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Faculty of Environmental Sciences

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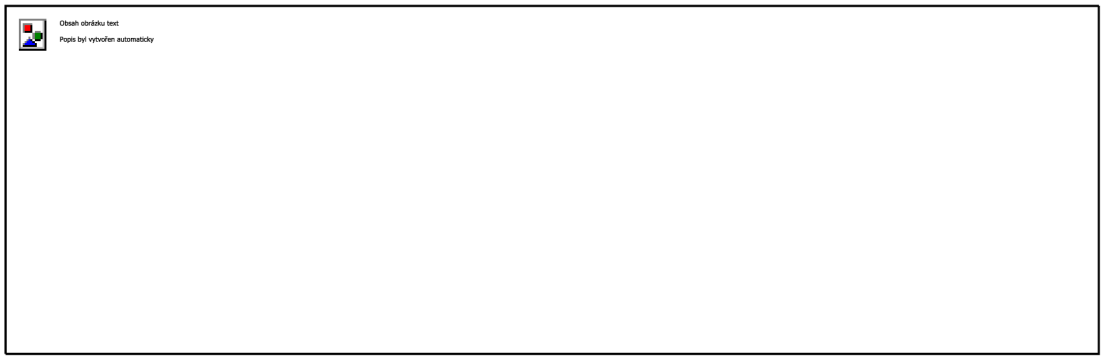

Chemical Composition and Antimicrobial Effect of *Thymus Vulgaris* Essential Oil Against Bacterial Pathogens Causing Pneumonia in Vapour Phase

BACHELOR'S THESIS

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Environmental Sciences
Applied Ecology

Thesis title

Chemical Composition and Antimicrobial Effect of Thymus Vulgaris Essential Oil Against Bacterial Pathogens Causing Pneumonia in Vapour Phase

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Objectives of thesis

The main aim of the study is to analyse chemical composition and antibacterial activity of T. vulgaris EO vapours against pathogens causing pneumonia
Specific objectives are:
- Determination of in vitro growth-inhibitory effect of T. vulgaris EO against H. influenzae, S. aureus and S. pyogenes in vapour and liquid phase using broth microdilution volatilization method
- Analysis of chemical composition of T. vulgaris EO and its vapours using GC-MS and HS-SPME sampling technique.

Methodology

Dried plant material will be grinded, homogenized. Essential oil will be obtained by hydrodestilation. Plant volatile compounds with potential antimicrobial effects in liquid and vapour phase will be determined using broth microdilution volatilization method. Chemical analysis of EO's will be performed using Gas Chromatography/ Mass Spectrometry (GCMS) for liquid phase and HS-SPME sampling technique for vapour phase.

The proposed extent of the thesis

40

Keywords

Antibacterial activity, Thymus vulgaris, Essential oil, Vapor, Volatilization method

Recommended information sources

Houdková M., Kokoška L. Volatile antimicrobial agents and in vitro methods for evaluating their activity in vapour phase: A review. Planta Medica, 2020, 12(86): .822-857

Houdková M., Rondevaldová J., Doskočil I., Kokoška L. Evaluation of antibacterial potential and toxicity of plant volatile compounds using new broth microdilution volatilization method and modified MTT assay. Fitoterapia 2017, 118 56–62.

Kokoška L., Klouček P., Leuner O., Nový P. Plant-derived products as antibacterial and antifungal agents in human health care. Current Medicinal Chemistry 2019, 26(29): 5501-5541.

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Dean

Prague on 31. 03. 2022

Declaration

I declare that I elaborated this Bachelor Thesis *"Chemical Composition and Antimicrobial Effect of Thymus Vulgaris Essential Oil Against Bacterial Pathogens Causing Pneumonia in Vapour Phase"* alone, and that I have used only literature, web resources and other resources mentioned in references at the end of the thesis.

I agree with placing this work in the library of CZU Prague and make it accessible for study purposes.

Prague 31.3.2022: Nicole Robin:

_____ _____

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Abstract

Pneumonia is a serious public health problem worldwide and it is caused by bacterial pathogens. *Thymus vulgaris* is used in traditional medicine to relieve pulmonary symptoms during infections. Essential oils obtained from *T. vulgaris* have been studied for years for its antimicrobial activity caused by volatile compounds contained in aerial parts of the plant. The objectives of this study were to determine *in vitro* growth-inhibitory effect of *T. vulgaris* EO against common bacterial strains of *H. influenzae*, *S. aureus* and *S. pyogenes* in vapour and liquid phase using broth microdilution volatilization method. *T. vulgaris* EO sample presented antimicrobial activity with minimum inhibitory concentrations (MIC) 512 μg/ml. Second objective was to determine chemical profile of headspace with sampling technique GC-MS: solid-phase microextraction (HS-SPME) using the HP-5MS column. Thymol, a monoterpene which is well known for its antibacterial and antimicrobial activities appeared with the highest percentage (48.09 %) of its content. These results suggest that *T. vulgaris* EO is a perspective plant derived product for further pharmaceutical applications.

