Antimicrobial Activity of Selected Essential Oils

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Abstract
Essential oils are natural, aromatic and volatile liquids, which are frequently obtained by steam or water distillation from plants. In general, they have a pleasant odor and their proven antimicrobial, antiviral, antifungal, antioxidant, antifungal, insecticidal and antiparasitic properties have been known for several years. The aim of this work was to monitor antimicrobial activity against pathogenic microorganisms using two methods. The antimicrobial activity of essential oils was determined using the disk diffusion method and the broth dilution method. We found that wild thyme and peppermint essential oils were the most effective against the gram-negative bacterium *Pseudomonas aeruginosa* CCM 3955 in both cases with size of an inhibition zone 20.66 mm measured by disk diffusion method. Coriander essential oil had the best antimicrobial activity against all tested pathogenic microorganisms in the range of concentrations from 0.75 µg.ml^{-1} to 13.35 µg.ml^{-1} with broth dilution method.

Keywords: antimicrobial activity, essential oils, bacteria, minimal inhibitory concentration, disk diffusion method

1. Introduction

Essential oils are classified as natural, aromatic and volatile liquids, which are obtained by extraction from plants [1]. About 300 species of essential oils are produced industrially, but it is estimated that there are about 18,000 species of oil plants on Earth. The use of essential oils has expanded significantly to this date [2]. In general, essential oils have a pleasant odor and their proven antimicrobial, antiviral, antifungal, antioxidant, antifungal, insecticidal and antiparasitic properties have been known for several years [1].

Citrus limon belongs to the family Rutaceae with always green leaves and yellow edible fruits. It is rich in vitamin C, and the juice has long been used to treat fever, scurvy, sore throat, high blood pressure and more [3].

Thymus serpyllum L. belonging to the family Lamiaceae is an aromatic flowering plant from which essential oils rich in polyphenolic compounds - flavonoids or phenolic acids - are obtained. Essential oils from this plant have antimicrobial, insecticidal, antibacterial and antifungal effects. Essential oils are known to exploit these effects on the growth and development of microorganisms, which is especially important in the prevention of pathogenic microorganisms and contamination by spoilage. Wild thyme essential oils also have an inhibitory effect against biofilm-forming microorganisms [4].

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Coriandrum sativum is an aromatic annual plant belonging to the family Apiaceae. It has a long history of nutritional and therapeutic use, and its use can be found in the preparation of various dishes, while serving as a spice. Its essential oils can be used mainly in food as a flavoring or preservative, and in pharmaceuticals as well as perfumes. Coriander leaves and seeds are a relatively good source of essential oils [5].
Mentha x piperita L. is a perennial plant belonging to the family Lamiaceae. It has already been known in Egyptian, Roman, and Greek medicine for its broad benefits to human health, especially for digestive and diuretic problems, and also as a cough and cold remedy [6].
Salvia sclarea is included in the family Lamiaceae [8]. It is a biennial or perennial herb distributed worldwide from the Middle East to Southern Europe [8].
Origanum vulgare is a medium-sized aromatic herb native to the Lamiacae family. Oregano essential oils show anti-inflammatory, antimicrobial, antibacterial, antioxidant, antifungal and analgesic activity [9].
Essential oils from *Citrus aurantium* var. *dulce* is one of the important natural essential oils that have an attractive orange flavor. Antistress, anticarcinogenic, antifungal and radical scavenging properties have been demonstrated [10].
Rosmarinus officinalis has been used for culinary and ornamental purposes since ancient times. It is a plant native to the Lamiaceae family. It is cultivated worldwide for the production of extracts, spices and vegetable oils for its beneficial biological activities. Essential oils from this plant have significant pharmacological properties [11].
Matricaria recutita is an annual plant belonging to the family Asteraceae. It occurs naturally in many parts of the world and is one of the most popular and widely used medicinal plants in the world. It is grown mainly for cosmetic and pharmaceutical purposes [12,13].
Cinnamomum cassia is a common ingredient in spices, sauces, pastries, confectionery and beverages, and is recognized as a safe food ingredient. The genus *Cinnamomum* is included in the family Laureaceae [14].
The aim of this work was to evaluate the antimicrobial activity of various types of essential oils against selected species of pathogenic microorganisms using various methods.

