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*Małgorzata Nabrdalik, Katarzyna Grata

Antibacterial activity of *Ocimum basilicum* L. essential oil against Gram-negative bacteria

Independent Chair of Biotechnology and Molecular Biology, University of Opole
Head of the Chair: Professor Adam Latała, DVM, PhD

AKTYWNOŚĆ PRZECIWBAKTERYJNA OLEJKU ETERYCZNEGO Z BAZYLII POSPOLITEJ (OCIMUM BASILICUM L.) WOBEC BAKTERII GRAM-UJEMNYCH

STRESZCZENIE

Wstęp. *Bazylia pospolita (Ocimum basilicum L.) jest powszechnie stosowaną w medycynie ludowej rośliną zielną. Ziele bazylii zawiera do 2,5% olejku eterycznego, a składnikami dominującymi są: linalol, 1,8-cyneol, metylochawikol oraz eugenol. Prozdrowotne właściwości olejku bazyliowego związane są między innymi z jego działaniem: przeciwbakteryjnym, przeciwgrzybiczym, przeciwwirusowym oraz przeciwutleniającym.*

Cel pracy. *Celem pracy było określenie aktywności przeciwbakteryjnej olejku bazyliowego wobec bakterii Gram-ujemnych.*

Materiał i metody. *W pracy oceniono wpływ olejku bazyliowego oraz czas jego działania na przeżywalność Gram-ujemnych bakterii. Badanie wrażliwości bakterii na olejek bazyliowy, w stężeniach 0,25-4,0% (v/v), przeprowadzono metodą rozcieńczeń w podłożu bulionowym. Inkubację prowadzono przez okres 4-168 godz., określając liczbę żywych bakterii w 1 ml hodowli. Za MIC uznano najniższe stężenie olejku, które całkowicie hamowało wzrost bakterii.*

Wyniki. *Badania wykazały, że olejek bazyliowy wykazywał wysoką aktywność przeciwbakteryjną, w szczególności wobec Aeromonas hydrophila, Citrobacter freundii, Escherichia coli, Hafnia alvei i Klebsiella pneumoniae. Wartość MIC dla tych szczepów mieściła się w granicach 0,25-1,0% (v/v). Żadne z zastosowanych stężeń olejku bazyliowego nie hamowało całkowicie wzrostu Pseudomonas aeruginosa oraz Salmonella enteritidis.*

Wnioski. *Znacząca aktywność olejku bazyliowego wobec bakterii Gram-ujemnych pozwala uznać go za skuteczny preparat naturalny, który może być stosowany jako składnik preparatów farmaceutycznych i kosmetycznych.*

SŁOWA KLUCZOWE: OCIMUM BASILICUM – OLEJEK ETERYCZNY – BAKTERIE GRAM-UJEMNE – AKTYWNOŚĆ PRZECIWBAKTERYJNA

Introduction

Medicinal plants have been for ages a valuable and an indispensable source of natural products used in the treatment of human diseases and ailments. Their applicability initiated the production of today's pharmaceutical and para-pharmaceutical products. It has been assumed that plant extracts possess biocidal properties against bacteria, fungi and viruses (1-3). The growing interest in the antimicrobial activity of plant extracts results from the necessity of looking for the new products as an alternative to antibiotics, which have limited time of acting effectively. Other reason is the society, which has become more aware of the consequences it may have if antibiotics are applied too often. Moreover, the growing autonomy in the treatment process and an accessibility to herbal products contribute to an annual growth of interest in the plant extracts as an alternative way of therapy. Additional advantage of herbal products application is their lower toxicity to people and higher environmental safety due to the fact that their production process causes less pollution.

Many species of plants, are not only the alternative to synthetic medicines but they are also spices, flavoring agents and an addition to cosmetics. *Ocimum basilicum* L. is an example of such plant. The raw material is a herb containing 0.5-2.5% of essential oils of variable chemical constituents (4). Essential oils are very complex natural mixtures which can contain about 20-60 components at quite different concentrations. They are characterized by two or three major components at fairly high concentrations (20-80%) compared to other components present in a trace amount. Generally, these major components determine the biological properties of essential oils. The components include two groups of distinct biosynthetical origin. The main group is composed of terpenes or terpenoids and the other of aromatic and aliphatic constituents, all characterized by a low molecular weight (1, 5-6).The main components of the essential oils from *O. basilicum* depend on the geographical origin of the plant and include the following: methyl eugenol and eugenol, methyl chavicol, methyl cinnamate, linalool, α-cubebene, 1,8-cineole, nerol, geranial, estragole, *epi*-α-cadinol, α-bergamotene, α-muurolene, 3,7-dimethyloct-1,5--dien-3,7-diol, β-cubebene and β-elemene (7-16).

