# Optimization and Study of the Antibacterial Effects of Clary Sage Extracts and Thyme Essential Oils Against Respiratory Pathogens

PhD Thesis


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## 1 Introduction

The increasing problem of antibiotic resistance presents a continuous challenge for healthcare professionals. According to the direction of the latest research in the field, the failure of therapies is attributed to the biofilm-forming ability of several pathogens (e.g., *Pseudomonas aeruginosa*, *Staphylococcus epidermidis*, methicillin-resistant *Staphylococcus aureus* (MRSA), *Acinetobacter baumannii*, *Haemophilus* spp.), which enhances their resistance to antibiotics.

With the increasing prevalence of antibiotic resistance, natural compounds are becoming more prominent in various industry sectors. The composition of essential oils and plant extracts can be influenced by numerous factors: environmental conditions, growth period, collection techniques, type of extraction procedure, chemotype, and the collected parts of the plant too. These factors can affect the chemical composition, which in turn impacts the biological activity. To achieve optimal yield and the appropriate quality with antibacterial efficacy, it is essential to clarify the relationship between environmental conditions and microbiological potential. Therefore, standardized extraction procedures, analytical investigations, and in vitro biological effect assessments are necessary to evaluate both chemical composition and antibacterial activity. Currently, environmentally friendly extraction methods, such as steam distillation and supercritical fluid extraction (SFE), are preferred in various industrial sectors. SFE is a well-parameterized method where minor adjustments to key parameters can significantly influence not only the extraction yield but also the antibacterial effect of the extract. One possible approach to a deeper understanding and optimization of the process is statistical modeling. By applying response surface models, quality can be optimized, and both time and costs can be saved.

Two prominent members of the Lamiaceae family are clary sage (*Salvia sclarea* L.) and common thyme (*Thymus vulgaris* L.). Traditionally, both plants have been successfully used for centuries, particularly against respiratory pathogens (Leigh-de Rapper et al., 2021). Their application has been significant primarily within the frameworks of aromatherapy and herbal medicine. However, numerous studies have demonstrated that their essential oils owe much of their antibacterial activity to the oxygenated monoterpenes they contain (e.g., linalool, linalyl acetate, thymol) (Oliveira et al., 2020). In Hungary, the cultivation of common thyme is increasing, and valuable data on the scientific evaluation of plants grown in private areas could further its utilization. Therefore, our research group investigated the antibacterial effects of essential oil samples extracted from common thyme collected at different flowering phenophases

(beginning of flowering, full bloom, end of flowering) against respiratory pathogens. One possible approach for "recycling" conventional antibiotics is to test combinations with plant-derived compounds and explore various additive and synergistic effects. In our work, we tested combinations of aminoglycoside-type antibiotics with thyme essential oil against bacteria causing respiratory infections. The combinational experiments were evaluated by using an in vitro checkerboard titration method.

## 2 Objectives

Based on the introduction, the following objectives were established:

- Evaluation of thyme grown in a cultivation area near Szigetvár (Hungary, Baranya County, coordinates: 46°02'60.0000" N, 17°47'59.9900" E) in terms of the antibacterial activity of essential oils extracted from samples collected at different phenophases (pre-flowering, full flowering, post-flowering).
- Optimization of clary sage supercritical fluid extraction (SFE) for antibacterial effects using response surface modeling (RSM).
- Determination of the chemical composition of the thyme essential oils examined by GC-MS (gas chromatography-mass spectrometry) and GC-FID (gas chromatography with flame ionization detection) techniques.
- Investigation of the antibacterial activity of clary sage extracts and thyme essential oils using a bioautography method.
- Demonstration of the biofilm-inhibitory effect of thyme essential oil.
- Examination of the antibacterial effects and potential synergistic interactions of combinations of aminoglycoside-type antibiotics and thyme essential oil.

## 3 Materials and methods

### 3.1 Plant materials

#### 3.1.1 Clary sage (*Salvia sclarea* L.)

The plant material was purchased from Naturix24 Ltd. (Dransfeld, Germany) in 2019. Within Europe, the company imports its clary sage from Italy and the south of France (Naturix24, 2019). Growing and harvesting crops complies with the recommendations of Good Agricultural Practice. After collection, the plant was air-dried and delivered to Germany. The whole plant has been processed. According to the statement of the merchant, the content of sclareol was relatively high, but the proportion of the compound in the essential oil was not higher than 12%.

#### 3.1.2 Thyme (*Thymus vulgaris* L.)

Thyme (*Thymus vulgaris* L.) was collected at three stages of flowering: at the beginning of flowering (May 23, 2019), full bloom (June 6, 2019), and the end of the flowering period (June 12, 2019). The collection site is located near Szigetvár (Hungary, Baranya County, coordinates: 46°02'60.0000" N, 17°47'59.9900" E). Meteorological data for the year are summarized in Table 1 (KSH, 2023). The plant material collected during the three flowering phenophases was divided into two portions. The first portion (fresh material) was used immediately, while the second portion was dried for one week. Drying occurred at room temperature (23°C) in the herbal dryer at the Department of Pharmacognosy, PTE GYTK (Pécs, Hungary). Essential oils from both fresh and dried plant samples were extracted using steam distillation, according to the specifications of the VIII$^{th}$ edition of the Hungarian Pharmacopoeia.

