Anti-biofilm and Antiradical Activity of Different Essential Oils

Miroslava Kačániová[1,2*], Lucia Galovičová[1], Eva Ivanišová[1], Attila Kántor[1], Simona Kunová[1], Margarita Terentjeva[3]

[1]*Slovak University of Agriculture, Faculty of Biotechnology and Food Sciences, Nitra 949 76, Tr. A. Hlinku 2, Slovakia*
[2]*University of Rzeszow, Faculty of Biology and Agriculture, 35-601 Rzeszow, Zelwerowicza St. 4, Poland*
[3]*Latvia University of Life Sciences and Technologies, Faculty of Veterinary Medicine, K. Helmaņa iela 8, LV-3004, Jelgava, Latvia*

Abstract
The formation of biofilms is determined by the type of microrganisms, availability of nutrients and the characteristics of substrate. The aim of the present study was to monitor *Stenotrophomonas maltophilia* biofilm development with matrix-assisted laser desorption ionization time-of-flight mass spectrometry profiling and evaluate the antibacterial, anti-biofilm and antioxidant activity of *Mentha piperita* and *Pimpinella anisum* essential oils against *S. maltophilia*. Biofilms were cultured in polypropylene tubes on the glass slide and wooden toothpick, and were cultivated for 3, 5, 7, 9, 12 or 14 days. Planktonic cells were used as a control. The biofilms were collected from glass slide and wooden toothpick after treatment with essential oils for MALDI-TOF experiment. The study shows that the MALDI profiling allow to recognize detachment of the biofilm cells and different stages of bioflm formation. In conclusion, the MALDI profiling is an effective method in control of biofilms formation.

Keywords: antioxidant activity, anti-biofilm activity, mass spectrometry, *S maltophilia*, essential oils

1. Introduction

Essential oils are volatile substances found of plant origin. They pharmaceutical and sensory abilities place them among the most economically important products with wide applications in pharmaceutical, cosmetic, and food industries. This special raw material is usually extracted by hydrodistillation or steam distillation methods, but essential oil also can be obtained directly by expression and centrifugation of citric fruits. Chemically, essential oils are mainly constituted of monoterpenes and sesquiterpenes, but they also can be constituted by aromatic substances, such as phenylpropanoids, and aliphatic substances [1].

* Corresponding author: Miroslava Kačániová, +42376414494, miroslava.kacaniova@gmail.com

The genus *Mentha* is represented by six species in India of which four species, *Mentha arvensis* L., *Mentha longifolia* (L.) Huds., *Mentha piperita* L., and *Mentha spicata* L. are common in Uttarakhand [2]. Among these *M. longifolia* (Horse mint) is exclusively temperate wild specie with considerable variation in morphology and three varieties of it *M. longifolia* (L.) Huds. var *longifolia*, *M. longifolia* var. *incana* (Willd.) Dinsm., *M. longifolia* var. *royleana* (Benth.) Hook.f. are known between 1200 and 3200 m altitude range in Uttarakhand. *Mentha arvensis* (corn mint), *M. piperita* (Peppermint), and *M. spicata* (Spearmint) are commonly cultivated up to 2500 m altitude as pot herbs, or sometimes occur in a semiwild state. All *Mentha* species have been known to the natives for a long time and are called *Podina* in Uttarakhand. These species are commonly used to provide flavoring and coolant,

