Investigation of Antibacterial Activity and Interaction Types of Essential oils and Their Volatile Components Against Respiratory Tract Bacteria

Ph.D. thesis

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1. Introduction

Respiratory tract infections (RTIs), which mostly occur from autumn to spring, affects people of all ages. Based on pathogenicity, bacteria, viruses, and fungi are the most common cause of these diseases. Therefore, their proper treatment requires complex therapy which can be influenced by secondary bacterial infections. The excessive use of antimicrobials leads to the growing appearance of antibiotic resistant pathogens producing serious problems in medicinal health care nowadays. As a result, efficacy of classic antibiotics is decreasing, they are becoming totally ineffective against these pathogens several times. Therefore, discovery of new alternative treatments, which could support the medical therapy of RTIs, is an important challenge nowadays.

Essential oils (EOs) are hydrophobic, natural extracts with complex composition whose antimicrobial effects have been known and used in medicine from ancient times. The application of EOs via inhalation is becoming more frequent nowadays, especially in the case of bacterial infections. Volatile components can easily reach the respiratory tract, the direct contact of fragrances and the infected surface is an advantage of this form of treatment. Multicomponent composition of EOs also gives them benefit against antibiotic-resistant strains.

The increasing interest about herbal medicines and their application by the patients requires updated and evidence based knowledge from health care professionals. We must note that the administration of EOs is mostly based on their tradicional use, in several cases more experiments are needed for the determination of their mode of action, dosage and toxicity. In the last decade, the antimicrobial potentials of EOs were studied by several *in vitro* techniques, but the results mostly focused on the antimicrobial activity of the EOs in liquid media. In the treatment of RTIs, the patients inhale the volatile components through their nose or mouth, hence investigation of the antibacterial activity of EOs' vapor would be highly important. For the detection of antimicrobial activity of EO volatiles, several *in vitro* vapor phase techniques exist, however, respiratory tract pathogens are used only in a few studies.

Besides, the discovery of new therapeutic alternatives and combination of the effective techniques are another possible solutions. Based on this possibility, in the last decade, several studies focused on the interaction of EOs, volatiles and antibiotics in combination.

2. Aims

Therefore, the aims of the present study were:

- the antibacterial evaluation of some commercially available EOs (cinnamon bark, clove, thyme, peppermint, citronella, scots pine, and eucalyptus) and the EO isolated from a Mongolian medicinal plant (*Artemisia adamsii* Besser) against respiratory tract pathogens.
- chemical analysis of the above mentioned oils with GC-FID, and GC-MS techniques. The EOs' vapor was planned to be analyzed with sHS-SPME-GC-MS method as well.
- application of new techniques besides the classical *in vitro* methods, such as vapor phase method and direct bioautography, which are more suitable for the detection of the antibacterial character of our samples.
- comparison of the antibacterial potency of EOs in liquid medium and vapor phase.
- combination of the most effective components and EOs to detect their interactions and activity especially against antibiotic-resistant strains. Besides checkerboard method, direct bioautography was used to detect its applicability in detection of interaction profiles between our samples.

We presume that our results may contribute to the expansion of the therapeutical application of EOs, moreover clinicians and pharmacists could use them for the proper treatment of RTIs. In the future, we hope that our data will be valuable in the development of new preparations against RTIs.

3. Materials and methods

3.1 Essential oil samples

We investigated the antibacterial activity of cinnamon bark (*Cinnamomum zeylanicum* Nees.), clove (*Syzygium aromaticum* (L.) Merill & Perry), thyme (*Thymus vulgaris* L.), eucalyptus (*Eucalyptus globulus* Labill.), scots pine (*Pinus sylvestris* L.), peppermint (*Mentha* × *piperita* L.), and citronella (*Cymbopogon nardus* (L.) Rendle). EO samples were purchased from Aromax Zrt. For the qualitative and quantitative analysis of the samples, gas chromatography (GC) was applied.

We also had an opportunity to test the EO of a mongolian medicinal plant called *Artemisia adamsii* Besser. The plant was collected and identified by Dr. Tserennadmid Rentsenkhand (National University of Mongolia, School of Biology) biologist. For the EO content determination, water-steam distillation was performed according to the guideline of the 7th Hungarian Pharmacopoeia. To 25 g dry herb, 500 ml distilled water was added, and the distillation was carried out for 3 hours after boiling.

3.2 Analitical assays

3.2.1 Parameters of gas chromatography

GC analysis was carried out by Dr. Andrea Böszörményi (Semmelweis University, Faculty of Pharmacy, Department of Pharmacognosy). During analyses, flame ionization detector (FID) and mass spectrometry (MS) were used. The data were evaluated using MSD ChemStation D.02.00.275 software (Agilent). The identification of the compounds was carried out by comparing retention times and recorded spectra with the data of authentic standards, and the NIST 2.0 library was also consulted, percentage evaluation was carried out by area normalization.