Keywords: antimicrobial activity, essential oils, pneumonia, thymol

Abstrakt

Zápal plic je celosvětovým, vážným, zdravotním problémem, který je způsoben bakteriálními patogeny. *Thymus vulgaris* se používá v tradiční medicíně ke zmírnění příznaků respiračních onemocnění. Esenciální oleje získané z *T. vulgaris* jsou již léta studována pro svou antimikrobiální aktivitu způsobenou těkavými látkami obsaženými, které jsou záskávané z nadzemních částech rostliny. Prvním cílem této studie bylo stanovit růst-inhibiční účinek *T. vulgaris* EO proti běžným kmenům bakterií *H. influenzae, S. aureus* a *S. pyogenes* v parní a kapalné fázi pomocí mikrodiluční metody těkání bujónu. Vzorek EO *T. vulgaris* vykazoval antimikrobiální aktivitu s minimálními inhibičními koncentracemi (MIC) 512 μg/ml. Druhým cílem bylo stanovení chemického profilu výparů, vzorkovací technikou GC-MS: mikroextrakce na pevné fázi (HS-SPME) použitím kolony HP-5MS. S nejvyšším procentem obsahu (48,09 %) se objevil thymol, monoterpen, který je dobře známý pro své antibakteriální a antimikrobiální účinky. Tyto výsledky naznačují, že *T. vulgaris* EO je perspektivní rostlinný produkt pro další farmaceutické aplikace.

Klíčová slova: antimikrobiální aktivita, silice, zápal plic, thymol

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List of Abbreviations

ATCC	American Type Culture Collection
CLSI	Clinical and Laboratory Standards Institute
DSMZ	German Resource Centre for Biological Material
EO	Essential Oil
IPNI	International Plant Name Index
MHB	Mueller-Hinton broth
MIC	Minimum inhibitory concentration
WCB	Wilkins-Chalgren broth

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1 Introduction

1.1 Pneumonia

Pneumonia is one of major lung illnesses that affects our respiratory system. It is an inflammatory condition of lungs, primarily affecting the small air sacs known as alveoli and is usually caused by infection with viruses, bacteria or less commonly by other micro-organisms (Fatima et al., 2021). It can affect the entire lobe (lobar pneumonia), one or more segments (segmental pneumonia) or transfer from the bronchi to the appropriate alveoli (bronchopneumonia) or it affects interstitial tissue (interstitial pneumonia). Pneumonia can be transmitted by the aspiration or inhalation of a pathogenic microorganism. They may also spread via air-borne droplets from a cough or sneeze (Cilloniz et al., 2016). As a result, pneumonia manifests as an acute inflammation of the respiratory bronchioles, alveolar spaces and interstitium which can lead to dry cough, fever, difficulty breathing and chest pain. Duration of pneumonia can vary from two to four weeks. As disease presentation varies from a mild illness that can be managed as an outpatient, to a severe illness requiring treatment in the intensive care unit (ICU). To determine the appropriate level of care is important for improving outcomes in addition to early diagnosis and appropriate and timely treatment (Regunath & Oba, 2022).

1.1.1 Epidemiology

Pneumonia has serious effect on children under age 5 years and elderly people over age 60 years (Turkington & Ashby, 2007). Globally, each year, pneumonia affects about 450 million people from which approximately 4 million cases can lead to death (Ruuskanen et al., 2011). Pneumonia killed more than 808 000 children under the age of 5 in 2017, accounting for 15% of all deaths of children under 5 years. Furthermore, in 2011, it was estimated that pneumonia caused the death of 1.3 million children, with 81% of that mortality rate occurring in children 0-2 years of age (Fischer Walker et al., 2013). People at-risk for pneumonia also include adults over the age of 65 and people with pre-existing health problems (World Health Organization, 2021). Moreover, in 2020 a Japanese study showed that pneumonia develops mainly in people aged 65 years and older in Japan, and treatment outcome is generally poor in elderly patients and the underlying conditions were seen to affect the 30-day mortality rate (Igari et al., 2020).

1.1.2 Bacterial pathogens

Streptococcus pyogenes

Viral infections of the upper respiratory tract are associated with a variety of invasive diseases caused by *S. pyogenes* or group A streptococcus (Lamoth et al., 2016). It is a gram-positive aerotolerant extracellular bacteria and it's made up of non-motile and non-sporing cocci that are 2 µm in length and forms chains or large colonies greater then 0.5 mm in size. *S. pyogenes* is the most common cause of a "strep" throat, which results in fever, enlarged tonsils, tonsillar exudate, sensitive cervical lymph nodes and malaise. It restricts to solely colonize humans. Outside of the human host, there is no known reservoir for this bacterium (Wilkening & Federle, 2017). Invasive infections caused by *S. pyogenes* include sepsis, bacteremic pneumonia, necrotizing fasciitis, and streptococcal toxic shock syndrome. If untreated, S. *pyogenes* can also lead to kidney inflammation or rheumatic fever during which, painful and inflamed joints can occur (Cunningham, 2008). Physicians commonly prescribe oral penicillin for 10 days. Patients who have penicillin allergy can be treated with macrolides and first-generation cephalosporins instead. This treatment is cost-effective and has a narrow spectrum of activity (Linder et al., 2005). Pneumonia caused by *S. pyogenes* is an uncommon cause, however it still shows a high mortality rate (Akuzawa & Kurabayashi, 2016).

