Investigation of the antibacterial effect of essential oils using *in vitro* methods

PhD Thesis

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Pécs, 2022

1. Introduction

Antibiotic resistance, which is related, among other things, to the biofilm-forming characteristic of bacteria, causes a lot of problems in healthcare today. In order to eliminate this problem, it is necessary to search new, effective agents. The antibacterial effect of essential oils is known, but in order to be able to use them with sufficient safety, it is vital to thoroughly study their mechanism of action, to determine the exact dose required for the antibacterial effect, and to verify this with appropriate microbiological tests. It is important to mention that most of the published publications so far did not investigate the antibacterial effect of essential oils and components against respiratory pathogens. The significant expansion of bacterial respiratory diseases is highly attributed to the spread of antibiotic resistance and the formation of bacterial biofilms. When bacteria form a biofilm, they are much more resistant to adverse environmental factors, e.g. compared to disinfectants and antibiotics, unfavorable changes in pH and extreme fluctuations in temperature are better "protected" this way. The biofilm is a protective structure that forms a mechanical and functional barrier surrounding the bacterial cells, which is difficult to eradicate with currently used therapeutic options (e.g. with administration of antibiotics). In addition, biofilm formation helps the development of antibiotic resistance to a great extent, since the antibacterial agents that break through the biofilm are sufficient for the given strain to develop resistance to it. The facts show that the above-mentioned factors can be used in the background of the development of chronic diseases (Singh et al., 2017). Next, it is absolutely important to examine natural substances in addition to synthetic active ingredients, which makes it possible to prevent and/or suppress the formation of bacterial biofilm. Using essential oils is not new, as they have been widely used since the Middle Ages. Therefore, they proved to be suitable for protection against epidemics, they were used to relieve fever, headache and cough, and they even played an important role in religious life (Burt, 2004). Nowadays, interest in essential oils is experiencing a renaissance, as more and more people are turning to the use of natural substances. However, in order to use essential oils with sufficient efficiency and safety, a thorough understanding of their effect on living cells and the body is essential.

Based on the above mentioned facts, we focused on the antibacterial and antibiofilm effect of peppermint, cinnamon bark, thyme and clove essential oils using *in vitro* microbiological methods. The essential oils were selected on the one hand because of their frequent use, and on the other hand, based on the preliminary test results of the Essential Oil Research Group of the Department of Pharmacognosy of University of Pécs (Ács et al., 2018). Since essential oils are lipophilic, we have also carried out technological developments that improve the bioavailability and biological effect of essential oils. Our experimental results can provide a suitable basis for the use of essential oils as antibacterial agents.

2. Aims

At the beginning of our work, the following goals were aimed:

• Determination of the composition of the essential oil samples (peppermint, cinnamon bark, thyme, cloves) using GC-MS (gas chromatography-mass spectrometry), and GC-FID (gas chromatography coupled with flame ionization detector) techniques.

• Examination the antibacterial effect of essential oils and their components (menthol, cinnamic aldehyde, thymol, eugenol) in a direct bioautographic system. Since bacteria with special nutrient requirements were part of our research, it was also necessary to optimize this method.

• Performation preparative layer chromatography in order to precisely determine which essential oil components are responsible for the antibacterial effect.

• Determination of the minimum inhibitory concentration (MIC) of the tested essential oil samples in a microdilution system against the test bacteria included in the tests.

• Studying the effect of the four most frequently used essential oils included in our tests on biofilm formation.

• Since essential oils are lipophilic, testing them in water-based bacterial systems is difficult. Therefore, to increase the better bioavailability of our essential oil samples, we also aimed to make the nanotechnological formulation of them.

• Comparization the effect on biofilm formation of the essential oil samples packed with the nanotechnological method (Pickering emulsion) and the essential oil samples prepared with alcohol and Tween excipients.

• Bacteria can communicate with each other through quorum sensing systems, thus helping to increase their resistance. Our goals included testing the anti-quorum sensing effect of our essential oils.

• Making scanning electromicroscopic images, in order to clearly establish the effect of essential oil samples on biofilm formation.

3. Material and methods

3.1. Determination the chemical composition of essential oils

The gas chromatographic measurements were performed by Dr. Andrea Böszörményi, from Semmelweis University, Institute of Pharmacognosy. To determine the composition of the essential oils, the retention times obtained for each component and the mass spectra were taken into account, which were compared with the values of standards and the data of the NIST 2.0 library. Area normalization was used to determine the percentage values (Adams, 2001). The parameters of the GC-MS and GC-FID measurements are summarized in Table 1.