Essential oils seem to have no specific cellular targets because of the great number of their constituents. The antibacterial properties of these constituents are, inter alia, associated with their lipophilic

character (17). They pass through the cell wall and cytoplasmic membrane, disrupt the structure of their polysaccharides, fatty acids and phospholipids and permeabilize them. Cytotoxicity appears to include such membrane damage. In bacteria, the permeabilization of the membranes is associated with loss of ions and reduction of membrane potential and depletion of the ATP. Essential oils can also damage lipids and proteins. Damage to the cell wall and membrane can lead to the leakage of macromolecules and to lysis (1).

Plant-derived essential oils are known to be active against a wide variety of microorganisms. Cytotoxic effects were observed *in vitro* in most of pathogenic Gram-positive and Gram-negative bacteria generally by a disc diffusion method or a dilution method. In own research, the biocidal activity of *O. basilicum* against selected pathogenic Gram-negative strains was assessed with an application of a dilution method with some modifications. Applied method enabled an assessment of the survival rate of the Gram-negative bacteria under study in reference to the time length of basilium oil activity and its concentration.

Aim

The aim of conducted research was to determine antibacterial activity of basil essential oil against Gram-negative bacteria.

Material and methods

Extraction of the essential oil

For the experiment, the natural basil oil was obtained by a steam distillation of the herb *O. basilicum* L., containing linalool (amounting 62%) as its dominant component. Specimen sample was kept under refrigerated condition for future references.

Bacterial strains and growth conditions

Bacterial strains applied in the experiment had been isolated from infected skin. The identification of selected Gram-negative bacteria was based on their morphological and biochemical features with the use of ID32GN (Biomerieux) tests. In the experiment seven different Gram-negative bacterial strains were applied, as follows: *Aeromonas hydrophila*, *Citrobacter freundii*, *Escherichia coli, Hafnia alvei, Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Salmonella enteritidis*.

All the bacteria used in this study were grown at 37°C in nutrient broth for 24 h. Throughout the experiments, the strains were subcultured every week on TSA (tryptone soya agar) containing (g/l): pepton SP 5.0, pepton K 15.0, sodium chloride 5.0, agar 15.0. The cultures were stored at 4°C. Before their application, liquid cultures prepared from a single colony were transferred twice into fresh nutrient broth and incubated at 37°C for 24 h. to adapt the inoculum's density to ca. 10^8 cfu/ml (turbidity = McFarland barium sulfate standard 0.5).

Antibacterial activity

Antimicrobial activity of the essential oil from *O. basilicum* (EOOB) against bacteria was determined by the modified dilution method. The experiment was run in five replicates with the following concentrations of the essential oil from *O. basilicum* EOOB: 0.25, 0.5, 1.0, 2.0 and 4.0 % (v/v) prepared in the nutrient broth with an addition of 0.05% (v/v) of Tween 80. The solutions were inoculated with bacterial strains under study of 10^8 cfu/ml density obtained after one day of culturing. The control trial contained nutrient broth only, without tested strain, with the same concentration of Tween 80, in order to eliminate its potential inhibition of the growth of the microorganisms. The culturing media were incubated at 37°C for 4, 24, 48 and 168 h. After the incubation time the number of bacteria per 1 ml of the culturing medium (cfu/ml) was determined for both test trials and the control. The number of active bacteria was assessed with the culturing-plate method on TSA medium.

Minimum inhibitory concentration (MIC) was defined as the lowest concentration of EOOB expressed in % (v/v), which inhibits the colonial growth of tested bacteria on TSA medium.

Evaluation of antibacterial effects

The antibacterial activity of EOOB was also expressed as a percentage of the growth reduction. The obtained results underwent statistical analysis of variance (ANOVA) with Duncan's test. Values were considered significantly different at $p < 0.05$.