Table 1. Meteorological data during the collection period

| | May | June |
|---|---|---|
| Maximum temperature | 25.3°C | 34.7°C |
| Minimum temperature | 3.3°C | 12.9°C |
| Rainy days | 18 days | 12 days |
| Rainfall | 152 mm | 95 mm |
| Sunny hours | 202 | 336 |

### 3.2 Extractions

#### 3.2.1 Supercritical fluid extraction (SFE) of Clary Sage

The studies were conducted at the BKV Research and Development Center of Pannon University. For the supercritical fluid extraction, $CO_2$ with a purity of 99.97% (w/w) (Messer Ltd.) was used.

The extractions were performed using an SF2000 Able & Jasco device (Jasco Ltd.). A stainless steel column with dimensions of 30 cm × 20 mm and a volume of 94.2 cm³ was used as the extraction vessel. For each measurement, approximately 22 g of finely ground, dried clary

sage was loaded into the column. The exact mass of the sample was recorded each time. The mobile phase was 99.9% pure $CO_2$, and absolute ethanol (Molar Ltd.) was added as a co-solvent at a concentration of 1-2%. Each extraction lasted for 120 minutes, as preliminary experiments indicated that the extraction yield did not significantly change after two hours. The extracts were collected in 15 ml centrifuge tubes, and the weight of the tubes were recorded. The samples were stored at -10°C until further processing.

For thyme, the essential oil distillation from the collected plant material was carried out at the Department of Pharmacognosy, University of Pécs. Essential oils can be easily extracted from various plant parts using steam, making steam distillation one of the most commonly used techniques. After grinding, 100 g of the herb was placed in a glass flask, and 1 liter of distilled water was added to the sample. The flask, containing both water and plant material, was then placed in a heating basket. Continuous heating allowed the essential oil to evaporate from the plant with the steam and then condense upon contact with the cooled condenser. The essential oil obtained during the distillation (at 170°C for 3.5 hours) was stored in a dark glass container at 4°C until use.

### 3.3 Cultivation of bacteria used in experiments

The antibacterial effects of the plant extracts were tested against strains of *Haemophilus* spp. (*Haemophilus influenzae* DSM 4690; *H. parainfluenzae* DSM 8978), methicillin-resistant *Staphylococcus aureus* (MRSA ATCC 700698), and *P. aeruginosa* ATCC 27853. For the thin-layer chromatography with direct bioautography (TLC-DB) assays, the *Haemophilus* strains were cultured in a medium containing 100 ml Brain Heart Infusion (BHI) (Sigma Aldrich Ltd.), 1 ml B-Supplement (Diagon Ltd.), and 15 µg/ml NAD solution (1 mg/ml). MRSA and *P. aeruginosa* were grown in 100 ml BHI. Each bacterial culture was incubated at 37°C with shaking at 60 rpm for 12 hours in a shaking incubator (New Brunswick Scientific Ltd.) (Balázs et al., 2019).

### 3.4 Chromatographic analyses

#### 3.4.1 Determination of ethanol content in clary sage samples

The analyses were carried out with an Agilent 6890N GC-FID (Santa Clara, CA, USA) system equipped with a TR-WAX (Thermo Fisher Scientific, Massachusetts, USA) capillary column (30 m × 250 µm × 1.0 µm). The GC oven temperature was programmed to increase from 60ºC (5 min isothermal) – 240ºC at 30ºC/min (5 min isothermal). High-purity hydrogen (5.0) was used as carrier gas at 2.9 mL/min (29 cm/s) in constant pres-sure mode. Vials were crimped to minimize the loss of volatile species. Absolute ethanol (a.r., Molar Chemicals Kft., Halásztelek, Hungary) was used as a standard to identify the ethanol peak based on retention time. For FID quantification, the external standard technique was used. 100 mg absolute ethanol was diluted

with dimethyl sulfoxide (a.r., Molar Chemicals Kft., Halásztelek, Hungary) to achieve a final concentration of 10 mg/mL as a stock solution. The calibration curve covered the range of 0.5 2.0 mg/mL.