Table 1. Parameters of GC-MS and GC-FID analyses

	GC-MS	**GC-FID**
Equipment:	Agilent 6890N	Fisons 8000
Column:	30 m x 0.25 mm i.d, Agilent SLB-5MS (film thickness 0.25 µm)	30 m x 0.25 mm Rt-β-DEXm (film thickness 0,25µm)
Program:	60˚C 3 min, 8˚C/min 60-250˚C, 250˚C 1 min	8˚C/min 60-230˚C, 230˚C 5 min
Gas:	high pirity Helium 6.0, 1.0 mL/min, (37 cm/s), constant flow mode	Nitrogen, 6.8 mL/min
Injektor:	250˚C	210˚C
Injection:	Split ratio 1:50	0.2 ml, 0,1% solution, splitless
Detector:	5973N (MS)	FID, 240˚C

3.2. Microbiological tests

Respiratory bacteria included in our studies are: *Haemophilus influenzae* (DSM 4690), *H. parainfluenzae* (DSM 8978), *Streptococcus pneumoniae* (DSM 20566), *S. mutans* (DSM 20533), *Pseudomonas aeruginosa* (ATCC 27853).

The bacteria were cultured under the following conditions: In the case of *H. influenzae* and *H. parainfluenzae* strains, a special nutrient solution was used. 500 µl *Haemophilus* supplement B (Diagon Kft.) and 750 µl (1 mg/ml) NAD solution were added to 3750 µl of Mueller-Hinton II Broth (Reanal Laborvegyszer Kereskedelmi Kft.). For *S. pneumoniae* (DSM 20566), *S. mutans* (DSM 20533) and *P. aeruginosa* (ATCC 27853), we used Brain Heart Infusion Broth (Sigma-Aldricht Kft.) (Balázs et al., 2019).

3.2.1. Direct Bioautography

We detected the inhibition of essential oils and their main components on bacteria using the direct bioautographic method. The inhibition zone formed on the layer corresponds to the antibacterial effect of the substance examined. As a first step, the substance to be tested (antibiotic, essential oil, essential oil component) was applied to the silica gel layers cut to size (10 x 5 cm) using a Finnpipette pipette (Merck TLC Silica gel 60 F254). The stock solution of the essential oil was 200 mg/mL, dissolved in absolute ethanol, of which 0.5 and 1 µL were applied to the layer. The main components of the essential oils (eugenol-clove, cinnamic aldehyde-cinnamon bark, menthol-peppermint, thymol-thyme; Sigma Aldricht Kft.) were applied to the layers from a 20 mg/mL stock solution, 0.5 µL for each standard. Amikacin (Likacin 250 mg/ml solution for injection, Lisapharma S.p.A.) was used as a positive control for *Streptococcus* and *Pseudomonas* strains, of which 0.4 µL was applied to the layers. In order to inhibit *Haemophilus* strains, gentamicin (Sandoz 40 mg/mL solution for injection, Sandoz) was used as a positive control, 2.5 µL of which was applied to the layer. In order to exclude the activity of the solvent, 0.5 µL of absolute ethanol was applied to the layers.

In addition to the total essential oil test (when the layers had not been developed), we were curious about which components of the essential oils had an antibacterial effect. We have developed the layers (developing agent: in the case of peppermint, thyme, clove essential oil: toluene: ethyl acetate 95:5 (v/v); in the case of cinnamon bark essential oil: dichloromethane). The layers that were not subjected to microbiological examination were detected at 256 nm (Camag UV lamp) and evaluated in visible light after incubation at 105°C (5 min) using vanillin-sulfuric acid developer (Wagner & Blandt, 1996). The microbiological tests were performed with the layers that were not developed.

The first step of the microbiological tests is the exact setting of the germ count (4 x 10^7 CFU/mL) using optical density measurement at 600 nm. Afterwards, the layers were dipped in the bacterial suspension, and after drying, they were incubated in a vapour chamber (37ºC, 3 h). After that, an aqueous solution of MTT dye [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] (MTT, 0.05 g/90 mL) (Sigma-Aldricht Kft.) was applied in order to visualize the inhibition zones. The software Excel was used for further analysis of the data. In order to get an accurate picture of which components result in a microbiological effect, the component/components forming the inhibition zones were subjected to a preparative chromatography test. The bands resulting in inhibition zones were identified based on Rf values and color reaction with vanillin-sulfuric acid. Then, the essential oil samples were applied in an amount of 10 µL (stock solution: 200 mg/mL) to the thin layer, after development, the silica layer was "scraped off" in appropriate zones (based on previously determined Rf values), then the fractions were collected in a separate glass and sent for GC analysis.

3.2.2. Determination of minimum inhibitory concentration (MIC)

The minimum inhibitory concentrations (MIC) were determined using the microdilution method on 96-cell microtiter plates (Kerekes et al., 2013). The minimum inhibitory concentration was defined as the lowest concentration of the essential oil that, after the incubation period, was able to inhibit the growth of the bacteria by >90% compared to the control.