2. Materials and methods

Essential Oil

Citrus limon (lemon), *Thymus serpyllum* L. (wild thyme), *Coriandrum sativum* (coriander), *Mentha x piperita* L. (peppermint), *Salvia sclarea* (clary sage), *Origanum vulgare* (oregano), *Citrus aurantium* var. *dulce* (sweet orange), *Rosmarinus officinalis* (rosemary), *Matricaria recutita* (chamomile), *Cinnamomum cassia* (cinnamon) essential oils (EOs) were purchased from Hanus, s.r.o. (Nitra, Slovakia). EOs were stored in the dark at 4 °C before the analyses.

Tested bacteria

The *Candida albicans* CCM 8261, *Candida glabrata* CCM 8270, *Candida krusei* CCM 8271, *Candida tropicalis* CCM 8223, *Bacillus subtilis* CCM 1999, *Enterococcus faecalis* CCM 4224, *Staphylococcus aureus* CCM 3953, *Pseudomonas aeruginosa* CCM 3955, *Salmonella enterica* CCM 4420, *Yersinia enterocolitica* CCM 7204 were collected from Czech collection of microorganisms (CCM, Brno).

Antimicrobial activity with disk diffusion method

Antimicrobial activity of EOs were determined using the disk diffusion method. Bacteria were aerobically cultivated on Mueller Hinton broth (MHB, Oxoid, Basingstoke, UK) at 37 °C for 24 h and yeasts on Sabouraud dextrose broth (SDB, Oxid, Basingstone, UK) at 25 °C for 24 h. An inoculum with an optical density of 0.5 McFarland standard (corresponded to 1.5×10^8 CFU/mL) was prepared and an amount of 100 µL of Mueller Hinton agar (MHA, Oxoid, Basingstoke, UK) and 100 µL of Sabouraud dextrose agar (SDA, Oxoid, Basingstoke, UK) was used for inoculation. Clean discs with 6 mm diameter were saturated with 10 µL of EOs and placed on the agar. Bacteria were incubated aerobically at 37 °C for 24 h and yeasts at 25 °C for 24 h. Each test was repeated 3 times. Antibiotic ticarcillin was used as positive control.

Minimum inhibitory concentrations (MIC)

Bacteria and yeasts were aerobically cultured for 24 h in MHB at 37 °C resp. SDB at 25 °C. The 50 µl of microbial suspension with optical density 0.5 McFarland standard was applied to a 96-well microtiter plate. 100 µL of MHB with EOs in concentrations from 400 µL/mL to 0.2 µL/mL, prepared with serial dilution, was added to sample. The contents of the wells were mixed by pipetting. MHB and EOs were used as a negative control, and MHB with inoculum was used as positive control of maximal growth. Absorbance was measured at 570 nm (Glomax spectrophotometer, Promega Inc., Madison, WI, USA). Each test had three replications.

Statistical analysis

The basic variation (disk diffusion method) – statistical values from obtained data were calculated with Statgraphic,

3. Results and discussion

C. cassia and *Mentha x piperita* had the greatest antimicrobial effects on *Staphylococcus aureus* CCM 3953. Their zone of inhibition was 16.33 mm (Tab. 1).

Enterococcus faecalis CCM 4224 achieved the highest sensitivity with an inhibition zone size of 15.66 mm in the following five EOs (Tab. 1).

The greatest antimicrobial effects on *Bacillus subtilis* CCM 1999 were shown by *C. cassia* and *T. serpyllum* with an inhibition zone size of 16.33 mm. Clary sage had only 4% lower sensitivity. Peppermint EO was the least sensitive to this bacterium with a zone of inhibition of 13.33 mm, which was 19% lower compared to the most effective EO, but this bacterium was sensitive to all tested EOs (Tab. 1).

Singh et al. [15] investigated the antibacterial effects of *Mentha x piperita* EO against multiple gram-positive and gram-negative bacteria using agar well diffusion and compared the results with the antibiotic gentamicin. *Staphylococcus aureus* was sensitive to 1 µl of the EO with an inhibition zone diameter of 11.4 mm and at 10 µl it was 17.2 mm, while gentamicin at 10 µg/ml had an inhibition zone of 14.7 mm.