Parameters of the GC-MS and GC-FID analysis:

The injector temperature was 250°C and the split ratio was 1:50 (Aromax oils). In the case of *A. adamsii* the injection was performed at 280°C with 0.7 mg/ml, in splitless mode. The analyses were carried out with an Agilent 6890N/5973N GC-MSD (Santa Clara, CA, USA) system equipped with an Agilent SLB-5MS, Agilent HP-5MS capillary column (30 m × 250 µm × 0.25 µm, GC-MS), and Rt-β-DEXm column (30 m × 250 µm × 0.25 µm; GC-FID). In the case of Aromax oils, the GC oven temperature was programmed to increase from 60ºC (3 min isothermal) to 250ºC at 8ºC/min (1 min isothermal). In the case of *A. adamsii*, the oven temperature was programmed to increase from 60ºC (3 min isothermal) to 200ºC at 8 ºC/min, which was followed by a 200-250˚C section at 10˚C/perc (15 min isothermal). High purity helium (6.0) was used as carrier gas at 1.0 ml/min (37 cm/s) in constant flow mode. The mass selective detector was equipped with a quadrupole mass analyser and was operated in electron ionization mode. The FID temperature was 240ºC, we used nitrogen (6.8 ml/min) as a makeup gas.

3.2.2 Static headspace solid phase microextraction (sHS-SPME) technique

0.1 mL of EOs was put into vials (20 mL headspace) sealed with a silicon/PTFE septum prior to SPME-GC/MS analysis. Sample preparation using the static headspace solid phase microextraction (sHS-SPME) technique was carried out with a CTC Combi PAL (CTC Analytics AG, Zwingen, Switzerland) automatic multipurpose sampler using a 65 μm StableFlex polydimethyl siloxane/divinyl benzene (PDMS/DVB) SPME fibre (Supelco, Bellefonte, PA, USA). After an incubation period of 5 min at 40ºC and 100ºC, extraction was performed by exposing the fibre to the headspace for 20 min at 40ºC and 100ºC. The fibre was then immediately transferred to the injector port of the GC/MS, and desorbed for 1 min at 250ºC. Injection was performed in split mode, split ratio was 1:30. The SPME fibre was cleaned and conditioned in a Fibre Bakeout Station in pure nitrogen atmosphere at 250ºC for 15 min. In the case of *A. adamsii* EO, SPME analysis was not performed.

3.3 Antibacterial activity of essential oils

3.3.1 Microorganisms

Bacteria, which cause RTIs, were involved in our experiments. Moreover, we complemented our studies with resistant strains as well. Strains isolated from clinical samples were as follows: methicillin-resistant *Staphylococcus aureus* (MRSA, 4262) multiresistant *Pseudomonas aeruginosa* (R-*P. aeruginosa*, 34205), and *Streptococcus pyogenes* (116). *P. aeruginosa* (ATCC 27853), *S. pneumoniae* (DSM 20566), *S. mutans* (DSM 20533), *Haemophilus influenzae* (DSM 4690), *H. parainfluenzae* (DSM 8978), and *Moraxella catarrhalis* (DSM 9143) were purchased from American and German culture collections.

3.3.2 Antibiotic susceptibility of microorganisms

For the determination of antibiotic sensitivity of our pathogens, Kirby-Bauer disc diffusion method was used according to the guidelines of CLSI and the Manual of Clinical Microbiology. After the discs were placed onto the surface of the inoculated broth, Petri dishes were incubated at 35±2^{o}C for 16-18 hours. Then, inhibition zones were evaluated in visible light. Discs were impregnated with the following antibiotics: amikacin amoxicillin/clavulanic acid, ceftazidime, cefepime, ciprofloxacin, erythromycin, gentamicin, imipenem, colistin, levofloxacin, meropenem, oxacillin, penicillin, piperacillin/tazobactam, trimethoprim/sulfamethoxazole, tobramycin, vancomycin (OXOID Ltd.).

3.3.3 *In vitro* antibacterial assays

Antibacterial activity was tested with macrobroth dilution, direct bioautography and vapor phase techniques. The first and the second methods investigate the effect of EOs in liquid phase, however, vapor phase could detect the activity of volatile components in gas phase. The results obtained from liquid phase tests were compared with the activity of antibiotics. Due to the small amount of the EO of *A. adamsii*, this sample was analysed only with direct bioautography.

3.3.3.1 Direct bioautography system

The antibacterial activity of *A. adamsii* was investigated with or without separation. TLC separation was prepared according to the following protocol: from a 200 μL/mL stock soultion 3 and 5 μL were applied to the plate (Merck TLC Silica gel 60 $F_{254,}$ 5 x 10 cm). As a control, we used 4 μL from 1 mg/mL vancomycin solution. After separation, 1, 3, and 5 μL of the above mentioned EO solution were applied to the 10 x 10 cm plate with Minicap capillary

pipets (Hirschmann Laborgerate GmbH & Co. KG, Eberstadt, Germany) to detect the activity of EO components. Thujone and 1,8-cineole standards (Sigma Aldrich Ltd.) were dissolved in ethanol to give solutions of 10 µL/mL. 2 µL of standard solutions were also spotted to the plate. After sample application, the plates were developed with toluene–ethyl acetate (93:7, v/v%). Ascending development was used in a saturated twin trough chamber (CAMAG, Muttenz, Switzerland).