After setting the appropriate germ count (10^5 CFU/ml), 100-100 µL of both the bacterial suspension and the tested essential oil were measured into the wells of the microplate. The test substance was added dissolved in a nutrient solution. The essential oil samples were prepared using three different methods. Tween80 emulsifier (1%), absolute ethanol, and Pickering's emulsion were used to be dissolved in a water-based bacterial suspension. Thus, for each essential oil sample, we performed the tests with all three types of solutions with six repetitions. After the addition of the test sample and incubation (24 hours, 37°C), absorbance at 600 nm was measured (BMG Labtech, Bio-Tek Kft.). The cell suspension medium without essential oils served as a positive control, and the cell-free medium containing essential oils served as a negative control. In order to eliminate the wall effect, we did not use the outermost holes forming the frame of the microplate. The mean was calculated from the six replicates, and then the average of the negative control was subtracted from the obtained value. The MIC value was considered to be the concentration at which values reduced to ± 10% compared to the absorbance of the positive control were measured. During our tests, we used antibiotics as a positive control. For *P. aeruginosa*, gentamicin (Gentamicin Sandoz 80 mg/2ml injection, Sandoz), for *S. pneumoniae*, imipenem (Imipenem/Cilastatin Kabi 500 mg/500 mg powder solution for infusion; stock solution: 0.4 mg/ml), for *S. mutans* amoxicillin/clavulanic acid (Aktil 1000 mg/200 mg powder for solution infusion, Gedeon Richter) and for *Haemophilus* strains, amikacin (Likacin 250 mg/ml solution injection, Lisapharma S.p.A.) antibiotics were used.

3.2.3. Production of Pickering emulsions

The preparation of O/W (Oil/Water) type Pickering emulsions stabilized by silica nanoparticles and containing essential oil was prepared by the Department of Pharmaceutical Technology and Biopharmacy based on the following description (Horváth, 2021). In the first step, hydrophilic silica nanoparticles with a size of 20 nm were produced by Stöber synthesis. In order to achieve adequate stability, based on preliminary experimental work, the surface modification of the silica nanoparticles was carried out with ethyl functional groups (20ET). To formulate the emulsions, an aqueous suspension of 20ET nanoparticles was first prepared, and then the appropriate amount of essential oil was added. After 2 minutes of ultrasonic pre-emulsification, the emulsions were homogenized with an UltraTurrax (IKA Werke) device at a speed of 13.500 rpm. The droplet size of the resulting Pickering emulsions was determined by DLS measurement (Malvern Zetasizer NanoS), and the change in droplet size was followed in time to establish their stability. All of the Pickering emulsions used for biofilm inhibition were stable for at least 1 week. Conventional emulsions were also prepared analogously to the above mentioned, whose droplet size and stability were also investigated. In this case, the stabilizing agent of the emulsions was Tween80 (Polysorbate80) surfactant. The independent application of absolute ethanol, Tween80 and Pickering emulsions without essential oil was part of their negative control test. Six replicates of each measurement were performed.

3.2.4. Investigation of the effect of essential oils and their components on biofilm formation

Bacterial biofilms were formed on 96-cell polystyrene microtiter plates (VWR, International Kft., Debrecen, Hungary). 200 µL of a cell suspension with a cell count of 10^8 CFU/ml was measured into one cell. After incubation (4 h, 37°C), the non-adherent cells were washed with physiological saline solution (9 g NaCl, 1000 ml distilled water), and the sample to be tested was added to the adhered cells. After incubation (24 h, 37°C) and washing with physiological saline, 200 µL of 99% v/v methanol was measured for each sample site. After waiting for 15 minutes, 200 µL of 0.1% crystal violet solution was measured into the cells. After 20 minutes, the dye bound to the biofilms was dissolved with a 33% acetic acid solution and absorbance was measured at 595 nm (BMG Labtech, Bio-Tek Kft.) using a plate reader. Crystal violet binds to negatively charged surface molecules and polysaccharides within the extracellular matrix of biofilms, thus allowing measurement of the total biomass of the biofilm in the cell of the microtiter plate (Peeters et al., 2008). The essential oils were used at a concentration of MIC/2.