in sauces, and are medicinally used in indigestion, vomiting, malarial fever, or as culinary herbs [3].
Pimpinella anisum or anise is one f the most important essential oils-containing plant species, known since ancient times, initially in Egypt. Anise essential oil is usually extracted from dried ripe fruits by hydrodistillation, but it also can be obtained by supercritical fluid extraction. It is mainly constituted by anethole, an aromatic substance that appears as the major compound of the oil, usually corresponding to more than 80% (w/w) of the oil [4-8]. Other substances, such as methyl chavicol, himachalene, linalool, and α-terpineol, are also found as important constituents of this oil [9].
The essential oils have been utilized as a natural medicine to fight against bacterial infection and other diiseases for hundreds years. Several essential oils have been documented for their antibacterial, antifungal, antimutagenic and anti-quorum sensing activities [10-15]. However little efforts have been made to assess their anti-biofilm activity and therapeutic potential against bacterial infection [11-16]. Antimicrobial action of essential oils ensured by the alteration of cell wall and membrane leading to cell lysis leakage of cell contents and inhibition of proton motive force. Many essential oils are relatively low-toxic to mammals with quick degradation making them safe and eco-friendly for applications.
Biofilms formation is a adaptive reaction of microorganisms. They protect bacteria from aggressive environmental factors and predator [17]. In some biofilms the associated express the resistant to antibiotics, contributing the global antimicrobial resistance problem [18-19]. Thus, the research to find the alternative to fight the resistant bacteria is emerged worldwide [20]. In particular, new substances which could be used in alternative treatment of bacterial infections minimizing necessity of traditional antimicrobial therapies. The approach of the present study was to investigate the local medicinal plants with antimicrobial and anti-biofilm properties [21].
Thus, the present study aims to evaluate the anti-biofilm and antioxidant activity of *Mentha piperita* L. and *Pimpinella anisum* L. essential oils against biofilm producing strain *Stenotrophomonas maltophilia*.

2. Materials and methods

2.1. Microorganisms

Stenotrophomonas maltophilia with biofilm formation from milk company was acquired.

2.2. Essential oils

Mentha piperita and *Pimpinella anisum* essential oils from Slovak company Hanus were evaluated. *Mentha piperita* L. essential oil contained limonene (1-3.5%), cineol (3.5-8%), menthon (14-32%), menthofuran (1-8%), isomenthon (1.5-10%), menthylacetate (2.8-10%), isopulegol (max. 0.2%), menthol (30-55%), pulegol (max. 3%), carvone (max. 1%), cineol (min. 2%) and *Pimpinella anisum* L. obtained anethol (80%).

2.3. Free radical scavenging activity

Free radical scavenging activity of samples was measured using 2,2-diphenyl-1-picrylhydrazyl (DPPH). The sample (0.4 mL) was mixed with 3.6 mL of DPPH solution (0.025 g DPPH in 100 mL methanol). Absorbance of the reaction mixture was determined using the spectrophotometer Jenway (6405 UV/Vis, England) at 515 nm. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (10-100 mg/L; R^2=0.989) was used as the standard, and the results were expressed in μg/mL Trolox equivalents [22].

2.4 Minimum biofilm inhibitory concentration (MBIC) assay

The microtiter plate assay and Minimum biofilm inhibitory concentration (MBIC) were used to detect antibiofilm activity of essential oils against *S. maltophilia* biofilm formation [23]. The MBIC test was performed according to Adukwu et al. [24]. The bacterial culture was cultured overnight in Muller Hinton broth (MHB). Then, a 100 μL of suspension with density of 10^8 CFU/mL) was used for inoculation of 96-well plate. A 100 μL of essential oils in concentrations ranging from 0.3125 to 10 μL were added to wells. The MHB was the negative control, while the bacterial suspension without essential oils was used for positive control. The supernatant of the wells was removed and 300 μL of sterile distilled water was used for rinsing. Water was discarded after incubation at 37 °C for 24 h. The microplates were dried for 30 min and stained with 0.1 % (w/v) of crystal violet at room temperature for 30 min,

repeatedly washed with distilled water and dried. Later, 96 % ethanol was added to each plate and the absorbance was read in microplate reader Biotek EL808 with shaker (Biotek Instruments, USA) at 570 nm. MBIC was the concentration of essential oils at which the absorbance was equal to or less than the negative control. The test was done in triplicate and the mean (n=3) was used for further calculation.