Antibacterial evaluation of Aromax oils was also carried out on TLC plates without separation. Preparation of plates was performed with the following protocol: EOs were diluted in absolute ethanol (Molar Chemicals Ltd., Halásztelek) to give 5-10-20-30 mg/mL stock solutions, and 5-5 µL were applied to the 5 x 10 cm plate. As reference, we used 1 µL from the 0.25-0.5 mg/ml solutions of vancomycin, polymyxin B, and ciprofloxacin. As solvent control, 5 µL of absolute ethanol was also tested.

Direct bioautographic method:

Firstly, the cultivation of test bacteria for dipping was prepared. The bacterial suspension was diluted with fresh nutrient broth to an OD_{600} of 0.4, which corresponds to approximately 4×10^7 CFU/mL.

The developed plates were dipped into the bacterial suspension for 10 s, then they were incubated in a water vapor-saturated chamber (chamber dimension: 20 × 14.5 × 5 cm) at 37°C for 17 h. After incubation, plates were dipped into an aqueous solution of MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide, Sigma Aldrich Ltd.) for 10 s which was followed by further 2 hours incubation. The diamaters of inhibition zones were detected in visible light with Motic Images Plus 2.0 program. All bioautographic experiments were performed in triplicate.

3.3.3.2 Broth macrodilution test (BDT)

5% emulsions of EOs were made. A serial twofold dilution was prepared from 50 to 0.0075 µL/mL. EOs were preliminary filtered through Millex-GV filter (filter unit: 0.22 µm, Millipore, Ireland). As control of the bacterial growth, neither EO nor detergent was added to the tubes. Test media containing 20 µL from 10% solutions of Polysorbate 80 or DMSO (Reanal Kft., Budapest) were also used separately as emulsifier controls. DMSO was applied only in the case of *M. catarrhalis*, considering that this bacterium cannot tolerate Polysorbate 80. In the case of *Haemophilus* spp., we used a special test medium supplemented with 15 µg/mL hematin, NAD (Bacto Supplement B, DIFCO, USA) and 5% of yeast extract. 10 µL of an overnight bacterial culture (~ 4×10^7 cells/mL) was added to each tube and incubated at 37°C for 24 h. Then, in the case of *Streptococci* and *M. catarrhalis* the tubes were plated out on 5% sheep blood agar and incubated again for 48 hours. Chocolate agar was used for *Haemophilus* spp. The number of bacterial colonies was compared to the controls and then the values of the minimum bactericidal concentrations (MBC) and minimum inhibitory concentrations (MIC) were determined. All tests were carried out in triplicate.
Antibacterial activity of control antibiotics (vancomycin, gentamicin, imipenem amoxicillin/clavulanic acid and amikacin) was tested with 100 µg/mL initial concentration. In this assay, detergents were not applied. There were no antibiotics in control tubes.

3.3.3.3 Vapor phase test (VPT)

The *in vitro* VPTs were based on the method described by Kloucek et al. (2012) with some modifications. Test system was developed in a four-section Petri dish (PD, diameter 90 mm, VWR, Debrecen, Hungary) containing 5 mL of 5% sheep blood agar in the case of

Streptococci and *M. catarrhalis*. *Haemophilus* spp. required chocolate agar with 15 µg/mL NAD supplementation. Mueller-Hinton broth was used for the cultivation of MRSA and *Pseudomonas* spp. Test medium was not added into the upper lid of the PD. All bacteria were grown in solid test medium before the assay and then 10^5 CFU/mL suspension was made from each strain. Three sections of the PD were inoculated with 20 µL of the selected bacterium. Different strains were spread into each section. The fourth compartment was left uninoculated as contamination control. EO samples were diluted in absolute ethanol (0.5-195 µL/mL) and distributed on the surface of a sterile filter paper disc (Albet-Hahnemühle, Germany). The disc was placed on the separating wall of the PD after solvent evaporation. Therefore, there was approximately 2 mm distance between the disc and the inoculated agar surface. PDs were hermetically closed with parafilm adhesive tape (Sigma Aldrich Ltd., Budapest, Hungary), and were incubated at 37°C for 48 h. After incubation, filter papers were removed and MIC (expressed as µL of EO/free atmosphere above the growing microorganism) values were determined. Filter paper discs containing absolute ethanol or left untreated were used as solvent and growth controls. All tests were carried out in triplicate.

3.4 Assays for investigating the interaction between EOs and their volatile components

For detecting the interactions between the active oils and their components direct bioautography and checkerboard assays were used. Considering our previous results the combinations of cinnamon bark, clove, thyme, citronella, and peppermint oil and their main components, namely, *trans*-cinnamaldehyde, eugenol, thymol, menthol, geraniol, citronellal and citral (mixture of geranial and neral) (Sigma Aldrich Kft.) were tested against MRSA (4262), R-*P. aeruginosa* (34205), and *P. aeruginosa* (ATCC 27853).