3.2.5. Examination of biofilms using a scanning electron microscope (SEM)

SEM was used to visualize the structural modifications of biofilms after treatment of EO samples. For biofilm formation, 5 ml of each bacterial culture (10^8 cfu/mL) was added into sterilized bottle. Sterile coverslips were placed in the bottle and served as the attaching surface for the cells. The plates were incubated for 4 h at 37°C, then the planktonic cells and BHI were washed out. For treatment of developing biofilms, 5 ml from MIC/2 EO was added. The untreated coverlips were used as control. After incubation (24 h, 37°C), the supernatant was removed, and the bottles were washed with physiological saline. The preparation of the samples for electron microscopy was performed with 2.5% glutaraldehyde for 2 h at room temperature (RT) to fix the biofilms formed on the coverslips. For dehydration of biofilms, different ethanol concentrations (50, 70, 80, 90, 95, 98%) were used at room temperature for 2 x 15 min. Finally t-butyl-alcohol: absolute ethanol mixed in 1:2, 1:1 and 2:1 ratios were added to the samples (each case for 1 h, RT). Then the samples were dehydrated with absolute t-butyl alcohol for 2 h (RT). The samples were stored at 4°C for 1 h and freeze-dried overnight. The sample was coated with a gold membrane and observed with Hitachi S4700 scanning electron microscope. The investigation took place at the Department of Applied and Environmental Chemistry Department of SZTE.

3.2.6. Examination of the quorum sensing (QS) mechanism

In order to detect the anti-quorum sensing (anti-QS) effect, a paper disc diffusion method was used. The inhibition of the quorum sensing mechanism can be clearly illustrated with pigment-producing bacteria, so we performed our tests with the model bacterium Chromobacterium violaceum (SZMC 6269). In order to detect QS inhibition, sterile filter paper discs with a diameter of 6 mm were placed on Petri dishes containing previously inoculated BHI medium with C. violaceum 85 WT strain. 2 µL of concentrated essential oil (peppermint, cinnamon bark, thyme, clove) was applied to these, and 2.5 and 10 mg/mL dilutions were also prepared, of which 2 µL was also applied to the disks. We were also curious about the activity of the main components of the essential oils, so we prepared 10 mg/ml stock solutions for all four of the main components of the essential oil detected in the largest quantity, (of which 2 µL was placed on the disks (Kerekes et al., 2013; Kerekes, 2017). In the case of both the diluted essential oils and some of the main components, the solvent was absolute ethanol (Molar Chemicals Kft.), as a negative control, we used paper discs soaked in absolute ethanol. We could observe the formation of two circular zones around the paper discs in the case of all four test substances. The inner area can be said to be a complete purification zone, since we could not detect bacterial cells in the given area, while in the outer zone the bacterium grows, but the pigment production shows an inhibition. The colorless zones caused by depigmentation were measured (mm), because the production of pigment by

the bacteria is under QS control. The treatments were carried out with six parallel measurements (Kerekes, 2017).

4. Results and conclusions

4.1. Chemical analysis of the tested essential oils

The components of the essential oils and the percentages measured with the MS detector are summarized in Table 2, in which the components above 1% are listed.

Table 2 Analytical analysis of the essential oils included in the study in case of liquid injection (GC-MS)

		Percentage value of essential oil components (%)			
Components	**RI**	peppermint	cinnamon bark	thyme	clove
α-pinene	939	1.1	5.1	-	-
camphene	951	-	-	2.0	-
ß-myrcene	992	-	-	1.0	-
α-terpinene	1017	-	-	3.2	-
p-cimol	1026	-	1.9	19.2	-
limonene	1044	1.4	1.8	-	0.5
1,8-cineole	1046	5.5	2.8	4.6	-
γ-terpinene	1060	-	-	6.7	-
linalool	1104	-	4.0	5.6	-
isopulegon	1150	1.0	-	-	-
menton	1156	19.8	-	-	-
isomentone	1159	7.0	-	-	-
menthol	1172	50.4	-	-	-
isomenthol	1183	4.3	-	-	-
α-terpineol	1190	-	2.2	-	-
transz-cinnamic aldehyde	1266	-	64.7	-	-
borneol	1289	-	-	1.0	-
thymol	1297	-	-	39.8	-
carvacrol	1300	-	-	5.9	-
isomenthyl-acetate	1305	5.5	-	-	-
eugenol	1373	-	4.6	-	78.8
ß-caryophyllene	1417	-	4.2	4.2	13.5
cinnamyl acetate	1446	-	9.4	-	-
α-humulene	1452	-	-	-	4.6
ß-cadinene	1473	-	-	-	1.1
Sum		**98.1**	**99.7**	**93.2**	**98.5**