Results and discussion

The paper presents antibacterial activity of EOOB against selected strains of Gram-negative bacteria. When conducting the research, the concentration of EOOB and the length of its activity on the growth inhibition of tested bacteria were taken into account. It has been noted that every strain reacted differently to EOOB in the medium. After 4 h of culturing on the medium with EOOB at the concentration of 0.25 (% v/v) a slight stimulation of bacterial growth was observed for *E. coli*,

Tab. 1. An effect of basil oil on the amount (log cfu/ml) of Gram-negative bacteria

Bacteria	Control	Essential EOOB (% v/v)				
		0.25	0.5	1.0	2.0	4.0
	Time of action 4 h					
A. hydrophila	8.7190 f	8.2570 e	7.8097 d	2.0000 c	1.7000 b	0.0000 a
C. freundii	8.5800 c	8.6680 c	8.6497 c	6.7310 b	6.7323 b	2.3383 a
E. coli	8.8273 e	8.8417 e	7.7263 d	5.7767 c	4.4617 b	3.8373 a
H. alvei	8.0293 e	8.5440 f	4.9390 d	3.6387 c	1.5143 b	0.0000 a
K. pneumoniae	8.6757 e	7.8510 d	7.7043 d	6.5717 c	5.7240 b	5.4293 a
P. aeruginosa	7.3447 d	7.2703 d	7.2657 d	6.7457 c	6.1470 b	5.1623 a
S. enteritidis	7.6383 c	7.3960 c	7.2557 c	6.3713 b	5.7313 a	5.6243 a
	Time of action 24 h					
A. hydrophila	8.1453 d	3.3180 c	1.5440 b	0.0000 a	0.0000 a	0.0000 a
C. freundii	9.0210 c	1.4217 b	0.0000 a	0.0000 a	0.0000 a	0.0000 a
E. coli	8.9093 b	0.0000 a	0.0000 a	0.0000 a	0.0000 a	0.0000 a
H. alvei	8.9040 d	2.3350 c	2.0907 b	0.0000 a	0.0000 a	0.0000 a
K. pneumoniae	8.8753 c	6.0433 b	0.0000 a	0.0000 a	0.0000 a	0.0000 a
P. aeruginosa	7.6547 d	6.3390 c	6.2560 c	5.6497 b	5.5710 b	3.5960 a
S. enteritidis	7.9357 b	6.0287 a	5.8783 a	5.5903 a	5.6037 a	5.6007 a
	Time of action 48 h					
A. hydrophila	8.3657 b	0.0000 a	0.0000 a	0.0000 a	0.0000 a	0.0000 a
C. freundii	8.9057 b	0.0000 a	0.0000 a	0.0000 a	0.0000 a	0.0000 a
E. coli	9.1450 b	0.0000 a	0.0000 a	0.0000 a	0.0000 a	0.0000 a
H. alvei	9.0513 b	0.0000 a	0.0000 a	0.0000 a	0.0000 a	0.0000 a
K. pneumoniae	9.1340 c	0.0000 a	0.0000 a	0.0000 a	0.0000 a	0.0000 a
P. aeruginosa	8.0257 d	6.2267 bc	6.3877 c	6.2723 bc	6.0863 b	2.7700 a
S. enteritidis	7.4410 c	5.8603 b	5.7923 b	5.5973 a	5.5967 a	5.4660 a
	Time of action 168 h					
A. hydrophila	8.2467 b	0.0000 a	0.0000 a	0.0000 a	0.0000 a	0.0000 a
C. freundii	8.5677 b	0.0000 a	0.0000 a	0.0000 a	0.0000 a	0.0000 a
E. coli	8.6140 b	0.0000 a	0.0000 a	0.0000 a	0.0000 a	0.0000 a
H. alvei	9.1400 b	0.0000 a	0.0000 a	0.0000 a	0.0000 a	0.0000 a
K. pneumoniae	8.6113 b	0.0000 a	0.0000 a	0.0000 a	0.0000 a	0.0000 a
P. aeruginosa	8.0113 c	6.6913 a	6.7923 ab	7.2077 b	7.2400 b	6.3337 a
S. enteritidis	7.4503 e	5.8123 d	4.7837 c	4.1433 ab	4.2413 b	4.0430 a

Note: Different letters indicate significant differences (ANOVA, $p < 0.05$, Duncan's test)