### 3.4.2 Analysis of Thyme essential oil Samples using GC-MS and GC-FID techniques

The measurements were assisted by colleagues from the University of Messina, Italy. The samples (10 µL) were solubilized in 990 µL n-heptane (1:100 dilution) (Merck Ltd.) and then injected into the GC-MS and GC-FID systems for complete identification and quantification. The separation and identification of terpene and terpenoid compounds were carried out using a GCMS-QP2020 instrument (Shimadzu Ltd.) equipped with a split-splitless injector at 280°C and an AOC-20i automatic sampler. A non-polar capillary column, specifically SLB-5ms 30 m × 0.25 mm ID × 0.25 µm (Merck Ltd.), was used for the separation of analytes. Quantitative analyses were performed using a GC-2010 instrument (Shimadzu Ltd.) with a split-splitless injector (280°C), FID detector, and AOC-20i automatic sampler. The chromatographic conditions included: a volumetric injection of 0.5 µL in split mode (1:10) and a temperature program from 50°C to 300°C at a rate of 3.0°C/min. Helium was used as the carrier gas with a linear velocity of 30 cm/s. The MS parameters were: mass range 40-550 amu; ion source temperature: 220°C; and interface temperature: 250°C. The FID detector parameters included: detector temperature set to 300°C (sampling rate 40 ms), with a gas flow of 40 ml/min for hydrogen, 30 ml/min for make-up gas (nitrogen), and 400 ml/min for air. Data collection and processing were conducted using GCMS solution software (version 4.50, Shimadzu Ltd.), and compound identification was performed using the FFNSC mass spectrum library (version 4.0, Shimadzu Ltd.). Two identification parameters were applied: MS spectral similarity and linear retention index (LRI) matching (Pandur et al., 2022). C7-C40 saturated alkanes were used for LRI calculation. The standard mixture concentration was 1000 µg/ml, with all components dissolved in hexane (Merck Ltd.). GC-FID analyses were performed and processed using LabSolution software (version 5.92, Shimadzu Ltd.). Each sample was analyzed in triplicate to increase data accuracy (Micalizzi et al., 2020).

## 3.5 Thin-layer chromatography – Direct bioautography (TLC-DB)

The microbiological experiments were conducted at the Department of Medical Microbiology and Immunology, Medical School, University of Pécs.

For the clary sage samples, the analysis was conducted without chromatographic development, as we investigated the antibacterial effect of the total extract. In the case of thyme, we studied the antibacterial effect both without thin-layer chromatographic (TLC) separation and after TLC separation (Balázs et al., 2019). For thyme essential oils, the most effective samples in terms of antibacterial activity were selected during the non-separation examination. Only the oils

with the highest activity were subjected to further analysis, so only thyme oils distilled from fresh plant material were included in the TLC separation-related bioautography experiments. Chromatography was performed on 10x10 cm silica gel 60 F254 aluminum plates (Merck Ltd.).

For clary sage samples: Since the ethanol content of the extracts varied, we determined the solvent content using GC-FID. During chloroform dilution, we corrected for the different concentrations, ensuring that the applied sample solutions had a concentration of 10 mg/ml in all cases. We spotted 3.0 µL on the thin-layer chromatography (TLC) plate, with chloroform and absolute ethanol (Molar Ltd.) used as solvent controls. The positive control for MRSA was vancomycin (Pharmacologic) (stock solution: 50 mg/ml; 0.6 µL was applied to the plate), and gentamicin (Sandoz) was used for *P. aeruginosa* (stock solution: 40 mg/ml; 0.75 µL was applied to the plate).

For thyme samples: the samples were dissolved in absolute ethanol (the stock solution was 200 mg/ml), and 1.0 µl was applied to the thin-layer chromatographic plate using Finnpipette pipettes (Merck Ltd.). Absolute ethanol was used as the solvent control, and antibiotics were used as positive controls. For *Haemophilus* strains, ceftriaxone (Hospira, stock solution: 40 mg/ml) was applied, and gentamicin (Sandoz, stock solution: 40 mg/ml) was used against *P. aeruginosa*. 1-1 µl of the antibiotic solutions was applied to the TLC plate. During the TLC separation, the antibacterial activity of thymol, as the main component of thyme essential oil, was also investigated using TLC-DB. Thymol (Spektrum-3D Ltd.) was dissolved in absolute ethanol (20 mg/ml). 0.2 µl (4 µg) of the stock solution was applied to the plates. After applying the samples, the TLC plates were developed with toluene acetate (95:5 v/v) as the mobile phase (Horváth et al., 2018). Chromatographic development was performed in a twin-chamber (Camag Ltd.) and at room temperature (22°C) following saturation of the chamber with the eluent. After the thin-layer chromatographic separation, the adsorbent layers were dried under a safety hood for 5 minutes to completely remove the eluent. Ethanol vanillin-sulfuric acid reagent was used to visualize the compounds in the thyme essential oils. The separated compounds were detected based on the standard Rf value and color. The thin-layer chromatographic plates were also evaluated under UV light at 254 nm. The TLC plates intended for bioautography were not treated with ethanol vanillin-sulfuric acid reagent, as this step interferes with the microbiological steps of TLC-DB..

In the analysis of thyme essential oil, the detection of separated components was also performed. The total essential oil layers, as well as the developed layers containing thyme essential oil without prior elution, were dipped into 100 ml of bacterial suspension (4×10^7 CFU/ml). After dipping, the layers were placed in a shallow horizontal chamber (chamber dimensions: 20 x 14.5 x 5 cm) and incubated at 37°C for 4 hours. To visualize the antibacterial effect, indicated by clear zones of inhibition, the thin-layer chromatographic plates were dipped into a water solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 0.05 g/75 ml) (Sigma Aldrich Ltd.) for 5 seconds and then incubated at 37°C for 12 hours.