4.2. Results of bioautographic studies

Based on our bioautographic tests, it can be said that among the essential oils, the essential oils of thyme and cinnamon bark resulted in the largest inhibition zones, whereas peppermint was the least effective. *H. influenzae* reacted most sensitively to peppermint essential oil, as we could detect the largest inhibition zones for this bacterium (Table 3). This was followed by *S. mutans*, which also showed more significant inhibition. Clove essential oil follows the activity of peppermint in terms of effectiveness. The largest zone of inhibition was observed for *S. pneumoniae*, followed by *S. mutans*, so it can be concluded that *Streptococcus* species are more sensitive to clove essential oil compared to *Haemophilus* strains and *P. aeruginosa*. Of the bacterial strains we examined, the growth of *H. parainfluenzae* was inhibited the least by clove essential oil. In the case of thyme essential oil, it can be observed that the essential oil treatment was able to inhibit the growth of all test bacteria. *S. mutans* was the most sensitive to thyme essential oil, followed by *S. pneumoniae*. Similar to the essential oil of cloves, it can be concluded that the growth of *Streptococcus* species can be inhibited more effectively with thyme essential oil, compared to *Haemophilus* strains. Of the two *Haemophilus* species, *H. influenzae* proved to be more sensitive. It is important to mention that among the four essential oils involved in our study, with the exception of *Haemophilus* species, both *Streptococcus* and *P. aeruginosa* showed the greatest inhibitory effect against thyme. In the case of *Haemophilus* species, we used this method for the first time in order to test the effectiveness of essential oils (Balázs et al., 2019).

Table 3. Antibacterial effect of essential oil samples in a direct bioautographic test system, inhibition zones in mm

Concentrations of essential oils and their components		**Bacterial strains**				
		H. inf.	*H. parainf.*	*P. aerug.*	*S. mutans*	*S. pneu.*
peppermint	0.1 mg	4.26 ± 0,6	2.99 ± 0.6	3.17 ± 0.5	4.12 ± 0.5	3.02 ± 0.7
	0.2 mg	5.07 ± 0,5	3.61 ± 0.9	4.71 ± 0.4	4.91 ± 0.4	4.25 ± 0.5
menthol		2.14 ± 0,9	1.03 ± 0.5	1.69 ± 0.2	2.07 ± 0.2	1.14 ± 0.4
cinnamon bark	0.1 mg	9.55 ± 0,5	8.26 ± 0.7	7.43 ± 0.6	6.97 ± 0.7	6.13 ± 0.7
	0.2 mg	**10.98 ± 0,7**	9.06 ± 0.9	7.53 ± 0.8	6.01 ± 0.8	6.51 ± 0.5
cinnamic aldehyde		4.06 ± 0.7	4.92 ± 0.4	3.51 ± 0.4	3.07 ± 0.8	2.99 ± 0.8
thymus	0.1 mg	4.60 ± 0.6	4.39 ± 0.5	7.73 ± 0.3	10.08 ± 0.9	9.49 ± 0.9
	0.2 mg	8.13 ± 0.4	4.95 ± 0.2	8.18 ± 0.9	**11.23 ± 0.7**	9.63 ± 0.7
thymol		2.20 ± 0.6	2.10 ± 0.5	3.52 ± 0.8	5.36 ± 0.2	4.66 ± 0.9
clove	0.1 mg	7.39 ± 0.9	4.26 ± 0.5	5.52 ± 0.5	8.71 ± 0.5	8.06 ± 0.3
	0.2 mg	8.40 ± 0.7	4.39 ± 0.7	6.36 ± 0.4	8.97 ± 0.8	9.37 ± 0.8
eugenol		3.51 ± 0.5	2.19 ± 0.6	2.58 ± 0.2	4.63 ± 0.9	4.15 ± 0.5
antibiotic	0.1 mg	24.61 ± 0.9	24.55 ± 0.9	19.45 ± 0.8	21.22 ± 0.7	19.05 ± 0.9
abs. etanol		0	0	0	0	0

Legend: *H. inf.*: *Haemophilus influenzae, H. parainf.*: *Haemophilus parainfluenzae, P. aerug.*: *Pseudomonas aeruginosa, S. mutans*: *Streptococcus mutans, S. pneu.*: *Streptococcus pneumoniae*

In order to reveal the activity of the essential oil components, chromatographic development and separation of the essential oil samples was performed.