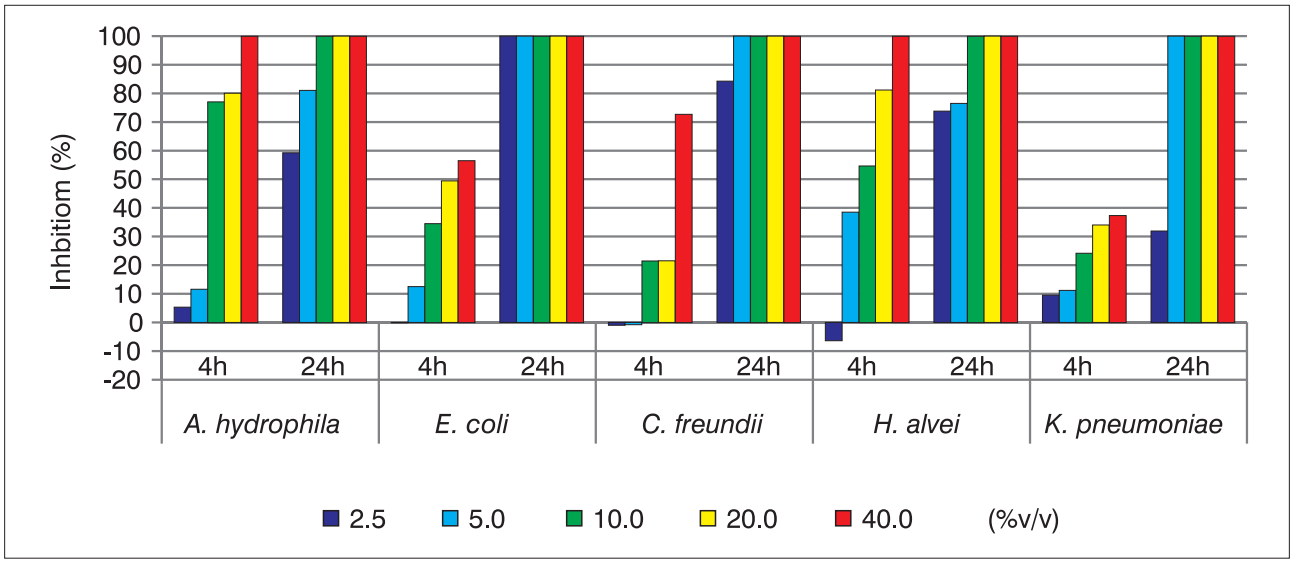

Fig. 1. The time length of EOOB activity against the growth inhibition of Gram-negative sensitive strains

C. freundii and *H. alvei*, amounting 0.16, 1.0 and 6.41%, respectively (tab. 1, fig. 1).

The higher concentration of EOOB resulted in a significant reduction of the bacteria amount in all the trials against control trials. However, the antibacterial activity of EOOB at its highest concentration 4.0 (% v/v) was noted after 4 h only for the following strains: *A. hydrophila* and *H. alvei.* When the time was exceeded to 24 h, EOOB at the concentration of 0.25 (% v/v) inhibited completely the growth of *E. coli,* while *C. freundii* and *K. pneumoniae* at the concentration of 0.5 (% v/v), whereas *A. hydrophila* and *H. alvei* at the concentration of 1.0 (% v/v). The growth inhibition of all sensitive strains have been observed after 48 and 168 h of EOOB treatment even at its lowest concentration. However, regardless of the time, the antibacterial activity of EOOB has not been noted at any of the concentrations for *P. aeruginosa* and *S. enteritidis.* For the both strains, the reduction after 4 h has been recorded between 1 and 30% for *P. aeruginosa* and between 3 and 26% for *S. enteritidis.* After one week the results changed and recorded values were between 16 and 21% and between 22 and 46%, respectively (fig. 2).

On the basis of obtained results, the Gram-negative strains under study were divided into two categories. Strains *A. hydrophila*, *C. freundii*, *E. coli*, *H. alvei* and *K. pneumoniae* may be described as sensitive