2.5. Biofilm development stages by MALDI-TOF MS

For the analyses by MALDI-TOF MS Biotyper two experiments were performed. The first analyze was evaluated to assess whether or not this method would be able to discriminate the stages of biofilm development as a function of the growth time. The second analyze aimed to use MALDI-TOF MS Biotyper to evaluate if biofilms grown in different substrates and essential oils would exhibit any detectable phenotypical distinction. Growing planktonic cells and biofilms: Five polypropylene plastic tubes of 50 ml capacity received 20 ml each of Mueller Hinton (MH) culture medium. A rectangular microscope glass slide and wooden toothpick were placed vertically inside the tube and experimental group with 0.1% EOs. A pre-inoculum of *S. maltophilia* was incubated in MH culture medium at 37 °C during 24 h. Ten ml of the pre-inoculum and EOs were added to the 50 ml polypropylene tubes, which were placed in a shaker with an inclination of 45 °C with a shaking speed of 170 rpm at 37 °C. The biofilms formed in each of the tubes were collected 3, 5, 7, 9, and 12 days after the inoculation. The culture medium of the remaining tubes was replaced in the same intervals. For the analysis of the biofilm, the slide and toothpick were washed two times with ultrapure water and bacteria from the glass slide and wooden toothpick were collected with a sterile toothpick. Planktonic cells were collected by centrifuging 300 ml of the medium of the 5-day tube at 3000 g for 3 min. The supernatant was removed and the pellet was resuspended in ultrapure water and centrifuged again. This procedure was repeated and the resulting pellet was resuspended in 25 ml of ultrapure water (one ml of this suspension was used for each well of the MALDI target plate). MALDI-TOF MS Biotyper: The intact materials (biofilm and planktonic cells) were then spread in 24 wells for each sample in a polished 96-well MALDI target plate (Bruker Daltonics, Germany). One µL of a-cyano-4-hydroxycinnamic acid saturated matrix solution (10 mg/ml) was used to cover every sample and dried at room temperature prior to MALDI-TOF MS Biotyper analysis. After crystallization, the samples were analyzed onto a commercial MALDI-TOF mass spectrometer MicroFlex (Bruker Daltonics, Germany) in the linear and positive mode for a range of m/z 2000-20000. The spectra were acquired automatically using a standard procedure. MALDI Biotyper approach: The similarities among the acquired spectra of the same sample were used for generating a standardized global spectrum (MSP), so all stages of biofilm development were represented by 40 spectra, using the software MALDI Biotyper 3.0 (Bruker Daltonics). From the MSPs of samples, it was generated a dendrogram by the MALDI Biotyper method following standard procedures [25].

2.6 Use of MALDI-TOF MS to evaluate bacterial biofilm growth on glass and on toothpick surfaces

Eighteen propylene tubes of 50 ml were prepared in the same way as described in the previous section. Bacteria materials of three tubes were gathered in each interval of 3, 5, 7, 9, 12, and 14 days. The culture media of the remaining tubes was replaced in the same intervals. However, in this experiment, the biological materials grown on glass and toothpick were collected separately with a sterile toothpick, and spread over 12 wells of the MALDI-TOF target plate. Furthermore, from the tubes of the 14th day, only planktonic cells were collected in the same way as described in the previous section, and one ml of the material was spread over each of the 36 wells. All MALDI target plate wells were always covered with one ml of a-cyano-4-hydroxycinnamic acid and analyzed within 24 h. In summary, 36 spectra were acquired in every group, and one was chosen from these as the most representative based on its common features and displayed for comparison among the experimental groups using FlexAnalysis 3.0 software (Bruker Daltonics). With the MALDI Biotyper 3.0 software, 11 MSPs were created, and subsequently clustered by a dendrogram using Euclidean distances [25].

3. Results and discussion

3.1 Antioxidant activity of EOs

The reduction ability of DPPH radicals' formation was the decrease in absorbance at 517 nm induced by antioxidants. The highest antioxidant activity was observed at *Mentha piperita* (59.56±2.75µg TEAC/ml) and lower at *Pimpinella anisum* (28.45±3.44 µg TEAC/ml). The same results with same essential oils was described in study Kačániová et al. [26].

3.2 Anti-biofilm activity with MBIC

The MIC values of essential oil of *Mentha piperita* was 2.5 µl/mL and essential oil of *Pimpinella anisum* was 2.5 µl/mL. The same results of *M. piperita* was found in study of Tutar et al. [27] against *A. baumani*.

In the study of Bazargani and Rohloff [28] the anti-biofilm activity against *E. coli* and *S. aureus* was 17.4%.