Metabolically active bacteria on the TLC plates converted MTT into a formazan dye. White spots (zones of inhibition) appearing against a blue-purple background indicated a lack of dehydrogenase enzyme activity, reflecting the antibacterial activity of the tested samples or their main compound (Siddiquee et al., 2023). The zones of inhibition for samples without separation were measured in millimeters using the Motic Images Plus 2.0 software (version 2.0, Motic Ltd.). Thyme essential oil samples were further subjected to checkerboard titration assays, as the musk sage extracts contained ethanol used as a solvent during extraction, which might affect the test results.

### 3.6 MIC determination by microdilution method (BMD)

Based on the TLC-DB results, the tests were conducted exclusively with thyme essential oil samples distilled from fresh plant material. This examination, as well as the biofilm inhibition studies, were performed using the microdilution method.

The minimum inhibitory concentrations were determined by the microdilution method.

After incubation (24 hours at 37°C), the absorbance of the samples was measured at 600 nm (BMG Labtech, Bio-Tek Kft.). The average of six repetitions was calculated, and then the negative control average was subtracted from the obtained value. The minimum inhibitory concentration (MIC) was determined as the lowest concentration of the essential oil that was able to inhibit bacterial growth by >90% compared to the control after the incubation period.

### 3.7 Investigation of Biofilm Inhibition Effect

The investigation was conducted using thyme essential oil. For the biofilm inhibition experiments, essential oil samples were distilled from fresh plant material collected at three different stages of flowering: early in the flowering period, during full bloom, and at the end of the flowering period. The Minimum Inhibitory Concentration (MIC) values were determined for each stage and used at a concentration of MIC/2 for the experiments.

For biofilm formation, a 96-well microtiter plate was used. After treatment, biofilms were stained with a crystal violet solution. Absorbance was then measured at $\lambda$ = 595 nm using a microtiter plate reader (BMG Labtech SPECTROstar Nano Kft.). Each experiment was conducted in sextuplicate (Balázs et al., 2019).

### 3.8 Scanning Electron Microscopy (SEM) Analysis

The morphological investigations were conducted at the Szentágothai János Research Centre of PTE. The experiments involved the use of thyme essential oil.

The morphological investigations were conducted at the Szentágothai János Research Centre of PTE. *P. aeruginosa*, *H. influenzae*, and *H. parainfluenzae* were used in the experiments. Scanning electron microscopy (SEM) was employed to examine the structural modifications of the biofilms. Based on the biofilm analysis results, the thyme essential oil sample distilled from fresh plant material collected before flowering was examined. For biofilm formation, 5 ml of *P. aeruginosa*, *H. influenzae*, and *H. parainfluenzae* BHI cultures (10^8 CFU/ml) were transferred into sterile glass containers. The biofilms were prepared according to the SEM protocol. Subsequently, the samples were coated with a gold layer and examined using a JEOL JSM IT500-HR scanning electron microscope (Jeol Ltd.) (Kerekes et al., 2013).

### 3.9 Checkerboard-titration

To observe the interactions between thyme essential oil and antibiotics, checkerboard titrations were performed. The measurements were also carried out on 96-well microtiter plates. Each well contained 50-50 µl of the test substance (A and B substances) dissolved in BHI broth at a given concentration, and 100 µl of a suspension of cells in the early stationary phase at $10^5$ CFU/ml..

Concentrations: 2×MIC, MIC, MIC/2, MIC/4. The combinations of the agents on the microtiter plate were arranged in decreasing order of concentration in opposite directions. After preparing the plate, it was incubated for 24 hours at 37°C. Subsequently, absorbance values were measured at 600 nm using the previously mentioned microtiter plate reader. The untreated bacterial suspension was used as the positive control, and the cell-free medium was used as the negative control. The results were obtained from two independent parallel measurements.

### 3.10 Statistical analysis

#### 3.10.1 Statistical analysis for thyme oil

Statistical analyses were performed using R software version 4.0.2 (R Development Core Team 2020). The measured inhibition zones were analyzed using a linear model. In our model, independent variables (bacterial strains, different phenophases of thyme) were treated as fixed factors. Due to the normal distribution of the data, no data transformation was applied. The necessity of transformation was checked based on graphical evaluation. Since F-tests confirmed the equality of variances, statistical hypothesis testing was conducted using ANOVA. Pairwise comparisons were performed using Tukey post-hoc tests with the multcomp package (Hothorn et al., 2008), which allowed for comparisons between all experimental arrangements.

#### 3.10.2 Response Surface Modeling (RSM) for Nutmeg Sage Extracts

Statistical modeling was performed in the Designe-Expert program version 12 (Stat-Ease Inc. 2020). A complete factorial model was used to determine the factors of the experiment. With

its help, the extraction process can be optimized to achieve the greatest possible antimicrobial effect against the tested respiratory pathogens. Factors are parameters that greatly influence the process, and factor levels are values that can be assumed by the factors. Extraction pressure *x1* is the ratio of extraction temperature *x2* and cosolvent *x3* (Jokić et al., 2018).