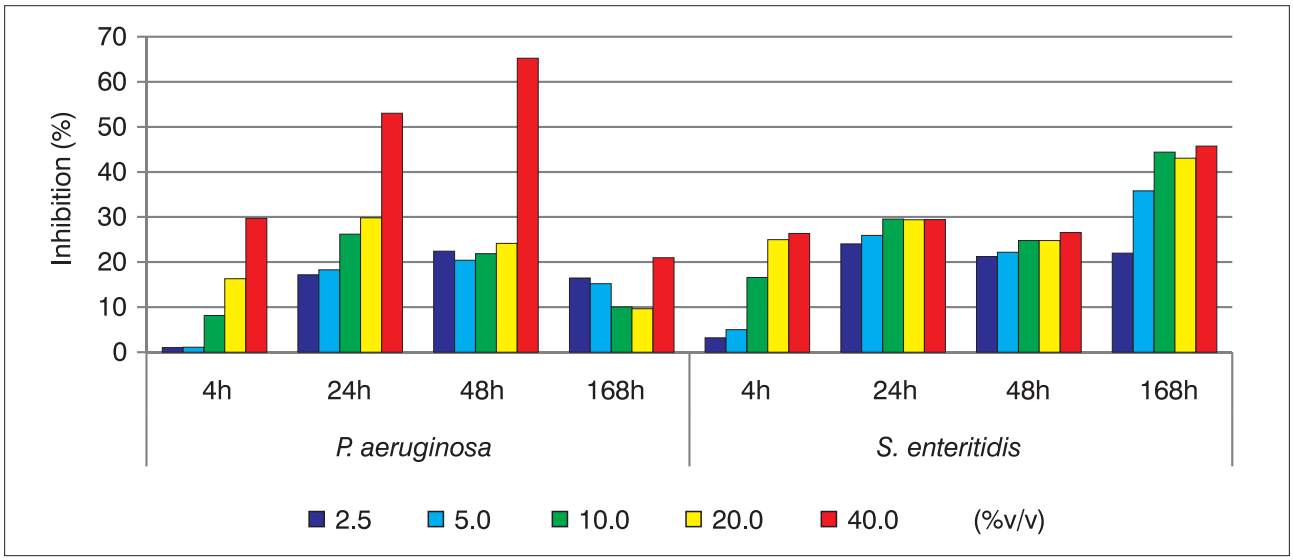

Fig. 2. The time length of EOOB activity against the growth inhibition of Gram-negative resistant strains

strains (fig. 1), whereas strains *P. aeruginosa* and *S. enteritidis* as resistant ones (fig. 2). It has been observed that even the lowest concentration of EOOB has antibacterial properties as long as the time of bacterial exposition to it is longer. However, in case of resistant strains even after one week of culturing on medium with EOOB only significant reduction in bacteria amount has been noted against control trial, instead of the complete growth inhibition. Therefore it can be assumed that the efficiency of essential oils may be determinated as soon as after 24 h when the value of MIC should be specified. The MIC values amount as follows: for *E. coli* 0.25 (% v/v), for *C. freundii* and *K. pneumoniae* 0.5 (% v/v), for *A. hydrophila* and *H. alvei* 1.0 (% v/v).

It is difficult, however, to compare obtained results with other authors' results. First of all, it is known that the composition of essential oils and plant extracts depend on local climatic and environmental conditions (8, 15). Moreover, some essential oils have the same common name while they are extracted from different kinds of plants. Secondly, as shown in other research papers there are different methods applied to determine antibacterial activity as well as different microorganisms employed in the process. Most common methods for determining antibacterial activity are agar disc diffusion and dilution methods. However, there are some limitations when applying the diffusion method, as hydrophobic compounds do not diffuse at the same pace. In many cases, the growth inhibition zones are not observed, due to the limitations of the diffusion method itself not the resistance of the tested strain against applied compound. The method is not recommended to verify the efficiency of poorly soluble compounds in water, such as essential oils or nonpolar extracts. The diffusion method is often restricted only to obtain preliminary qualitative data. Therefore, for the purpose of this research, a modified dilution method was applied and the efficiency of applied essential oils was assessed on the basis of the amount of live bacteria cells. Dilution in liquid medium (nutrient broth) is the most precise technique. This method is recommended for determination of MIC of an extract, essential oil or pure sample and it is the only method for determination of minimum bactericidal concentration.

In author's own research, the two strains *P. aeruginosa* and *S. enteritidis* proved to be resistant to essential oil applied. The analysis of the results noted by other authors concerning the a fore mentioned strains showed that they are most divergent. Similar results to the author's results were recorded by Hammer et al. (18), who have noticed that *P. aeruginosa* and *S. typhimurium* are resistant to most tested essential oils, including *O. basilicum.* The research conducted by Gupta et al. (19) have showed that *P. aeruginosa* and *S. typhi* strains were most resistant to different extracts of *O. basilicum.* However, Opalchenova and Obreshkova (20) have obtained a completely different result when testing the essential oil from *O. basilicum* against strains of *P. aeruginosa* ATCC 9034 and 652 for which the value of MIC determined after 24 h was very low and amounted 0.003% (v/v). Much higher value of MIC for *P. aeruginosa* has been determined by Adeola et al. (21) and amounted 50% (v/v) of applied volatile oil extracted from of *O. basilicum.* The same MIC values have been obtained for *K. pneumoniae* and *E. coli*. Values of MBC for these strains were the same and amounted ≥ 100% (v/v). Ahonkhai et al. (22) have recorded the MIC values for *P. aeruginosa* and *K. pneumoniae* at the level of 10.0 and 0.51% (v/v), respectively. Other authors have reported antibacterial properties of EOOB against strains, marked in author's research as sensitive: *A. hydrophila*, *C. freundii*, *E. coli* and *K. pneumoniae* (23-26). An addition of basil essential oils at the concentration higher than 0.125 (% v/v) (MIC) inhibited completely the growth of *A. hydrophila* in the research by Wan et al. (27). Other authors have noted varied values of MIC from 0.39% (v/v) in case of *C. freundii* (20) to 0.5-50.0% (v/v) in case of *E. coli* and *K. pneumoniae* (17, 18, 20-22). There were no references to antibacterial activity of EOOB against *H. alvei* strain, for which the value of MIC in the author's own research amounted 1.0% (v/v) after 24 h.