3.3 Anti-biofilm activity with MALDI-TOF MS

The spectra chosen as representative for each biofilm development stage in this experiment are shown in Figures 1-6. With the exception of the spectrum representing planktonic cells, which were obtained from the culture medium, the remaining spectra were grouped in pairs according to their time of growth, so that the biofilms obtained from different surfaces could be viewed together.

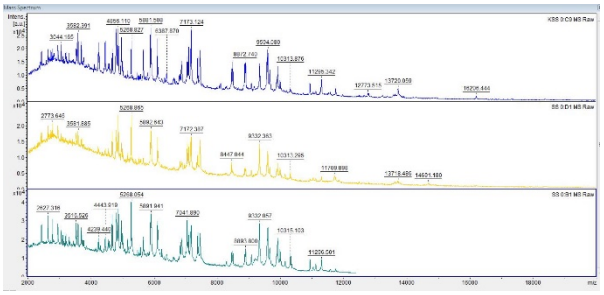

Figure 1 Representative MALDI-TOF mass spectra from the second experiment chosen for the different *S. maltophilia* biofilm formation stages after 3 days

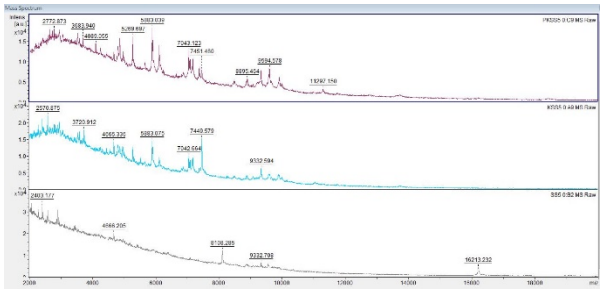

Figure 2 Representative MALDI-TOF mass spectra from the second experiment chosen for the different *S. maltophilia* biofilm formation stages after 5 days

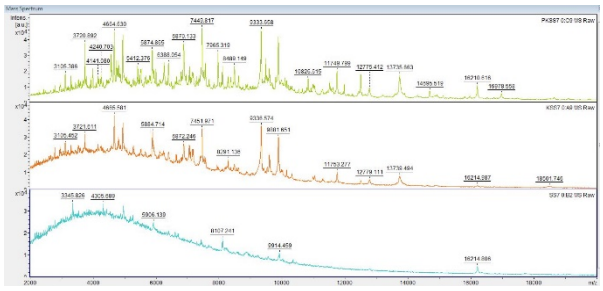

Figure 3 Representative MALDI-TOF mass spectra from the second experiment chosen for the different *S. maltophilia* biofilm formation stages after 7 days

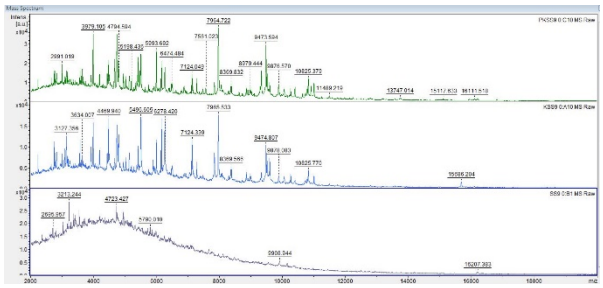

Figure 4 Representative MALDI-TOF mass spectra from the second experiment chosen for the different *S. maltophilia* biofilm formation stages after 9 days

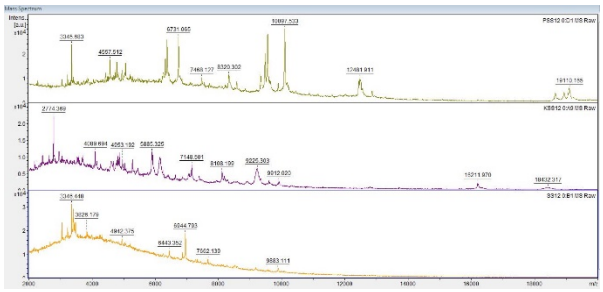

Figure 5 Representative MALDI-TOF mass spectra from the second experiment chosen for the different *S. maltophilia* biofilm formation stages after 12 days