Table 2. Factors Coding and Experimental Points in Design-Expert 12 Software

| Independent variable | Symbol | Levels | | |
|---|---|---|---|---|
| | | Low (-1) | Middle (0) | High (+1) |
| Pressure (Mpa) | *A* | 10 | 15 | 20 |
| Temperature (°C) | *B* | 40 | 60 | 80 |
| Cosolvent (%) | *C* | 1.0 | 1.5 | 2.0 |

## 4 Results

### 4.1 Antibacterial activity of clary sage extracts

The antibacterial activity of the extracts was examined without chromatographic separation using the TLC-DB method. *P. aeruginosa* and MRSA strains were selected as test bacteria. The results are summarized in Table 3.

Table 3. Extraction conditions and inhibition zones of SFE extracts

| **Samples** | ***P. aeruginosa* Diameter (mm)** | **[1]SD** | **MRSA Diameter (mm)** | **[1]SD** | **MPa** | **°C** | **EtOH (%)** |
|---|---|---|---|---|---|---|---|
| **1** | 5,86 | 0,72 | 5,46 | 0,50 | 20 | 40 | 2 |
| **2** | 5,50 | 0,56 | 4,85 | 0,78 | 15 | 40 | 2 |
| **3** | 7,51 | 0,85 | 7,57 | 0,62 | 10 | 40 | 2 |
| **4** | 6,18 | 0,64 | 4,69 | 0,46 | 20 | 60 | 2 |
| **5** | 5,43 | 0,42 | 4,80 | 0,38 | 15 | 60 | 2 |
| **6** | 0,00 | 0,00 | 0,00 | 0,00 | 10 | 60 | 2 |
| **7** | 4,29 | 0,71 | 4,66 | 0,39 | 20 | 80 | 2 |
| **8** | 4,83 | 0,29 | 4,32 | 0,59 | 15 | 80 | 2 |
| **9** | 0,00 | 0,00 | 0,00 | 0,00 | 10 | 80 | 2 |
| **10** | 6,50 | 0,42 | 4,59 | 0,65 | 20 | 40 | 1,5 |
| **11** | 6,91 | 0,20 | 4,77 | 0,69 | 15 | 40 | 1,5 |
| **12** | 6,27 | 0,33 | 3,29 | 0,42 | 10 | 40 | 1,5 |
| **13** | 5,41 | 0,47 | 3,41 | 0,36 | 20 | 60 | 1,5 |
| **14** | 5,71 | 0,80 | 4,25 | 0,35 | 15 | 60 | 1,5 |
| **15** | 0,00 | 0,00 | 0,00 | 0,00 | 10 | 60 | 1,5 |
| **16** | 6,37 | 0,78 | 3,50 | 0,40 | 20 | 80 | 1,5 |
| **17** | 6,56 | 0,55 | 3,29 | 0,46 | 15 | 80 | 1,5 |
| **18** | 0,00 | 0,00 | 0,00 | 0,00 | 10 | 80 | 1,5 |

| Samples | *P. aeruginosa* Diameter (mm) | [1.]SD | MRSA Diameter (mm) | [1.]SD | MPa | °C | EtOH (%) |
|---|---|---|---|---|---|---|---|
| **19** | 5,96 | 0,15 | 4,44 | 0,53 | 20 | 40 | 1 |
| **20** | 6,34 | 0,82 | 3,58 | 0,82 | 15 | 40 | 1 |
| **21** | 5,32 | 0,41 | 3,72 | 0,40 | 10 | 40 | 1 |
| **22** | 5,61 | 0,47 | 3,72 | 0,40 | 20 | 60 | 1 |
| **23** | 4,98 | 0,61 | 3,07 | 0,36 | 15 | 60 | 1 |
| **24** | 0,00 | 0,00 | 0,00 | 0,00 | 10 | 60 | 1 |
| **25** | 3,02 | 0,23 | 3,40 | 0,58 | 20 | 80 | 1 |
| **26** | 2,75 | 0,48 | 3,25 | 0,68 | 15 | 80 | 1 |
| **27** | 0,00 | 0,00 | 0,00 | 0,00 | 10 | 80 | 1 |
| **chloroform** | 0,00 | 0,00 | 0,00 | 0,00 | | | |
| **[2.]ch. + EtOH** | 0,00 | 0,00 | 0,00 | 0,00 | | | |
| **EtOH** | 0,00 | 0,00 | 0,00 | 0,00 | | | |
| **vancomycin** | - | - | 12,30 | 0,45 | | | |
| **gentamicin** | 8,26 | 0,81 | - | - | | | |

[1.]SD = standard deviation; [2.] ch. = chloroform

## 4.2 Optimization of the SFE Method

The statistical analysis of the results obtained during the extractions confirmed that the data were suitable for model development and for determining the optimal parameters.