3.4.1 Direct bioautography

Firstly, the microbiologically active compounds were detected against our pathogens. Then the minimal detectable dose (MDD) of the selected components was determined.

3.4.1.1 Detection of active components

To prepare stock solutions, 100 µL of EOs were diluted in 500 µL absolute ethanol. To determine the activity of main components, 40 mg/mL solutions were also prepared. 0.6 µL of each samples were applied to a 20 x 10 cm TLC plate with automatic pipette (Finnpipette, Thermo Fisher). Separation distance was 8 cm. All measurements were carried out in duplicate. Toluene-ethyl acetate (93:7, v/v%) was used as a mobile phase except for cinnamon bark oil. In this case, dichloromethane stabilized with methanol (Molar Chamicals Kft.) was applied. Ascending development was used in a saturated twin trough chamber (CAMAG, Muttenz, Switzerland). TLC separation and mobile phase evaporation were performed at room temperature (22°C). With one half of the layers direct bioautography assays was performed as described in Chapter 3.3.3.1. The first incubation time was reduced to 4 hours. The other half of the layers was dipped into ethanolic vanillin-sulfuric acid reagent to visualize the separated components.

3.4.1.2 Determination of MDD

0.2 to 1.5 µl of 10-60 mg/mL solutions of active components were applied to a TLC plate. Then, the same direct bioautography process was carried out as described in the previous chapter.

3.4.1.3 Interactions between EOs and their main components

Against MRSA eugenol-menthol, *trans*-cinnamaldehyde-menthol, thymol-menthol, geraniol-thymol combinations were tested. In the case of *P. aeruginosa*, eugenol-menthol, *trans*- cinnamaldehyde-menthol, thymol-menthol, geraniol-thymol, citral-thymol, citronellal-thymol, citronellal-citral, citral-geraniol, and citronellal-geraniol compositions were evaluated. Against R-*P. aeruginosa* the combinations of *trans*-cinnamaldehyde-thymol, eugenol-thymol, and *trans*-cinnamaldehyde-eugenol were examined.

According to the MDD values, the concentrations of the combined samples were applied to the TLC plates with automatic pipette. Then, direct bioautography described in Chapter 3.4.1.1. was performed. Four measurements were carried out in parallel. The diamaters of inhibition zones were measured with Motic Images Plus 2.0 program.

3.4.2. Statistical analysis

The statistical analysis of the combinations' data obtained by direct bioautography was performed with Mann-Whitney-Wilcoxon test of R Studio 1.1.383 program (R version: 3.4.3). The aim of the non-parametric test was to detect the positive effect of combinations in comparison with the inhibition zones (ZOI) of the individual components. Our null hypothesis was H_o:$\mu_{combinationZOI}$- $\mu_{componentZOI}$=0, the alternative hypothesis was H_1:$\mu_{combinationZOI}$-$\mu_{componentZOI}$>0.

3.4.3 Checkerboard assay
3.4.3.1. Determination of MIC values

To confirm our bioautographic results, experiments with cinnamon bark, clove, citronella, peppermint and thyme oils and their main components were performed against *P. aeruginosa* (ATCC 27853). Unfortunately, it was not possible to include resistant strains in this assay. Firstly, MIC values of components and EOs were determined according to the following protocol: the concentrations of the samples were in the range of 100-0.1 mg/mL. Sterile 96-well microtiter plates (VWR International Kft.) were used in this assay. Each well contained 100 µL of bacterial suspension (approximately 10^5 CFU/mL) and 100 µL sample mixed with nutrient broth. As control, nutrient broths with or without bacterial suspension were used. Plates were incubated for 24 h at 37°C. Then, the absorbance was measured by microtiter plate reader (SPECTROstar Nano Microplate reader, BMG Labtech) at 600 nm. The concentration was considered as MIC, when values showed 10% reduction of absorbance in comparison with the controls. All measurements were made in duplicate.

3.4.3.2. Parameters of checkerboard titration

All measurements were carried out in triplicates using sterile 96-well microtiter plates. Each well contained 50 µL of Mueller-Hinton broth in combination with samples and 100 µL of 10^5 CFU/mL bacterial suspension. As an emulgent 1% solution of Tween 40 (Sigma Aldrich Kft.) was used. Combinations of the samples were made according to the following concentrations: 2 x MIC, MIC, MIC/2, MIC/4, and MIC/8. Plates were incubated for 24 hour

at 37°C. Absorbance was measured by microtiter plate reader at 600 nm. As control, nutrient broths with or without bacterial suspension were used. For determination of the interaction between the components, fractional inhibitory concentration index (FICI) was calculated. A FICI of 0.5 or less is regarded to be synergistic; between 0.5 and 1: additive. If the value of FICI is between 1 to 4, no interaction could be observed.

4. Results and discusion

4.1 Essential oil content of *Artemisia adamsii*

EO yield was 0.56 mL/100 g of dried plant material. In contrast to other members of *Artemisia* genus, the color of the oil was pale yellow with characteristic odor. Therefore, we presumed that the EO does not contain any proazulenes which are responsible for the bluish color of the EO of other *Artemisia* species. Our hypothesis was confirmed by GC measurements.