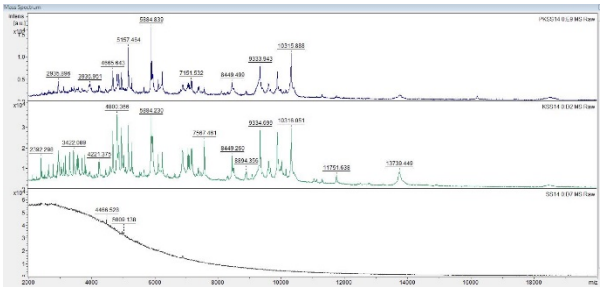

Figure 6 Representative MALDI-TOF mass spectra from the second experiment chosen for the different *S. maltophilia* biofilm formation stages after 14 days

3.4 Biofilm development stages by MALDI-TOF MS

Figures 7-9 presents the dendrogram generated by the first MALDI-TOF MS Biotyper experiment. These results indicated that the MSPs of the groups were distinguishable by the use of MALDI profiling approach, since they have been separated into different clusters. It can be observed that the planktonic stage of *S. maltophilia* showed the lowest difference, in terms of MSP distance level, from the 7- and 12-day biofilms on wooden toothpick and 7- and 14-day biofilms on glass surface.

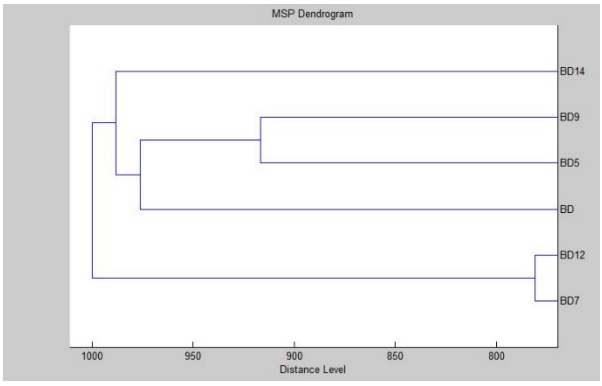

Figure 7. Dendrograms generated using the MSPs of *S. maltophilia* biofilms at different developmental stages collected from the glass surface

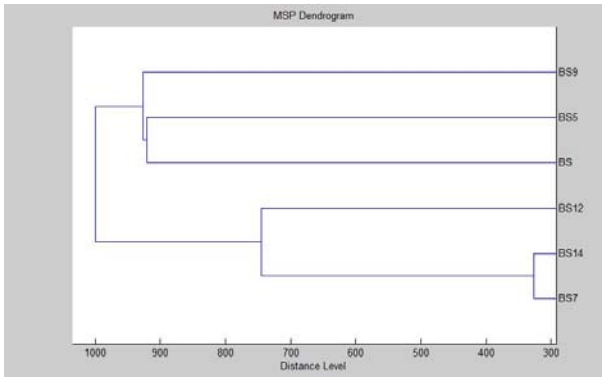

Figure 8. Dendrograms generated using the MSPs of *S. maltophila* biofilms at different developmental stages collected from the wooden toothpick

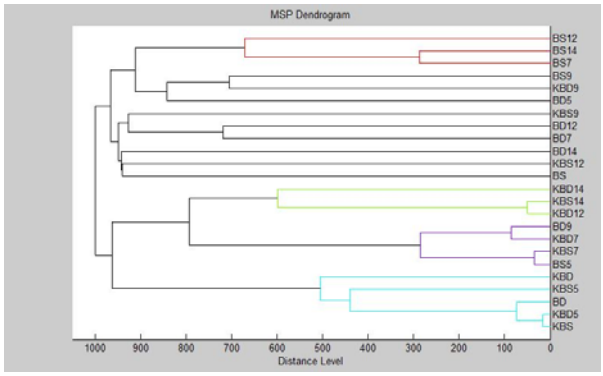

Figure 9. Dendrograms generated using the MSPs of *S. maltophila* biofilms at different developmental stages collected from both the glass surface and wooden toothpick

Pereira et al. [25] studied bacterial biofilm of *Pseudomonas aeruginosa* representing clinical relevance. The initial adhesion of cells to surfaces is of crucial importance in formation of biofilm. The characteristics of the contact surface for biofilm formation have great influence on adhesion. In the present study, we applied the MALDI-TOF technology for assessment of *S. maltophilia* biofilm development. In the present study, two different experiments involving MALDI-TOF were performed by growing and collecting of biofilms from glass slide, toothpick in polypropylene tube. The results showed that MALDI-TOF technology allowed to recognized different biofilm development stages. MALDI-TOF could serve as a tool for prediction of formation and control of biofilms.