The optimal extraction parameters were determined by considering the antibacterial effect on both bacteria during the calculation. In both cases, the goal was maximization. No minimum value was set for yield in this calculation. The following results were obtained: the calculated optimal setting parameters were 18.6 MPa, a temperature of 40°C, and an EtOH ratio of 2%. Under these parameters, the predicted inhibition zone diameters were *P. aeruginosa*: 7.95 mm and MRSA: 7.57 mm, with a yield of 3.64 m/m%.

## 4.3 Thyme oil results

### 4.3.1 Results of steam distillation

The quantities of essential oils obtained from fresh and dried plants collected at different phenophases are presented in Table 4.

Table 4. The results of the distillation of thyme samples

| | Collection periods | | |
|---|---|---|---|
| | Beginning of flowering | Fool bloom | End of flowering |
| Fresh thyme (g) | 1826 | 1464 | 764 |
| Distilled essential oil (µl) | 8450 | 6350 | 3920 |
| Yield (ml/100 g) | 0,46 | 0,43 | 0,51 |
| Dried Thyme (g) | 963 | 964 | 1440 |
| Distilled essential oil (µl) | 7520 | 6080 | 7550 |
| Yield (ml/100 g) | 0,78 | 0,63 | 0,52 |

4.3.2 Chemical composition of thyme essential oil

The results are summarized in Table 5.

Table 5. Percentage composition of essential oil samples extracted from fresh and dried thyme collected at different flowering phenophases

| **Compounds** | **MS Sim (%)** | **LRI Exp** | **LRI Ref** | **1** | **2** | **3** | **4** | **5** | **6** |
|---|---|---|---|---|---|---|---|---|---|
| *α*-Thujene | 98 | 925 | 927 | 0,99 | 1,09 | 0,99 | 0,46 | 0,21 | 0,37 |
| Myrcene | 96 | 988 | 991 | 1,45 | 1,34 | 1,28 | 1,17 | 0,71 | 0,84 |
| *α*-Terpinene | 98 | 1017 | 1018 | 1,40 | 1,09 | 0,82 | 1,15 | 0,74 | 0,79 |
| *p*-Cimén | 96 | 1025 | 1025 | 12,89 | 17,44 | 20,64 | 12,46 | 15,02 | 22,78 |
| *γ*-Terpinene | 95 | 1058 | 1058 | 15,18 | 7,38 | 6,01 | 13,67 | 5,06 | 5,49 |
| Linalool | 97 | 1099 | 1101 | 1,46 | 1,56 | 2,15 | 1,69 | 1,67 | 2,17 |
| Thymol | 94 | 1294 | 1293 | 55,81 | 57,10 | 54,21 | 56,39 | 62,46 | 52,33 |
| Carvacrol | 94 | 1302 | 1300 | 2,30 | 2,92 | 2,90 | 2,98 | 3,48 | 3,11 |
| (*E*)- Caryophyllene | 97 | 1421 | 1424 | 0,99 | 2,05 | 0,99 | 2,15 | 2,50 | 2,44 |
| Total | | | | 99,77 | 99,76 | 99,65 | 99,69 | 99,69 | 99,49 |

Abbreviations: MS Sim, MS spectral similarity; LRI Exp, experimental linear retention index; LRI Ref, reference linear retention index; 1. Fresh plant material collected at the beginning of flowering, 2. Fresh plant material collected at full flowering, 3. Fresh plant material collected at the end of flowering, 4. Dried plant material collected at the beginning of flowering, 5. Dried plant material collected at full flowering, 6. Dried plant material collected at the end of flowering. The table does not include components present in less than 1%.

4.3.3 Results of the TLC-DB investigations

We investigated the antibacterial effect of thyme essential oils using the TLC-DB method against *Haemophilus* spp. and *P. aeruginosa* bacterial strains. The experiments were initially

conducted without chromatographic separation, as we were interested in the antibacterial effect of the "whole" essential oil (thyme essential oil). The inhibition zone diameters were expressed in mm. From the essential oil stock solution, 1 µl (equivalent to 0.2 mg of undiluted oil) was applied to the TLC plate. *Haemophilus* spp. was more sensitive to the thyme oil than *P. aeruginosa*. Absolute ethanol, as a negative control, did not inhibit the growth of any of the bacterial strains. The antibiotic sample's 1 µl solution (ceftriaxone against *Haemophilus* spp., gentamicin against *P. aeruginosa*) was effective against the tested bacteria. In general, essential oils derived from fresh plant material exhibited greater antibacterial activity compared to those from dried plant material. The thyme essential oil distilled from fresh plants and collected at the beginning of flowering proved to be the most effective against the tested pathogens: *Haemophilus influenzae* - 7.04 mm, *H. parainfluenzae* - 6.5 mm, and *P. aeruginosa* - 5.5 mm. Essential oils from fresh plant material collected during full bloom and at the end of blooming also demonstrated antibacterial effects (full bloom: *H. influenzae* - 6.3 mm, *H. parainfluenzae* - 5.2 mm, *P. aeruginosa* - 4.9 mm; end of blooming: *H. influenzae* - 6.15 mm, *H. parainfluenzae* - 4.8 mm, *P. aeruginosa* - 4.5 mm). The smallest inhibition zones were obtained with essential oils distilled from dried material and collected at the end of blooming (*H. influenzae* - 5 mm, *H. parainfluenzae* - 4.8 mm, *P. aeruginosa* - 3.7 mm). Overall, it can be concluded that essential oils distilled from fresh plant samples were more effective than those from dried plant material. Additionally, concerning the flowering phenophase, the essential oil from plant material collected at the beginning of blooming was the most effective. The antibiotic controls were more effective than the thyme essential oil samples at the concentrations used in the study. The antibacterial effects of thymol and *p*-cymene were investigated by Gömöri et al. both separately and in combination. Thymol alone showed greater antibacterial activity than *p*-cymene alone or any combination of the two components. However, essential oils with reduced thymol and increased *p*-cymene and *γ*-terpinene content exhibited higher antimicrobial activity in some cases (Gömöri et al., 2018). Our results support these findings.