For each bacterial strain, several components of peppermint resulted in an inhibitory effect. Menthol (Rf=0.31) inhibited all bacterial strains. In addition, 1,8-cineole (Rf=0.4), isomenthone (Rf=0.51) and menthone (Rf=0.68) showed an inhibitory effect. Members of the *Haemophilus* genus reacted the most sensitively, as we detected the best clear-up zones here. In addition to the main component, *P. aeruginosa* was slightly inhibited by 1,8-cineole and isomenthone. In the case of *S. mutans*, we could observe fainter zones, the most intense component was menthol (Rf=0.31). *S. pneumoniae* also resulted in a clearing zone for several components. Among the components of the essential oil of cinnamon bark, cinnamic aldehyde (Rf=0.62) was the most active, and α-terpineol (Rf=0.35) resulted in a small inhibition for all bacterial strains. The effect of eugenol (Rf=0.76) was also shown for *Haemophilus* species. In the case of thyme essential oil, we could observe the pronounced inhibition of the main component (thymol: Rf=0.56), and linalool (Rf=0.33) showed a significant inhibitory effect on bacterial growth. In the case of clove essential oil, the most effective inhibition was achieved by eugenol (Rf=0.51), but the effect of limonene (Rf=0.42), which showed the least in the case of *S. mutans*, cannot be neglected either. Based on the literature data so far, we found that the novelty of our work is that we were the first to optimize the bioautography system for respiratory pathogens, among which *Haemophilus* spp. members should be highlighted separately, as they represent a group of bacteria with special nutrient requirements. Furthermore, we were the first to detect the antibacterial effect of peppermint and its main component, menthol, against respiratory pathogens in this test system. Most studies attribute their antibacterial effect to the main component of essential oils, however, our studies confirmed that the minor components also play an extremely important role in the antibacterial effect (Balázs et al., 2019).

4.3. Results of determination of MIC values

From our results (Table 4), we did not detect any significant differences between the *Haemophilus* species. Both *H. influenzae* and *H. parainfluenzae* reacted most sensitively to cinnamon bark essential oil (MIC: 0.06-0.08 mg/mL). Antibiotics used as positive controls proved to be more effective compared to essential oil samples.

Table 4 MIC values of the tested essential oils and antibiotics (in case of essential oils: mg/mL; in case of antibiotics: µg/ml)

	Bacterial strains				
Essential oils	*H. influenzae*	*H. parainfluenaze*	*P. aeruginosa*	*S. mutans*	*S. pneumoniae*
peppermint	0.21	0.21	1.5	1.96	0.35
cinnamon bark	0.06	0.06	0.4	0.78	0.06
thyme	0.11	0.11	1.4	0.46	0.12
clove	0.25	0.25	1.6	1.02	0.25
amikacin	0.8	0.8	-	-	-
gentamicin	-	-	2	-	-
amoxicillin	-	-	-	0.3	-
imipenem	-	-	-	-	0.4

4.4. The results of biofilm inhibition tests of essential oils

We illustrate our results in the form of a deviation from the control by indicating the percentage of the inhibition rate (Yanwei et al., 2018). Based on our results, it is striking that for all bacteria, the Pickering emulsion resulted in the smallest deviation from the control (untreated bacteria) (1.35-1.81%). Absolute ethanol alone did not produce a significant inhibitory effect either (3.12-4.52%), but the inhibitory effect of the emulsifier Tween80 proved to be the strongest among the three controls (6.35-11.8%). In contrast, it was not the essential oil samples produced with the Tween80 emulsifier that resulted in the most effective inhibition, but rather the nanotechnologically formulated samples. Our tests confirmed that all four essential oils have a biofilm-degrading effect in the case of the bacterial strains included in the study. We found that nanotechnologically formulated essential oil is much more effective than solutions formed with Tween and alcohol.

In the case of ***H. influenzae***, thyme proved to be the most effective essential oil (73.64%), followed by clove (69.59%). For both essential oils, the nanotechnologically formulated samples resulted in the most intense inhibitory effect (ethanolic extract for thyme: 42.31%; extract emulsified with Tween80 for thyme: 54.08%; ethanolic extract for cloves: 36.92%; emulsified with Tween80 extract for cloves: 39.79%). The effectiveness of thyme essential oil was greatly influenced by different formulation techniques. It can be observed that the nanotechnologically formulated thyme essential oil resulted in a one and a half times increase in the inhibitory effect compared to the ethanol extract. Overall, our results show that all four essential oils proved to be effective against the *H. influenzae* bacterium, the nanotechnologically formulated samples being the most effective. Similar correlations were observed in the case of *H. parainfluenzae*, as the nanotechnologically formulated essential oils proved to be extremely active. In the case of ***H. parainfluenzae***, cinnamon bark exerted the most significant inhibitory effect (ethanolic solution: 46.92%, emulsion made with Tween80 emulsion: 43.87%, Pickering emulsion containing cinnamon bark essential oil: 76.35%). The effect of the nanotechnologically formulated thyme and clove essential oil is also not negligible, as they resulted in a 60-65% difference compared to the control biofilm formation. In the case of the ***P. aeruginosa*** bacterium, it can be observed that cinnamon bark showed the most significant biofilm degrading effect. During the treatment with the absolute ethanol solution, the essential oil of the cinnamon bark resulted in 62.11% inhibition compared to the control. When using the emulsifier Tween80, this value increased by almost 10% (70.17%), which can be attributed to the effect of Tween80, and the Pickering emulsion containing cinnamon bark essential oil resulted in an 80.22% biofilm inhibition effect. In the case of ***S. mutans***, the essential oil of cinnamon bark (85.96%) also proved to be the most effective, but a significant inhibitory effect was also detected in the case of the essential oil of cloves (73.06). In the case of ***S. pneumoniae***, the essential oil of cinnamon bark was also able to most effectively degrade the biofilm formed by the bacterium (inhibition rate ethanolic solution: 57.21%, emulsion produced using Tween80 emulsifier: 69.62%, Pickering's emulsion: 78.22%) , but the effect of thyme essential oil is also not negligible (Pickering emulsion: 73.31). In the case of *S. pneumoniae*, it can be observed that compared to the other bacteria, it reacted much more sensitively to the treatment with peppermint essential oil (42.83-63.11%). During the biofilm degradation test, it can be observed for the solvents that, while Tween80 alone produces an inhibitory effect compared to the medium control, the effects of Pickering emulsion and absolute ethanol are negligible. The use of the Pickering emulsion proves to be promising for the formulation of essential oils, since the largest biofilm can be characterized by a degradation effect, but on its own (without essential oil) it does not have an inhibitory effect.