4. Conclusions

MALDI-TOF MS is a reliable method for microorganisms identification. In the present study, instead of focusing on different bacteria species, we decided to apply a MALDI-TOF profiling method to evaluate different stages of *S. maltophilia* biofilm development and compare such results with the morphological behavior of this bacteria biofilm. The results showed not only that the approach is sensitive enough to detect phenotypical changes in the biofilm progression, but also is able to detect some distinct characteristics related to the surface on which the bacteria were grown.

Acknowledgements
The study was supported by the European Community project No 26220220180: Building Research Centre „AgroBioTech" and by the grants APVV-15-0544.

References

1. Bakkali, F., Averbeck, S., Averbeck, D., Idaomar, M., Biological effects of essential oils – a review, Food and Chemical Toxicology, 2008, 46, 446-475
2. Uniyal, B.P., Sharma, J.R., Choudhery, U., Singh, D.K., Flowering Plants of Uttarakhand (A Checklist), Bishen Singh Mahendra Pal Singh, Dehradon, 2007, 404 p.
3. Gaur, R.D., Flora of the District Garhwal Northwest Himalaya (with Ethnobotanical Notes), Kew Bulletin, 2001, 56(1), 251
4. Brasil, Farmacopeia Brasileira. Agência Nacional de Vigilância Sanitária, Anvisa, Brasília. 2010, 2, 808 p.
5. Samojlik, I., Mijatović, V., Petković, S., Škrbić, B., Božin, B., The influence of essential oil of aniseed (*Pimpinella anisum*, L.) on drug effects on the central nervous system, Fitoterapia, 2012, 83, 1466-1473
6. Özel, A., Anise (*Pimpinella anisum*): changes in yields and component composition on harvesting at different stages of plant maturity, Experimental Agriculture, 2009, 45, 117-126
7. Rodrigues, V.M., Rosa, P.T.V., Marques, M.O.M., Petenate, A.J., Meireles, M.A.A., Supercritical extraction of essential oil from aniseed (*Pimpinella anisum* L) using CO2: solubility, kinetics, and composition data, Journal of Agriculture and Food Chemistry, 2003, 51, 1518–1523.
8. Ullah, H., Honermeier, B., Fruit yield, essential oil concentration and composition of three anise cultivars (*Pimpinella anisum* L.) in relation to sowing date, sowing rate and locations, Industrial Crops and Products, 2013, 42, 489-499
9. Matos, F.J.A., Constituintes químicos ativos e propriedades biológicas de plantas medicinais brasileiras, Editora UFC, Fortaleza, 2004, 448 p.
10. Nelson, R.R.S., *In vitro* activities of five essential oils against methicillin-resistant *Staphylococcus aureus* and vancomycin-reistant *Entrococcus faecium*, Journal of Antimicrobial Chemotherapy, 1997, 40, 305-306
11. Nostro, A., Marino, A., Blanco, A.R., Cellini, L., Di Giulio, M., Pizzimenti, F., Roccaro, A.S., Bisignano, G., *In vitro* activity of Carvacrol against Staphylococcal preformed biofilm by liquid and vapour contact, Journal of Medicinal Microbiology, 2009, 58, 791
12. Khan M.S.A., Zahin, M., Hasan, S., Husain, F.M., Ahmad, I., Inhibition of quorum sensing regulated bacterial functions by plant essential oils with special reference to clove oil, Letters of Applied Microbiology, 2009, 49, 354-360
13. Khan, M.S.A., Ahmad, I., *In vitro* antifungal, anti-elastase and anti-keratinase activity of essential oils of Cinnamomum, Syzygium and Cymbopogon-species against *Aspergillus fumigatus* and *Trichophyton rubrum*, Phytomedicine, 2011, 19, 48-55
14. Kavanaugh, N.L., Ribbeck, K., Selected antimicrobial essential oils eradicate *Pseudomonas* spp. and *Staphylococcus aureus* biofilms, Applied Environmental Microbiology, 2012, 78, 4057-4061
15. Husain, F.M., Ahmad, I., Asif, M., Tahseen, Q., Influence of clove oil on certain quorum sensing regulated functions and biofilm of *Pseudomonas aeruginosa* and *Aeromonas hydrophila*, Journal of Biosciences, 2013, 38, 1-10
16. Singh, J., Chhikara, B.S., Comparative global epidemiology of HIV infections and status of current progress in treatment, Chemical and Biological Letters, 2014, 1(1), 14-32
17. Batoni, G., Maisetta, G., Brancatisano, F.L., Esin, S., Campa, M., "Use of antimicrobial peptides against microbial biofilms: Advantages and limits," Current Medicinal Chemistry, 2011, 18(2), 256-279
18. Gupta, P., Sarkar, S., Das, B., Bhattacharjee, S., Tribedi, P., "Biofilm, pathogenesis and prevention—a journey to break the wall: a review," Archives of Microbiology, 2016, 198(1), 1-15
19. Takaine, M., Imada, K., Numata, O., Nakamura, T., Nakano, K., "The meiosis-specific nuclear passenger protein is required for proper assembly of forespore membrane in fission yeast", Journal of Cell Science, 2014, 127(20), 4429-4442
20. Petrovska, B.B., "Historical review of medicinal plants' usage," Pharmacognosy Reviews, 2012, 6(11), 1-5
21. Bakkali, F., Averbeck, S., Averbeck, D., Idaomar, M., "Biological effects of essential oils—a review", Food and Chemical Toxicology, 2008, 46(2), 446-475
22. Sánchés-Moreno, C., Larrauri, A., Saura-Calixto, F., A procedure to measure the antioxidant afficiency