After chromatographic separation using the TLC-DB method, it is possible to examine the effects of antibacterial active components individually. The TLC-DB experiments without separation were considered as preliminary screening. Based on these results, only the essential oils distilled from fresh plant material were included in the bioautographic assays with chromatographic separation. In the thyme essential oils, the component thymol (Rf = 0.56) and its standard showed activity against all the tested bacteria. Additional biological activity was detected at Rf = 0.33. This corresponds to the Rf value of linalool according to Wagner and Bladt (Wagner & Bladt, 2001). Since the relative amount of linalool in the samples was low (1.56%–2.17%), co-elution with other antibacterial active components is suspected.

4.3.4 Results of minimum inhibitory concentration (MIC) determination

Based on the TLC-DB results, only the thyme essential oil samples distilled from fresh plant material were used in this study and in the biofilm inhibition experiments.

Table 6. MIC and MIC/2 Values of Thyme Essential Oils (mg/ml) and Antibiotics (Gentamicin and Amikacin) (µg/ml)

| | **Collection period** | **1** | **2** | **3** |
|---|---|---|---|---|
| **MIC value** | At the beginning of flowering | 0,156 | 0,156 | 1,50 |
| | In full bloom | 0,187 | 0,187 | 1,750 |
| | At the end of the flowering period | 0,187 | 0,187 | 1,750 |
| **MIC/2 value** | At the beginning of flowering | 0,078 | 0,078 | 0,75 |
| | In full bloom | 0,093 | 0,093 | 0,87 |
| | At the end of the flowering period | 0,093 | 0,093 | 0,87 |
| **MIC value** | Gentamicin | --- | --- | 6,30 |
| | Amikacin | 3,10 | 1,60 | --- |
| **MIC/2 value** | Gentamicin | --- | --- | 3,15 |
| | Amikacin | 1,55 | 1,30 | --- |

**1**: *H. influenzae*, **2**: *H. parainfluenzae*, **3**: *P. aeruginosa*

4.3.5 Biofilm test results

Our results showed that all freshly collected thyme essential oil samples inhibited the biofilm formation. The distilled essential oil of fresh thyme collected at the beginning of flowering was the most effective against all tested bacteria. This oil showed the highest inhibition of 72,93% against *P. aeruginosa*. In the case of *H. influenzae* and *H. parainfluenzae,* we calculated an inhibition rate of 72,32% and of 64,88%.

### 4.3.6 Results of SEM Analysis

Based on the results of the TLC-DB and biofilm inhibition studies, only the essential oil distilled from fresh *Thymus vulgaris* collected at the beginning of the flowering period was included in the SEM analysis.

The images of the control samples (without essential oil treatment) showed characteristic morphological features of a mature, three-dimensional biofilm. Essential oil treatment resulted in bacteria adhering to the surface but not forming biofilm-specific structures. Additionally, cell wall degradation was also observed.

### 4.3.7 Checkerboard-titration

Based on the measurements of minimal inhibitory concentrations (MICs), we conducted combination experiments using the checkerboard-titration method. The antibacterial effects of combinations of antibiotics from the aminoglycoside group (gentamicin, amikacin) and thyme essential oil were investigated on Gram-negative respiratory pathogens. For the study, we used essential oil samples collected at the beginning of flowering and freshly distilled. The summary of the results is presented in Table 7.

Table 7. Results of combination studies

| **Combination** | | **MIC mg/ml** | | **MIC µg/ml** | | **FICI** | **Bacteria** |
|---|---|---|---|---|---|---|---|
| | | **Component 1** | | **Component 2** | | | |
| **Component 1** | **Component 2** | **$MIC_A$** | **$MIC_B$** | **$MIC_A$** | **$MIC_B$** | | |
| Thyme oil | Amikacin | 0,156 | 0,078 | 3,100 | 1,550 | 1,00 | *H. influenzae* |
| Thyme oil | Amikacin | 0,156 | 0,039 | 1,600 | 0,800 | 0,75 | *H. parainfluenzae* |
| Thyme oil | Gentamicin | 1,500 | 0,375 | 6,300 | 1,575 | 0,49 | *P. aeruginosa* |

FICI: fractional inhibitory concentration index; The combination was considered additive in the case of a value between 0.5<FICI<1, and antagonistic in the case of FICI>4. If the FICI is between 1 and 4, no interaction between the components was detected.