4.2 Result of analytical assays

4.2.1 Results of GC-FID and GC-MS analysis

Evaluation of the A. adamsii EO:

α-Thujone was the main component (64.4%), while the amount of β-thujone was lower (7.1%.). The presence of 1.8-cineole (15.2%) was also confirmed by GC/MS. Minor components were *p*-cymene (1.5%), terpinen-4-ol (1.5%), linalool (0.4%), and spatulenol (1.8%). In contrast with previous publications, our samples did not contain limonene, probably due to the differences in the habitat of the plant and the circumstances of plant collection.

Evaluation of the Aromax EO:

In all samples, the amount of the detected components was above 96%. In accordance with the literature, the main component of cinnamon bark oil was the *trans*-cinnamaldehyde (74.0%), but in lower amount (1.4-5.3%) eugenol, limonene, linalool, 1,8-cineole, and cinnamyl acetate also occured. Citronellal (36.2%) was detected as the main compound in citronella oil, but in realtively high amount (13.6-25.3%) citronellol and geraniol were also present in the oil. Eugenol (88.6%) occured in clove, and 1,8-cineole (84.8%) in eucalyptus oil. In comparison with previous reports, small differences were observed in *p*-cymene and *α*-pinene content in eucalyptus oil. However, menthol (50.4%) was detected as the main compound of peppermint, the amount of isomenthol (4.3%) was above the previously described limit (0.2-1.2%). The camphene and limonene content in scots pine also differed from previous observations. According to the literature, *α*-pinen in pine oil (39.4%), and thymol (46.3%) in thyme oil were evaluated as the main components. In thyme, *p*-cymene and linalool were detected in higher amount, but carvacrol and γ-terpinene were identified in lower amount compared to previously published data.

4.2.2 Results of sHS-SPME-GC-MS analysis

The measurements were carried out at 40°C and 100°C. At 40°C, results are modelling the vapor phase composition what we used in our VP tests. In accordance with the literature and our previous observations, the main volatile compound in the vapor pase of cinnamon bark was *trans*-cinnamaldehyde (45.9%-52.1%) at both temperature. Above 5%, *p*-cymene, *α*-

pinene, 1,8-cineole, limonene, linalool and *β*-caryophyllene were also detected. Cinnamyl acetate (1.9% and 3%) was presented in relatively small amount in both systems.

Besides citronellal (40.0-42.3%), nerol and limonene was found over 10% at 40°C incubation. In relatively high amount, citronellol was identified at 100°C. In contrast with GC-FID, geraniol was detected in lower concentration (0.8% and 0.9%). In 1-3% sesquiterpenes (*β*-elemene, *β*-muurolene, *β*-cadinene) were also observed at 40°C and 100°C incubation temperatures.

In eucalyptus oil, 1,8-cineole (91.0% and 91.7%) was found as the main component, *γ*-terpinene was detected only in smaller amount (3.6% and 4.4%).

Similarly to the GC-FID observations, eugenol, *β*-caryophyllene, and *α*-humulene were detected in clove oil. At both temperature, the percentages of these components were similar.

Besides *α*- and *β*-pinene, limonene and *δ*-3-carene were detected above 10% in the vapor phase of scots pine at 40°C. We found *α*-terpineol and *β*-caryophyllene in lower amount at both temperature.

Vapor of thyme oil was characterised by thymol and *p*-cymene. Additionally, the presence of 1,8-cineole, linalool, and *γ*-terpinene was also observed from 3% to 6%. In peppermint EO, menthol derivatives and 1,8-cineole (16.4% and 17.4%) also occured besides the main component (menthol: 27.2% and 33.2%).

In the case of main components of the EOs, the result was similar to GC-FID. However, it must be noted that some components, which were identified in higher amount with GC-FID, were under 1% in sHS-SPME-GC-MS analysis at both temperature. Small differences in EO composition were observed at 40°C and 100°C incubation times. We suppose that these differences could be due to the different volatility of the individual components.

4.3 Antibiotic susceptibility of microorganisms

The aim of the experiment was to determine the most effective antibiotics against our microorganisms. Furthermore, we could detect the resistance profile of clinically isolated strains as well.

According to our result, vancomycin and gentamicin were used against MRSA. *Haemophilus* spp. showed sensitivity to amikacin. Imipenem inhibited the growth of *Streptococci* spp. and *M. catarrhalis*.

In the case of *Pseudomonas* spp., polymyxin B, ciprofloxacin, and gentamicin were tested.

4.4 Antibacterial evaluation of essential oils

4.4.1 Results of direct bioautography

Direct bioautography of A. adamsii:

Considering the results of GC, we presumed that the EO could not be used via inhalation without serious side effects due to its high *α*-thujone content. However, we wanted to investigate the antibacterial potency of our oil against MRSA which may cause serious problems especially in nosocomial infections.

We observed that the unseparated oil was effective against MRSA. The undiluted EO (in 0.558 mg and 0.903 mg quantities) produced 4.5 and 5.5 mm of inhibition zones. The 0.004 mg of positive control, vancomycin, performed 7 mm of inhibition zone as well. In comparison with the antibiotic, higher amount of EO should be applied to achive the similar inhibition.