of polyphenols, Journal of Science and Food Agriculture, 1998, 76, 270-276
23. Ceri, H., Olson, M.E., Morck, D.V., Storey, D.G., Minimal biofilm eradication (MBEC) assay: susceptibility testing for biofilms, In: Pace, J.L., Rupp, M.E., Finch, R.G., editors, Biofilms, infection, and antimicrobial theraphy, CRC Pres, Boca Raton, 2006, pp. 257-269
24. Adukwu, E.C., Allen, S.C., Phillips, C.A., The anti-biofilm activity of lemongrass (*Cymbopogon flexuosus*) and grapefruit (*Citrus paradisi*) essential oils against five strains of *Staphylococcus aureus*, Journal of Applied Microbiology, 2012, 113(5), 1217-1227
25. Pereira, F.D.E.S., Bonatto, C.C., Lopes, C.A.P., Pereira, A.L., Silva, L.P., Use of MALDI-TOF mass spectrometry to analyze the molecular profile of *Pseudomonas aeruginosa* biofilms grown on glass and plastic surfaces, Microbial Pathogenesis, 2015, 86, 32-37
26. Kačániová, M., Terentjeva, M., Vukovic, N., Puchalski, C., Roychoudhury, S., Kunová, S., Kluga, A., Tokár, M., Kluz, M., Ivanišová, E., The antioxidant and antimicrobial activity of essential oils against Pseudomonas spp. isolated from fish, Saudi Pharmaceutical Journal, 2017, 25(8), 1108-1116
27. Tutar, U., Çelik, C., Karaman, I., Ataş, M., Hepokur, C., Anti-biofilm and antimicrobial activity of *Mentha pulegium* L. essential oil against multidrug-resistant *Acinetobacter baumannii*, Tropical Journal of Pharmaceutical Research, 2016, 15(5), 1039-1046
28. Bazargani, M.M., Rohloff, J., Anti-biofilm activity of essential oils and plant extracts against *Staphylococcus aureus* and *Escherichia coli* biofilms, Food control, 2016, 61, 156-164.