4.5. SEM results

We were able to take scanning electromicroscopic (SEM) images of *P. aeruginosa* and *S. mutans*. SEM images were used to demonstrate the effect of essential oils on the biofilm. We chose the two bacteria mentioned above because we could gain insight into the biofilm examination of both Gram-negative and Gram-positive bacteria.

In the case of *P. aeruginosa*, peppermint resulted in the smallest biofilm-inhibiting effect, the bacterial cells remained intact, but they were no longer able to stick together to the extent that can be observed in the case of the untreated control. As a result of the treatment with the essential oil of cinnamon bark, the cell structure of the bacteria was completely deformed, and it can be assumed that they are functionally inactive. In the case of *P. aeruginosa*, after the essential oil of cinnamon bark, clove can be mentioned as the most active oil. After peppermint, thyme proved to be the least effective, although biofilm formation did not occur, on the other hand, cells with a suitable structure were observed around the alginate mucus.

S. mutans showed the most intense resistance to peppermint essential oil, as a smaller *S. mutans* biofilm was also formed in this case. As a result of the treatment with the essential oil of cinnamon bark, the number of cells decreased, the essential oil of clove and thyme was also able to inhibit the formation of the biofilm. The cells adhered and microcolonies formed, but no coherent, three-dimensional biofilm could be observed.

4.6. Results of the investigation of the QS mechanism

The bacterial strain *Chromobacterium violaceum* WT85 is an indicator strain that shows the quorum sensing (QS) inhibitory effect of the test substance through depigmentation. The colorless zones caused by depigmentation were measured (mm), because the production of the pigment by the bacteria is under QS regulation. The size of the inhibition zones (mm) is summarized in Table 5.

Among the tested essential oils, peppermint essential oil produced the largest zone of inhibition in both concentrated and diluted form. This was followed by the essential oil of thyme and cinnamon bark. A similar trend was observed in the case of the main components, menthol produced the largest zone of inhibition, followed by thymol, cinnamic aldehyde and eugenol. It is important to mention that the activity of the essential oil has always been more effective compared to the component. Clove essential oil showed the weakest activity. When the negative control was used, the bacterial colony completely overgrew the Petri dish, with the appearance of pigmentation, as it did not result in an inhibitory effect, so it was not indicated in the table. In the case of biofilm tests, we observed that peppermint essential oil proved to be the least effective for the bacteria we tested. On the other hand, the quorum sensing mechanism can be said to be the most effective for inhibition. The background of this phenomenon may be that while other oils can inhibit the structural structure of the biofilm, the activity of peppermint essential oil lies in the inhibition of the QS mechanism (communication).

Table 5 The zones of inhibition produced by the essential oils involving in the QS study, expressed in mm. (The results are shown as mean ± SD.)

Samples	**Concentrations**		
	2.5 mg/mL	10 mg/mL	thick solution
peppermint	8.32±0.5	23.12±0.7	39.28±0.6
menthol	-	4.2±0.3	-
cinnamon bark	5.12±0.6	18.3±0.4	33.39±0.5
cinnamic aldehyde	-	2.6±0.2	-

thyme	6.95±0.6	20.2±0.5	36.32±0.9
thymol	-	1.54±0.2	-
clove	2.21±0.3	15.97±0.2	26.47±0.3
eugenol	-	1.28±0.5	-

From our results, it can be concluded that the concentrated essential oil resulted in the largest inhibition zones, and then, as the concentration decreased, the size of the inhibition zones also decreased, so we could observe the development of a concentration dependence. The main components resulted in a significantly smaller inhibition compared to the activity of the essential oils, so it can be said that the complexity of the essential oil is an extremely important factor during quorum sensing inhibition. In addition, we found that all four essential oils and their main components have a QS inhibitory effect.