The main components of EO did not show any activity against MRSA. Compounds, most likely terpene alcohols, showed antibacterial activity in the 0.15-0.45 R_f range, and the inhibition zones appeared as pale white spots around the separated constituents. Therefore, the

main components of the oil (thujone, and 1,8-cineole) have no apparent antibacterial activity at the concentration at which it was present in the oil.

It should be highlighted that the EO of *A. adamsii* showed activity against MRSA, the antibacterial effect is probably due to the minor components. However, in comparison with control antibiotic, the EO did not produce enhanced inhibition, but it could be an effective surface desinfectant especially in hospital environments. To specify the dosage and justify the activity of the EO more experiments are required in the future.

Direct bioautography of Aromax oils:

The activity of EOs was tested without TLC separation. It should be noted that for those pathogens which require 5% blood and chocolate agar, we could not optimise the test system. In the future, we would like to focus on this problem. Therefore, the antibacterial effect was tested only against MRSA, R-*P. aeruginosa* and *P. aeruginosa.*

In the case of MRSA, clove produced the highest inhibition zone (9.5 mm) among the EOs which was followed by eucalyptus, thyme, and cinnamon bark. Moderate activity of peppermint and citronella was detected (4.4-4.8 mm) only in higher concentrations. In contrast with other samples, scots pine oil did not show any activity against this pathogen.

Cinnamon bark produced the highest inhibitory effect, while thyme and clove oil showed similar activity (6.3 mm) in the case of R-*P. aeruginosa*. Other oils were found ineffective. Against sensitive strain of *P. aeruginosa*, cinnamon bark presented the highest inhibition as well (9.3 mm). Similarly to the antibiotic-resistant strain, thyme and clove oil produced activity (7.3-8.0 mm). In this case, we found peppermint and citronella equally active. Scots pine presented slight inhibition (3.8 mm).

It should be highlighted that absolute ethanol did not produce any activity in our test system; therefore, the antibacterial effect is attributed to our EOs' activity.

The positive controls were vancomycin in the case of MRSA as well as ciprofloxacin and polymyxin B against *Pseudomonas* spp.

0.25 µg of vancomycin produced 3.8 mm inhibition zone against MRSA. 0.5 µg of ciprofloxacin presented 7 mm inhibition zone in the case of *P. aeruginosa*. Ciprofloxacin was not effective against the resistant strain of *P. aeruginosa.* Unfortunately, polymyxin B did not show high activity (2 mm) and this effect did not alter after increasing its concentration.

It is concluded that our EOs were active against at least one of our pathogens. Clove produced the highest inhibition against MRSA, while in the case of *Pseudomonas* spp. cinnamon bark was the most effective. However, in comparison with antibiotics, the EOs presented lower activity, their inhibitory effect showed dose dependency.

4.4.2 Results of broth macrodilution tests

This method allowed us to detect the antibacterial activity of EOs in liquid media. Clove oil (MIC: 0.1 mg/mL), was the most effective against *S. pyogenes* which was followed by citronella and peppermint (MIC: 0.17 mg/mL and 0.35 mg/mL). Against this pathogen cinnamon and thyme was found equally active, while eucalyptus and scots pine showed activity only in higher concentrations. Citronella and cinnamon bark oil produced the lowest MIC values (MIC: 0.06-0.09 mg/mL) against *S. pneumoniae.* Thyme, clove, and peppermint oils showed (MIC: 0.11-0.35 mg/mL) activity as well. In the case of eucalyptus oil we must applied twenty times higher amount than cinnamon to achieve inhibition.

Among our tested materials thyme oil showed the highest inhibitory activity (MIC: 0.04 mg/ml) against *S. mutans*, which was followed by citronella, cinnamon bark, and clove. (MIC: 0.17-0.41 mg/mL). Unfortunately, scots pine oil showed activity only in relatively high concentrations.

Against *Haemophilus* spp. cinnamon bark (MIC: 0.06 mg/mL) oil was the most effective. The antibacterial activity of thyme, clove, and peppermint was similar against *Haemophilus* spp. In contrast, *H. parainfluenzae* was more susceptible for citronella and eucalyptus oils. Scots pine oil showed significant activity only against *H. parainfluenzae* and *M. catarrhalis* (MIC: 0.34 mg/mL).

Cinnamon bark was the most effective oil against *Pseudomonas* spp. In higher concentration thyme, clove and eucalyptus were found to be active against *P. aeruginosa* as well. EO of peppermint, citronella, and scots pine showed only bacteriostatic effect in our test system. In the case of R-*P. aeruginosa*, only the cinnamon bark oil showed inhibitory activity, the other EOs presented bacteriostatic effect.

Clove oil produced the highest inhibition against MRSA (MIC: 0.1 mg/mL), while peppermint and scots pine did not show any activity.