Pseudomonas aeruginosa CCM 3955 was the most sensitive to *Mentha x piperita* and *T. serpyllum* EOs with an inhibition zone size of 20.67 mm (Tab. 2). Gür et al. [16] focused on testing the antimicrobial activity of wild thyme and rosemary EOs against different strains of *Pseudomonas aeruginosa* in their study. They found that rosemary EO was only effective against two strains of *Pseudomonas aeruginosa* at high concentrations, where a zone of inhibition of 13 mm was noted in both cases.

The highest antimicrobial effect on *Salmonella enterica* CCM 4420 showed *Mentha x piperita* and *T. serpyllum* EOs with an inhibition zone size of 18.67 mm. *Yersinia enterocolitica* CCM 7204 was the most sensitive to *C. cassia, T. serpyllum* and *C. aurantium* var. *dulce* EOs with an inhibition zone of 16.67 mm. Kluga et al. [17] investigated pathogens isolated from fish, including *Yersinia enterocolitica*. They used 14 types of EOs in the research and they found strong activity of the EOs against pathogenic bacteria.

Hsouna et al. [18] tested the antimicrobial activity of lemon EO against food spoilage and foodborne pathogens - gram-positive *Bacillus cereus, Enterococcus faecalis, Staphylococcus aureus, Staphylococcus epidermidis*, *Bacillus subtilis,* and gram-negative *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella enteritidis* and *Klebsiella pneumoniae*. Inhibition zones ranged from 13 to 26 mm.

Micic et al. [19] investigated the antimicrobial activity of rosemary EO (Russian and Serbian), which they performed using the disk diffusion method. EOs were tested against *Escherichia coli, Pseudomonas aeruginosa, Bacillus cereus* and *S. aureus*. According to their results, it can be stated that EO obtained from Russia showed a much better antimicrobial potential compared to EOs from Serbia. In the case of Russian EO, a maximum inhibition zone of 40.00 mm was reported for all tested microorganisms, except *Pseudomonas aeruginosa*, whose inhibition zone was 21.33 mm. The inhibition zone of Russian rosemary EO was even higher than the positive controls. Serbian EOs showed high antimicrobial activity above 30.00 mm against *Escherichia coli* and *Staphylococcus aureus*.

The results showed that the worst antimicrobial effects on the investigated yeast *Candida albicans* CCM 8261 were shown by several EOs with the same inhibition zone of 6.33 mm (Tab. 3). Ghazanfari et al. [20] examined several effects of coriander EO, which plays an important role in preventing food spoilage and food preservation. This EO had a very strong effect on the yeast *Candida albicans*. EO extracted by microwave extraction (ME) showed better antimicrobial and antioxidant activity and higher phenol yields compared to coriander EO obtained by hydrodistillation. The most effective against *Candida glabrata* CCM 8270 was peppermint, chamomile and cinnamon EOs which had the largest inhibition zone of 8.33 mm. However, *Candida tropicalis* CCM 8223 also reacted sensitively to clary sage and sweet orange EO with a diameter of 8.00 mm, rosemary EO with an inhibition zone of 7.66 mm and lemon, wild thyme, coriander and oregano EOs with a zone of 7.33 mm (Tab. 3).

Table 1. Antimicrobial activity of EOs against G^+ bacteria in mm

EO/bacteria	*S. aureus*	*E. faecalis*	*B. subtilis*
C. sativum	15.33±0.58	15.67±0.58	14.67±1.15
S. sclarea	14.33±0.58	15.67±0.58	15.67±0.58
C. cassia	16.33±0.58	15.67±0.58	16.33±0.58
T. serpyllum	14.67±1.15	15.67±1.53	16.33±0.58
R. officinalis	15.67±0.58	15.33±0.58	15.33±0.58
M. recutita	15.33±0.58	15.33±0.58	15.33±0.58
O. vulgare	15.67±0.58	15.33±0.58	15.33±0.58
C. aurantium var. dulce	15.33±0.58	15.33±0.58	15.33±0.58
Mentha x piperita	16.33±1.15	15.67±0.58	13.33±0.58
C. limon	15.33±0.58	15.33±0.58	15.33±0.58