As shown in research papers, Gram-negative bacteria are more resistant to essential oils than Gram-positive bacteria (28-30), which is a natural consequence of their cell construction. All Gram-negative bacteria have an outer membrane (OM), which constitutes hydrophilic surface due to the presence of lipopolysaccharide molecule (LPS) (17). Hydrophilic soluble substances of small sizes are able to pass the OM through abundant porin proteins providing hydrophilic transmembrane channels. The OM constitutes, however, the barrier to macromolecules and hydrophobic compounds. Therefore, the Gram-negative bacteria are resistant to hydrophobic antibiotics and toxic medicine. The OM is not completely impermeable for hydrophobic compounds, as some may pass through the porins. Generally, if a compound is to be bacteriocidal against Gram-negative bacteria, it has to pass through the OM (31-32). It has been established that, the antibacterial activity of essential oils is related to their attack on the phospholipids present in the cell membranes, which causes increased

permeability and leakage of cytoplasm, or their interaction with enzymes located on the cell wall (33). What is more, chemical compounds of essential oils have the ability to disrupt the lipid structure of bacterial cell wall, leading to the destruction of cell membrane, cytoplasmic leakage, cell lysis and ultimately cell death. The decrease in pH that occurs due to the cell membrane disruption results in a loss of control of cellular process such as ATP biosynthesis, DNA transcription and protein synthesis (34).

When analysing the EOOB constituents, the above characteristic refers to terpenoids, such as linalool and eugenol. It is linalool and methyl chavicol, the main constituents of EOOB, that are responsible for antibacterial activity (35). The antimicrobial activity of linalool against several microorganisms has also been reported in studies conducted *in vitro.* Bassolé et al. (36) have confirmed, in their studies, antibacterial activity of linalool and eugenol. However, having compared the two compounds, they have found out that eugenol was 3 times stronger when acting against Gram-negative bacteria than linalool. Also Rattanachaikunsopon and Phumkhachorn (37) have proved that constituents of *O. basilicum*, such as linalool, eugenol, 1,8-cineole and α-terpineol have antibacterial properties. Kim et al. (38) have shown the antimicrobial activity of some essential oils components including linalool against 5 Gram-negative bacteria. It is necessary to investigate further and understand the relationship between the antibacterial activity and chemical structure of each compound in the extracts tested. However, it is very difficult to attribute the biological effect of a total essential oil to one or a few active principles, because in addition to the major compounds, also minor compounds may make a significant contribution to the oil activity.

Conclusions

Summing up, the essential oil from *O. basilicum* may be applied as a natural antimicrobial agent. The minimum bactericidal concentration was determined at different concentrations and varied for different species. Among all tested strains, the most sensitive to EOOB were: *E. coli*, *C. freundii*, *A. hydrophila* and *H. alvei* and the resistant strains were: *P. aeruginosa* and *S. enteritidis*. Obtained results suggest, that it is a strain feature, if bacteria is sensitive to essential oil. It also depends on the concentration of the essential oil, the time of its activity and its components. The major components of basil oil vary extensively, depending on genetic factors, geographical origins, nutritional status, the extracted plant materials, extraction methods, and others. The obtained results are useful in the search of more selective and naturally produced antimicrobial compounds.

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Conflict of interest
Konflikt interesów
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Address/adres:
*Małgorzata Nabrdalik
ul. Kard. B. Kominka 6A, 45-032 Opole
tel.: +48 774-016-056
e-mail: mnabrdalik@uni.opole.pl