Negative controls (solvent and detergent) did not influence the growth of the bacteria. In comparison with the MIC values of antibiotics, we found that our EOs were less effective. However, their efficacy, especially in the case of resistant pathogens should not be neglected. Because of their multi-component character and their mode of action on several receptors, we suppose that the advantage of these substances is that resistance hardly develops against them.

4.4.3 Results of the vapor phase technique

During this experiment, the antibacterial activity of EO's vapor could be evaluated directly. As controls, the solvent and filter paper did not influence the growth of the bacteria. After the incubation period, MIC values (µL/L) considering the amount of EOs (µL) were calculated referring to the free airspace (L) in our Petri dish. Contrary to our previous observations obtained in liquid phase, cinnamon bark oil produced the lowest MIC value (31.25 µL/L) against MRSA. In higher concentrations, thyme and clove inhibited the growth of this pathogen as well, while eucalyptus and scots pine did not show any activity. Differences between the activity of peppermint and citronella (MIC: 375 µL/L) were not observed.

The total inhibition of the growth of the resistant *P. aeruginosa* was only managed by cinnamon (MIC: 125 µL/L), which was in parallel with results of BDT. In the case of sensitive *P. aeruginosa*, cinnamon and thyme vapor acted equally (MIC: 31.25 µL/L). In contrast with our observations in the liquid phase, vapor of clove, eucalyptus, and peppermint showed weak activity.

Among *Streptococci*, *S. mutans* was the less sensitive to EO volatiles. In lower concentration, only cinnamon bark performed potent inhibition against this pathogen. Clove was active only in higher concentrations (MIC: 150-500 µL/L) against *Streptococcus* spp. Eucalyptus oil was effective only against *S. pneumoniae* (MIC: 1200 µL/L).

Vapor of scots pine did not show any inhibition, except in the case of *H. influenzae* (MIC: 500 µL/L). Therefore, we presume that scots pine has bacteriostatic effect, and its MIC is probably higher than 1500 µL/L. Vapor of other EOs also inhibited the growth of *Haemophilus* spp. *H. parainfluenzae* was less sensitive to our samples.

In the case of *M. catarrhalis*, we found citronella and cinnamon bark oils equally active (MIC: 25 µL/L), which was followed by peppermint, and thyme. Clove and eucalyptus vapor produced total inhibition in higher concentrations.

Advantage of this method is that we can detect the activity against several microorganisms at the same time. However only those pathogens could be tested together, which require the same growth medium. As a result, the most effective EO vapor was cinnamon bark against all investigated pathogens (MIC: 15.62-125 µL/L). Besides, thyme, clove, peppermint, and citronella showed potent activity as well. Eucalyptus oil performed activity against Gram-

negative strains, except *Pseudomonas* spp. as well. Scots pine produced total inhibition only in the case of *H. influenzae*. According to previously published results, Gram-negative pathogens are less sensitive to the EO treatment due to the structure of their outer membrane and antibiotic resistance (Burt 2004, Doran et al. 2009). Contrary to this hypothesis our experiments showed that Gram-negative strains (except *Pseudomonas* spp.) were more sensitive to EO vapors: we detected higher MIC values against Gram-positive bacteria. These findings were parallel to the observation published by Inouye et al. (2001).

4.5 Evaluation of interactions between essential oils and their components

4.5.1 Direct bioautography assay

4.5.1.1 Detection of active compounds, and calculation of their MDD

Trans-cinnamaldehyde, eugenol, menthol, thymol, and geraniol showed activity against MRSA. Same activity was observed with citronellal and citral against sensitive strain of *P. aeruginosa*. In the case of R.-*P. aeruginosa*, only main components of cinnamon bark, thyme and clove produced inhibition.

We prepared combinations considering the MDD values. As we expected in the case of R-*P. aeruginosa*, higher amount of the components was needed to achive appropriate inhibition (0.012-0.036 mg). In contrast with our previous observation, cinnamaldehyde produced weaker activity individually, therefore, we supposed that other minor components could enhance the activity of cinnamon oil.

4.5.1.2 Evaluation of interaction between essential oil components

According to our knowedge, it was the first time that direct bioautography was used to detect interactions between biologically active compounds. Since the direct bioautography proved to be suitable for rapid and effective testing of antibacterial activity, it was considered to explore the applicability of this test system from this point of view as well. The inhibition zones of combinations in parallel with the activity of individual components were detected. Data were statistically analysed with Mann-Whitney-Wilcoxon test.

In the case of *P. aeruginosa*, combination of thymol and menthol was active; against R-*P. aeruginosa*, *trans*-cinnamaldehyde combined with thymol was found to be effective. Against MRSA, mentol combined with *trans*-cinnamaldehyde, and eugenol also showed activity.

Combination of menthol with *trans*-cinnamaldehyde, as well as compounds of citronella oil with each other and thymol was tested firstly against the above mentioned microorganisms. In contrast with previous observations, we could not detect the antagonistic effect of menthol and thymol against MRSA, however, combination of eugenol and methol produced enhanced activity in this system. Mixture of geraniol and thymol did not show any positive effect.

There was also no significant difference in the combination of the less active components of citronella oil with each other and with geraniol, and therefore, it is assumed that the citronella and citral component contributes only to a lesser extent to the inhibitory activity.