Table 2. Antimicrobial activity of EOs against G^- bacteria in mm

EO/bacteria	*S. enterica*	*P. aeruginosa*	*Y. enterocolitica*
C. sativum	17.67±0.58	16.67±1.15	15.33±0.58
S. sclarea	18.33±0.58	17.67±0.58	16.33±0.58
C. cassia	16.33±0.58	17.00±1.00	16.67±0.58
T. serpyllum	18.67±0.58	20.67±0.58	16.67±0.58
R. officinalis	15.67±0.58	16.33±1.15	16.33±1.15
M. recutita	15.33±0.58	15.33±0.58	15.33±0.58
O. vulgare	18.33±0.58	17.67±0.58	16.33±0.58
C. aurantium var. dulce	16.33±0.58	17.00±1.00	16.67±0.58
Mentha x piperita	18.67±0.58	20.67±0.58	16.67±0.58
C. limon	15.67±0.58	16.33±1.15	16.33±1.15

Table 3. Antimicrobial activity of EOs against yeasts in mm

EO/yeast	*C. albicans*	*C. glabrata*	*C. krusei*	*C. tropicalis*
C. sativum	6.33±0.58	8.00±1.00	8.33±0.58	7.33±0.58
S. sclarea	6.33±1.15	8.33±0.58	8.33±0.58	8.00±1.00
C. cassia	5.33±0.58	7.33±0.58	8.00±1.00	8.33±0.58
T. serpyllum	5.33±0.58	7.67±1.15	8.33±0.58	7.33±0.58
R. officinalis	5.33±0.58	8.33±0.58	7.33±0.58	7.67±1.15
M. recutita	5.67±0.58	7.33±0.58	8.00±1.00	8.33±0.58
O. vulgare	6.33±1.15	8.00±1.00	8.33±0.58	7.33±0.58
C. aurantium var. dulce	5.33±0.58	8.33±0.58	7.33±0.58	8.00±1.00
Mentha x piperita	5.33±0.58	7.33±0.58	8.00±1.00	8.33±0.58
C. limon	5.33±0.58	8.00±1.00	8.33±0.58	7.33±0.58

Oregano EO generally seemed to be the most effective against majority of the microorganisms. Oregano EO was effective against *Enterococcus faecalis* CCM 4224 at MIC 50: 3.22 and MIC

90: 5.46 µl/ml, and against *Staphylococcus aureus* CCM 3953 at MIC 50: 1.21 and MIC 90: 3.18 µl/ml (Tab. 4).

Oregano EO also had the greatest inhibitory effect on the gram-negative bacterium *Pseudomonas aeruginosa* CCM 3955 with growth inhibition at MIC 50: 3.22 and MIC 90: 5.46 µl/ml. The reproduction and growth of *Salmonella enterica* CCM 4420 was most inhibited by coriander EO with a concentration of MIC 50: 3.22 and MIC 90: 5.46 µl/ml. The clary sage EO inhibited the growth of *Yersinia enterocolitica* CCM 7204 most effectively at MIC 50: 3.22 and MIC 90: 5.46 µl/ml (Tab. 5). The growth of the yeast *Candida albicans* CCM 8261 was most effectively inhibited by sweet orange EO with a concentration of MIC 50: 0.75 and MIC 90: 1.13 µl/ml. Coriander EO was effective with the same minimum inhibitory concentration against *Candida glabrata* CCM 8270: MIC 50: 0.75 and MIC 90: 1.13 µl/ml. The growth of *Candida krusei* CCM 8271 was most effectively inhibited by four EOs: coriander, peppermint, rosemary and sweet orange with the same concentration MIC 50: 5.56 and MIC 90: 7.23 µl/ml. The yeast *Candida tropicalis* CCM 8223 was inhibited the most by chamomile EO with the concentration of MIC 50: 3.22 and MIC 90: 5.46 µl/ml (Tab. 6). Oregano EO most effectively inhibited the growth of all three tested gram-positive bacteria. In *Bacillus subtilis* CCM 1999, inhibited growth at MIC 50: 5.56 and MIC 90: 7.23 µl/ml.

