoculated into each microplate well.

The test was performed in a volume of 200 µl with final essential oil concentrations of 50-0.025 µl/ml. Plates were incubated at 37°C for 24 hours. The same tests were performed simultaneously for growth control (MHB + test organism) and sterility control (MHB + test oil). The MIC was defined as the lowest concentration of the essential oil at which ≥ 99.9 percent of the inoculated microorganisms were killed. According to MBC definition, the presence of ≤2 cfu per inspected plate was tolerated.

RESULTS AND DISCUSSION

It is well known that both environmental and genetic factors are effective in observed variations among Origanum heracleoticum L. accessions with high accuracy (3). Because of this, yield and chemical composition of essential oil can vary among the populations of the same species from different localities.

From the collected plant material of Origanum heracleoticum L. total of 2.05 percent (v/w) of essential oil has been isolated by the process of hydro-distillation. In our previous investigation (4), twenty six components (92.86 percent) were identified as constituents of this essential oil by Gas Chromatography-Mass Spectroscopy (GC-MSD) analysis. The major components were carvacrol (69.00 percent), p-cymene (10.50 percent), thymol (7.94 percent) and γ-terpinene (2.86 percent). Except β-caryophyllene (1.53 percent) and β-bisabolene (1.01 percent) the amount of all remaining oil components was less than 1 percent.

Aromatic alcohol carvacrol was also dominant compound in Origanum heracleoticum oil analyzed by other authors (6, 20, 21). The predominant group of compounds in the oil were monoterpenes (95.77 percent), with significantly more oxidized compounds (79.21 percent) than hydrocarbons (16.56 percent). Sesquiterpenes were present at a low percentage in the oil (3.48 percent).

Numerous studies have demonstrated that the essential oils of Origanum species are among the most potent essential oils with regard to antimicrobial properties (2, 5, 6). This was confirmed in the present study.
According to the results of preliminary testing, disc diffusion method indicated generally strong antimicrobial activity of the oil against all tested strains of bacteria with the exception of the test strain of Pseudomonas aeruginosa. 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 inhibition zone is proportional to the logarithm of the concentration of the substance studied. The results obtained with all tested bacteria show 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, while it was not proportional at lower concentrations (Figure 1 and 2). 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. Because of that, during further investigation, we used more precise, broth micro-well dilution method. Using the broth microdilution method, the essential oil of Origanum heracleoticum L. showed antimicrobial activity against all tested strains of microorganisms. The tested oil had antibacterial effect on gram-positive bacteria in the range of MIC/MBC=0.2-0.39/0.78 μl/ml (Figure 3). The oil exhibited the highest activity against S. aureus ATCC /11632/ [MIC/MBC=0.2/0.78 μl/ml]. The obtained value for the MIC of the tested essential oil with 69 percent carvacrol against S. aureus is in agreement with previous studies in which the MIC of carvacrol against S. aureus ranged from 0.2 to 0.9 μl/ml (22, 23). E. faecalis ATCC/14506/ showed the lowest sensitivity of the tested gram-positive bacteria to tested oil at MIC/ MBC=0.78/0.78 μl/ml. The oil showed bacteriostatic and bactericidal effects against E. faecalis at the same concentration of MIC / MBC = 0.78 / 0.78 μl / ml.

The essential oil was active against tested gram-negative bacteria in the range from MIC/MBC=0.39 to 50/0.78 to 50 μl/ml (Figure 4). The oil showed the highest activity against strains of E. coli ATCC/10536/ and P. mirabilis ATCC/12453/ [MIC/MBC=0.39/0.78 μl/ml]. The obtained value for the MIC of the tested essential oil with 69 percent carvacrol against E. coli is in agreement with the results of Oussalah et al. (28) who showed the essential oil of Origanum heracleoticum L. with 54 percent carvacrol exhibited MIC against E. coli at a concentration of 0.25 μl/ml.
In previously published studies of carvacrol against E. coli MIC ranged from 0.2 to 5 μl/ml (22, 27). The effect of the tested oil was uniform, against S. enteritidis ATCC/13076/ and S. choleraesuis ATCC/10708/ at MIC/MBC = 0.78/1.56 μl/ml. The obtained value of MIC of the tested essential oil of Origanum heracleoticum L. is in agreement with previously published results of antimicrobial activity of essential oil of oregano against S. typhimurium in which the essential oil of oregano exhibited MIC concentrations ranging from 0.5 to 1.2 μl/ml (29, 28). In the research of Cosentino et al. (27) MIC value of carvacrol against S. typhimurium was 0.2 μl/ml. P. aeruginosa ATCC/10145/ showed the lowest sensitivity at MIC/MBC=50/50 μl/ml against the tested essential oil is a result of its outer hydrophylic membrane, which prevents the passage and effects of hydrophobic oils (30).

The surface of its cells forms selectively permeable pass, while hydrophobic macromolecules (such as those included in the composition of essential oil) remain on the outer side of the membrane. The present results demonstrate high activity of essential oil of Origanum heracleoticum L. against tested bacterial strains with exception of the test strain of Pseudomonas aeruginosa. High antimicrobial activity is explained firstly by the fact that the aromatic alcohol carvacrol is the main constituent of the oil, present in very high percentage (20, 23).

CONCLUSION

Origanum heracleoticum L. collected in Serbia is found to be (2.05 percent) of essential oil. The oil exhibited very high antibacterial activity, owing to high content of monoterpene carvacrol, which is well known antimicrobial compound. The tested oil had antibacterial effect on gram-positive bacteria in the range of MIC/MBC=0.2-0.39/0.78 μl/ml. The essential oil was active in the range from MIC/MBC=0.39 to 50/0.78 to 50 μl/ml against the tested gram-negative bacteria. P. aeruginosa ATCC/10145/ showed the lowest sensitivity with MIC/MBC=50/50 μl/ml. These values, together with high yield and lack of toxicity economically justify the use of essential oil derived from Origanum heracleoticum L for many purposes such as food preservation, active and intelligent packaging systems and also for the treatment of different human diseases.

Future research will be focused on application of the essential oil in food systems. The most interesting area of application for EOs is the inhibition of growth and reduction in numbers of the more serious food borne pathogens.

Extension of shelf-life and improvement of organoleptic qualities of meat and meat products may also be interesting from a commercial point of view. In view of their organoleptic properties, EOs could most readily be incorporated in the manufactured foods that are traditionally associated with herbs or with spices.

ACKNOWLEDGMENTS

These results are part of a research on the Project III046009, financed by the Ministry of Education and Science, Republic of Serbia.

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