4.5.2 Results of checkerboard titration

Based on the result obtained from direct bioautography, the interaction of the active components was tested using *P. aeruginosa.* EOs, which contain the active compounds, were also involved in our experiments. FICI values were calculated.

In parallel with our observation obtained from direct bioautography, there was synergistic effect between menthol and thymol (FICI: 0.38). In the case of menthol-eugenol (FICI: 0.38) combination, and peppermint oil-thyme oil mixture, a synergy (FICI: 0.25) was also observed.

Surprisingly, no interaction was detected between EOs of peppermint and clove (FICI: 1.50), while the combination of peppermint and cinnamon bark oils was evaluated as additive (FICI: 0.63). In contrast, the mixture of their main components (*trans*-cinnamaldehyde and menthol) did not show any activity. According to our findings, the minor components may play important role in the antibacterial effect. Unfortunately, the combination of citronella oil with thyme oil, and the mixture of citronellal with thymol did not manage any enhanced inhibition, therefore, their FICI values could not be calculated.

We concluded that against *P. aeruginosa*, the combinations involved menthol and thymol, as well as peppermint and thyme oil had synergistic effect.

5. Summary and novel findings

The results and novel findings of our research are summarized below:

1. We determined the EO composition of a less known Mongolian plant, *Artemisia adamsii,* with GC-MS and GC-FID analysis.
2. According to our knowledge, it was the first time when EO of *A. adamsii* was tested against MRSA. Our results revealed that the main components of the oil (thujone, and 1,8-cineole) have no apparent antibacterial activity. We presume that other terpenoids are responsible for the activity.
3. We detected the activity of citronella oil in direct bioautography system against MRSA first.
4. Antibacterial effect of our EO vapors was confirmed against resistant strains (MRSA and R-*P. aeruginosa*) first. Cinnamon bark vapor presented the best inhibition (MIC: 31.25-125 µL/L). Besides, thyme, clove, peppermint, and citronella vapor were also regarded as inhibitors of MRSA. In the case of R-*P. aeruginosa*, only cinnamon bark vapor showed activity.
5. In the case of pathogens, which require special growth medium, we optimised the vapor phase system. We detected the effect of thyme, clove, and cinnamon bark vapor against *H. parainfluenzae* first. The activity of citronella vapor was investigated first against respiratory pathogens as well. Activity of the vapor of EOs used in this study was determined first against *S. mutans*, and *M. catarrhalis.*
6. Effect of peppermint vapor was established at the first time in the case of *H. parainfluenzae* as well. We also detected the differences between the *Haemophilus* species. Contrary to our expectation, scots pine and eucalyptus vapors were ineffective against the Gram-positive strains and *Pseudomonas* spp. In higher concentrations, eucalyptus showed activity in the case of *Haempohilus* spp. and *M. catarrhalis*. Scots pine vapor inhibited only the growth of *H. influenzae*.
7. In liquid phase, we observed the inhibitory potency of citronella and scots pine oil against *H. influenzae* first. Besides the EOs mentioned above, thyme, clove, and peppermint showed activity against *H. parainfluenzae* as well.
8. Based on our result in BDT, we can conclude that clove and thyme oil should be applied in liquid form against MRSA, *Streptococcus* spp., and *M. catarrhalis*. In contrast, we suggest that lower concentration of cinnamon bark may be used via inhalation to achive the best inhibition against respiratory tract bacteria.
9. To investigate the interaction between EOs and their components, we applied direct bioautography for the first time. In combination with statistical analysis, it could be regarded to a cost effective, quick screening method. The disadvantage of this method is that additive effect can not be detected precisely.The interaction between peppermint and thyme, cinnamon, and clove were tested for the first time against *P. aeruginosa,* and the combination of thyme and citronella with checkerboard titration

as well. The combination of menthol and cinnamaldehyde, or the mixtures of citral, citronellal, and geraniol with thymol were evaluated first in the case of this pathogen. We detected the combinations of *trans*-cinnamaldehyde-thymol, *trans*-cinnamaldehyde-eugenol, and thymol-eugenol first against R.-*P. aeruginosa,* similarly to the activity of menthol-eugenol, menthol-*trans*-cinnamaldehyde against MRSA.

Our findings justified that EOs and their components used in this study have antibacterial effect against respiratory tract pathogens, especially in the case of multiresistant bacteria. Therefore, we suggest that EOs could be promising supplements in the therapy. In the future, further experiments are required to determine their mode of action and cytotoxicity as well. We suggest that our findings provide relevant data for the development of new therapeuticals against RTI's.

Based on previous results and our observations, we can recommend the modification of some preparations in Formulae Normales VII. In the future, the promising results of the combinations of active components should be extended to involve other respiratory pathogens and to use the results for designing *in vivo* studies. Due to the growing appearance of antibiotic resistant pathogens, it would be reasonable to focus on the combination of EOs and their main componenst with antibiotics as well.

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8. List of publications

8.1 Articles related to the thesis

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8.3 Abstracts

8.3.1 Oral presentations

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