El Atki et al. [21] also focused in their research on the minimum inhibitory concentration of cinnamon oil against three bacterial strains: *E. coli* ATCC 25922, *S. aureus* ATCC 25923 and *P. aeruginosa* ATCC 27853. EO inhibited all tested strains with a MIC concentration of 4.88, 4.88 and 19.53 µg/ml, respectively. De Azeredo et al. [22] tested the antimicrobial activity of oregano and rosemary EO. The results of MIC concentrations of these EO against the tested bacteria, often associated with minimally processed vegetables, showed values in the range of 1.25–5 µl/ml and 20–40 µl/ml. The highest MIC values were found against *P. fluorescens* for both mentioned EOs. They also concluded that the MIC concentrations of rosemary EO were 8 to 16 times higher than those found for oregano EO. The antimicrobial effect of oregano and wild thyme EOs was investigated by Fournomiti et al. [23] against clinical isolates of multidrug-resistant *Escherichia coli, Klebsiella oxytoca* and *Klebsiella pneumonia* using the minimum inhibitory concentration method. Their results showed that the most sensitive organism was *K. oxytoca* with an average MIC value of 0.9 mg/ml for oregano EO and 8.1 µg/ml for wild thyme EO. The second most sensitive strain was *K. pneumoniae* with average MIC values of 9.5 µg/ml for wild thyme and 73.5 µg/ml for oregano EO.

Kosakowska et al. [24] tested two types of oregano EOs against selected gram-negative bacteria - *E. coli* ATCC 25922, *E. coli* O157: H7 ATCC 700728, *S. enteritidis* ATCC 13076 and gram-positive bacteria - *S. aureus* ATCC 25923, *B. cereus* ATCC 11778, *L. monocytogenes* ATCC 7644. The antimicrobial activity of Greek oregano EO against the tested bacterial strains was at least three times higher compared to the activity of ordinary oregano EO. Greek oregano EO effectively inhibited the growth of both gram-negative and gram-positive bacteria with MIC concentration values ranging from 0.25 to 1 mg/ml. Common oregano EO showed higher values from 2 to 8 mg/ml.

Rezaei et al. [25] tested the antimicrobial activity of coriander EO against five strains of pathogenic bacteria transmitted by food: *Staphylococcus aureus, Escherichia coli, Vibrio cholerae, Salmonella enterica* and *Yersinia enterocolitica*. The antimicrobial activity against the five tested pathogens ranged from 2.5 to 320 µg/ml. The increase in the concentration of EO also caused a significant increase in inhibitory properties. Coriander EO has shown good antimicrobial activity against selected pathogenic bacteria and can also be used as an alternative to synthetic food preservatives without toxic effects. Tao et al. [26] focused on testing of the antimicrobial effects of orange EO on microorganisms commonly associated with spoilage and food safety. The effects were tested against the pathogens *Staphylococcus aureus, Bacillus subtilis, Penicillium chrysogenum, Aspergillus niger, Escherichia coli* and *Saccharomyces cerevisiae*. The results indicated that the EO showed different levels of antimicrobial activity against the tested microorganisms. The inhibitory properties of orange EO were observed in the concentration range from 4.66 µl/ml to 18.75 µl/ml. Maximum activity was observed against *S. aureus* with a MIC of 4.66 μl/ml. *B. subtilis* and *P. chrysogenum* showed MICs of 9.33 μl/ml. A weak inhibitory effect was observed against *S. cerevisiae* and *E. coli* with a MIC of 18.75 μl/ml. Pľuchtová et al. [27] used the minimum inhibitory concentration method as an extension to the disk diffusion method to determine the antimicrobial activity of peppermint EOs. They determined the effects of these oils against gram-negative bacteria *Enterobacter cloacae, Salmonella spp., Klebsiella pneumoniae, Escherichia coli* and gram-positive bacteria such as *Streptococcus pyogenes* and *Staphylococcus aureus*. MICs of both tested peppermint EOs for selected bacteria ranged from 0.625 to 2.5 μg/ml.