5. New results, Summary

Our new scientific findings are summarized below:

1. We successfully determined the composition of our essential oil samples using GC-MS and GC-FID techniques.

2. Using the method of direct bioautography, we were the first to demonstrate the inhibitory effect of cinnamon bark, thyme, peppermint and clove essential oils and their components against *Haemophilus* spp. against. In the case of clove oil tests, we observed that the bacteria reacted most sensitively to the treatment with cinnamon bark and thyme essential oil. *Haemophilus influenzae* should be highlighted, which showed an inhibition zone of 10.98 mm when treated with cinnamon bark essential oil. In addition, in the case of treatment with thyme essential oil, an inhibition zone of 11.23 mm was measured for *S. mutans*. We found that in the case of peppermint, menthol, 1,8-cineole, isomenthone, menthone and menthyl acetate also exert an inhibitory effect on *Haemophilus* spp. In the case of cinnamon bark essential oil, eugenol, cinnamic aldehyde, and α-terpineol exert an inhibitory effect. In the case of thyme, thymol and linalool, and in the case of clove essential oil, in addition to eugenol, the inhibitory effect of limonene was detected in the bioautographic system. In addition, the inhibitory effect of the components of all four tested essential oils was also supported in the case of *Pseudomonas aeruginosa* and *Streptococcus* strains using a bioautographic test system. Complemented by the preparative chromatography method, the method shows a detailed picture of which essential oil components can be said to be active for each bacterial strain.

3. We successfully used the microdilution method to determine minimum inhibitory concentrations. We found that peppermint essential oil proved to be the least effective of the tested essential oils. The essential oil of cinnamon bark proved to be the most effective, with an inhibitory concentration of 0.06 mg/ml against *Haemophilus* spp. and for *S. pneumoniae*.

4. Our tests covered the use of essential oils in the form of Pickering emulsion. The Pickering emulsion formulation of essential oils was found to be the most effective in biofilm inhibition compared to ethanolic and Tween stock solutions of essential oils.

5. We were the first to describe that, in terms of biofilm inhibition, *Haemophilus* spp. in this case, thyme essential oil can be said to be the most active (inhibition rates: 76.64% and 76.35%) among the essential oils included in our study.

6. We have determined that the essential oil of cinnamon bark is effective against *Streptococcus* sp. representatives (inhibition rates: 85.96%, 78.22%), and in the case of *Pseudomonas aeruginosa* (inhibition rate: 80.22%), the experiment proved to be more effective for coated essential oils.

7. We were the first to demonstrate the anti-QS effect of menthol and clove essential oil.

6. Acknowledgment

I would also like to thank all those who provided the conditions for my work and contributed to the realization of the objectives of the research with their cooperation.

First of all, I would like to thank my supervisor, Dr. Györgyi Horváth, for his far-reaching support, who guided my work with boundless patience and helpfulness and provided useful professional advice.

I express my gratitude to my consultant, Dr. Judit Krisch, who helped my work by sharing her professional experience.

I am grateful to Dr. Barbara Vörös-Horváth and her supervisor, Dr. Aleksandar Széchenyi, for increasing the novelty of my work by preparing nanotechnologically formulated samples.

I would like to thank Dr. Béla Kocsis and dr. Adorján Varga, that I could always count on them in the preparation of microbiological tests.

I would like to thank Dr. Erika Kerekes for introducing me to the mysteries of biofilm formation inhibition testing, so after learning the method, we were able to optimize this test method at the University of Pécs.

I owe a debt of gratitude to Tamás Petkovits, who helped me prepare the SEM images.

I owe a debt of gratitude to Dr. Kamilla Ács, who always helped my work with her encouraging attitude even in the face of difficulties.

I would also like to thank all the colleagues of the GYTK Pharmacognosy Institute of the University of Pécs, who contributed to the completion of my work with their patience and advice.

I would like to thank Dr. Ágnes Farkasné Papp for starting me on this path during my high school studies. Finally, I am indebted to my family and friends, without whose encouraging words my research would not have been possible.

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8. List of publications

8.1. Publications forming the basis of the thesis

Balázs V.L., Horváth B., Kerekes E., Ács K., Kocsis B., Varga A., Böszörményi A., Nagy D., Krisch J., Széchenyi A., Horváth Gy. (2019): Anti-*Haemophilus* activity of selected essential oils detected by TLC-Direct Bioautography and biofilm inhibition. **MOLECULES**. 24:3301. **[IF: 3,286]**

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The summary of the conference presentations is included in the dissertation.