## 5 New results, summary

1. We determined the antibacterial activity of sage extracts using the TLC-DB method against *Haemophilus* spp., MRSA, and *P. aeruginosa* strains. Generally, MRSA strains were less sensitive than *P. aeruginosa*. The largest average inhibition zone was observed for the triple sample, measuring 7.51 mm (SD = 0.85 mm) for *P. aeruginosa* and 7.57 mm (SD = 0.62 mm) for MRSA. The small variation in measurements provided a stable foundation for subsequent 3D modeling.

The *Haemophilus* strains were more sensitive to thyme oil than *P. aeruginosa*. The essential oil of thyme, distilled from fresh plants and collected at the beginning of flowering, was

found to be the most effective against the studied pathogens: *Haemophilus influenzae* - 7.04 mm, *H. parainfluenzae* - 6.5 mm, and *P. aeruginosa* - 5.5 mm. The TLC-DB experiments without separation were considered as preliminary screening. Based on the results, only essential oils distilled from fresh plant material were included in the chromatographic separation TLC-DB, during which thymol was identified as the main antibacterial component.

2. We first optimized SC-$CO_2$ extraction for antibacterial activity using response surface methodology (RSM). Our results clearly demonstrate that the correct selection of the combination of main operational parameters is critical for antibacterial activity. A major advantage of our method is that, in addition to determining antibacterial activity, the extraction yield remains calculable. The pressure and temperature applied during extraction had the greatest impact on the quality and quantity of the final product. RSM allows for the optimization of SC-$CO_2$ extraction and a deeper statistical understanding of the process.

3. We first evaluated the antibacterial effect of thyme essential oil from the cultivation area near Szigetvár (Hungary, Baranya County, coordinates: 46°02'60.0000" N, 17°47'59.9900" E), considering the flowering phenophases. The best antibacterial effect was observed in the samples collected at the beginning of flowering. Based on yield results, harvesting at the beginning of flowering does not result in yield loss in the area. The producer can gain a comparative advantage over competitors by starting the harvest earlier..

4. We succeeded in determining the chemical composition of *Thymus vulgaris* essential oils for each flowering phenophase. The flowering phenophases clearly affect the chemical composition of thyme essential oil and consequently its antibacterial activity. The ratio of *γ*-terpinene in the oils was almost three times higher at the beginning of flowering compared to the later flowering phenophases. The ratio of thymol was highest in samples collected during full bloom. Samples with reduced thymol and increased *p*-cymene and *γ*-terpinene content exhibited greater antibacterial activity.

5. Through biofilm analysis, we demonstrated that each freshly collected *Thymus vulgaris* essential oil sample inhibited biofilm formation. The essential oil distilled from fresh thyme collected at the beginning of flowering was the most effective against all tested bacteria. This oil showed the highest inhibition rate, 72.93%, against *P. aeruginosa*. In the case of *H. influenzae* and *H. parainfluenzae*, inhibition rates of 72.32% and 64.88% were recorded, respectively. SEM analysis confirmed that bacterial cells adhered to the surface but did not form biofilm-specific structures. Additionally, cell wall degradation was also observed.

6. We first investigated the *Thymus vulgaris* essential oil in combination with amikacin and gentamicin against *Haemophilus* spp. and *P. aeruginosa* strains. Through combination studies, we successfully demonstrated that thyme essential oil not only aids in enhancing the effectiveness of antibiotics through biofilm inhibition but also exhibits additive and synergistic

effects. A synergistic effect was observed with the combination of gentamicin and thyme essential oil against *P. aeruginosa*.

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## 8 Publication list

Publications forming the basis of the dissertation:

**Bakó Cs**, Balázs V.L., Kerekes E., Kocsis B., Nagy D.U., Szabó P, Micalizzi G., Mondello L., Krisch J., Pethő D., Horváth Gy. (2023): Flowering phenopases influence the antibacterial and antibiofilm effects of *Thymus vulgaris* L. essential oil. ***BMC Complementary Medicine and Therapies***. 23:168. **[IF: 2,838]**

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## 9 Conference list

**Bakó Cs.**, Balázs V.L., Takács Gy., Pallos J.P., Pál Sz., Kocsis B., Rippelné Pethő D., Horváth Gy. (2021) Muskotályzsályából nyert szuperkritikus folyadék extraktum biológiai aktivitásának optimalizálása analitikai és statisztikai módszerek kombinálásával. Fiatal Gyógynövénykutatók Fóruma. 2022. június 17. In: Book of Abstracts p. 6.

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