Table 4. Minimum inhibitory concentration of G^+ bacteria in μl/ml

G^+	***B. subtilis***		***E. faecalis***		***S. aureus***	
EO/MIC	**MIC50**	**MIC90**	**MIC50**	**MIC90**	**MIC50**	**MIC90**
C. limon	11.56	13.35	11.56	13.35	11.56	13.35
T. serpyllum	23.45	26.15	3.22	5.46	5.56	7.23
C. sativum	11.56	13.35	5.56	7.23	11.56	13.35
Mentha x *piperita*	52.36	55.18	5.56	7.23	5.56	7.23
S. sclarea	23.45	26.15	5.56	7.23	11.56	13.35
R. officinalis	11.56	13.35	23.45	26.15	5.56	7.23
M. recutita	23.45	26.15	52.36	55.18	5.56	7.23
O. vulgare	5.56	7.23	3.22	5.46	1.21	3.18
C. aurantium var. *dulce*	23.45	26.15	23.45	26.15	11.56	13.35
C. cassia	98.64	101.27	23.45	26.15	98.64	101.27

Table 5. Minimum inhibitory concentration of G^- bacteria in μl/ml

G^-	***P. aeruginosa***		***S. enterica***		***Y. enterocolitica***	
EO/MIC	**MIC50**	**MIC90**	**MIC50**	**MIC90**	**MIC50**	**MIC90**
C. limon	5.56	7.23	11.56	13.35	11.56	13.35
T. serpyllum	11.56	13.34	23.45	26.15	11.56	13.35
C. sativum	11.56	13.35	3.22	5.46	5.56	7.23
Mentha x *piperita*	11.56	13.35	23.45	26.15	11.56	13.35
S. sclarea	11.56	13.34	23.45	26.15	3.22	5.46
R. officinalis	23.45	26.15	23.45	26.15	11.56	13.35
M. recutita	11.56	13.35	23.45	26.15	3.22	5.46
O. vulgare	3.22	5.46	11.56	13.35	11.56	13.35
C. aurantium var. *dulce*	98.64	101.27	23.45	26.15	52.36	55.18
C. cassia	52.36	55.18	23.45	26.15	23.45	26.15

Table 6. Minimum inhibitory concentration of yeasts in μl/ml

Yeast	***C. albicans***		***C. glabrata***		***C. krusei***		***C. tropicalis***	
EO/MIC	**MIC50**	**MIC90**	**MIC50**	**MIC90**	**MIC50**	**MIC90**	**MIC50**	**MIC90**
C. limon	1.21	3.18	3.22	5.46	11.56	13.35	11.56	13.35
T. serpyllum	3.22	5.46	3.22	5.46	11.56	13.35	11.56	13.35
C. sativum	5.56	7.23	5.56	7.23	5.56	7.23	5.56	7.23
Mentha x *piperita*	5.56	7.23	5.56	7.23	11.56	13.35	11.56	13.35
S. sclarea	1.21	3.18	0.75	1.13	5.56	7.23	5.56	7.23
R. officinalis	3.22	5.46	11.56	13.35	5.56	7.23	5.56	7.23

M. recutita	11.56	13.35	11.56	13.35	23.45	26.15	3.22	5.46
O. vulgare	3.22	5.46	11.56	13.35	11.56	13.35	11.56	13.35
C. aurantium var. *dulce*	0.75	1.13	5.56	7.23	5.56	7.23	11.56	13.35
C. cassia	98.64	101.27	11.56	13.35	11.56	13.35	11.56	13.35

4. Conclusions

Based on our results, we can conclude that all tested microorganisms were sensitive to EOs. When evaluating the results obtained by the disk diffusion method, we concluded that the largest inhibition zone was achieved by the *Mentha x piperita* and *Thymus serpyllum* EOs against the gram-negative bacterium *Pseudomonas aeruginosa* and gram- positive bacterium *Bacillus subtilis*. The most effective among all tested EOs was *C. aurantium* var. *dulce* EO against *Candida albicans* and *C. sativum* EO against *Candida glabrata*.

Acknowledgements

This research was funded by the grant APVV-20-0058 “The potential of the essential oils from aromatic plants for medical use and food preservation”.

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