Haemophilus influenzae

The second most common cause of bacterial pneumonia is *H. influenzae* (World Health Organization, 2021), which is another gram-negative coccobacillus that is facultatively anaerobic and capnophilic. This bacterium that forms chains of immobile coccoids (Morens et al., 2010), is one of the major human pathogens. Some strains of *H. influenzae* are encapsulated whilst others are non-encapsulated. This bacterium is only found in humans and forms colonises in the nasopharynx and in the throat. Transmission occurs through the spread of respiratory droplets or contact with respiratory secretions (Slack, 2015). There are six capsulated types of *H. influenzae* (a, b, c, d, e, f) and type b (Hib) is considered the most common one as it leads to severe invasive infections among children under 5 years old severe such as meningitidis or pneumonia (Bessen, 2009). Approximately around 3 million serious infections and up to 700,000 childhood deaths are cause because of Hib

(Nascimento-Carvalho & Andrade, 2006). The incidence of *H influenzae* type b infections has significantly declined since the introduction of a highly effective conjugate vaccine. The signs and symptoms of *H. influenzae* pneumonia are not very different from those of other pathogens causing pneumonias. This pathogen is considered an important cause of pneumonia in children, especially in developing countries. To treat infections caused by *H. influenzae*, antibiotics such as ampicillin are administered (Kärpänoja et al., 2004). Those antibiotics are being administered in the stable state of the patient, to clear infection from colonizing bacteria. However as mentioned above frequent antibiotic treatments can lead to the development of antibiotic resistance, which can occur by mutation (Maddi et al., 2017).

Staphylococcus aureus

One of a the most common pathogens in all pneumonia types is *S.areus* is frequently found in the upper respiratory tract and on the skin (Kollef et al., 2005). *S. aureus* is a gram-positive round shaped bacterium, which cocci gathers in clusters and are non-sporulating, immobile and mostly unencapsulated. Further, this pathogen is now increasingly recognized as an important cause of community-acquired pneumonia, displaying the capacity to infect a population of otherwise healthy adults and children (Ragle et al., 2010). An international study of the prevalence and outcomes of infection in ICU found that *S. aureus* was the most common cause of infection (Vincent et al., 2009). The only therapies available to treat pneumonia cause by this human pathogen are antibiotics such as oxacillin or nafcillin (David & Daum, 2017), also vancomycin and linezolid have similar efficacy. The selection of antibiotics should be based on patient tolerance, antibiotic allergy profile, renal function, drug interaction, and intravenous access (Wang et al., n.d.). *S. aureus* is also cause of cystic fibrosis (Dyon-Tafani et al., 2021) which is a condition where bacterial pathogens don't affect only lungs but also intestines, livers, and pancreas (Hodson et al., 2007). Newly, *S. aureus* was found in resistance to other groups of antibiotics. Therefore, it is important for different approaches of treatments of respiratory infections.

1.1.3 Inhalation Therapy

As mentioned above administrating antibiotics is an adequate treatment for these three bacteria. Both ampicillin and oxacillin are affecting gram-positive and gram-negative, aerobic, and anaerobic bacteria (Modnicki & Balcerek, 2009). However oral treatment can have its negatives. Before drugs are absorbed to the systematic circulation, it must pass through gastrointestinal tract and hepatic portal vein and then the liver which can reduce bioavailability and therapeutic efficacy, such as poor gastrointestinal absorption and first-pass metabolism in the liver (Steiropoulos et al., 2021). Inhaled therapy delivers drugs directly into the airways, which helps to pass high local drug concentration and limits systematic toxicity at the same time. Delivery of high concentrations of the active drug is also minimizing systematic side effects and its time, from drug administration to clinical response onset (Lavorini et al., 2015). Inhalation of essential oils (referred to as EOs) or their volatile components plays a significant role in controlling the central nervous system.

2 Essential oils

2.1 Distribution in Plants

Volatile aromatic compounds found in EOs are extracted from the bark, flowers, leaves, roots, seeds, stems, and other parts of plants (Konyak, 2018). EOs are localized in the cytoplasm of certain plant cell secretions, which lies in one or more organs of the plant (Dhifi et al., n.d., 2016). An estimated 3000 EOs are known, of which about 300 are commercially important, they are distilled chiefly for the flavours and fragrances market. Plants from *Lamiaceae* family are very important for EOs studies. For example, lavender EO is widely used in the aromatherapy industry and research has shown that they possess a range of biological properties that may make them useful in a medical environment. For example, research of *Lavandula angustifolia* has been targeted to the antimicrobial properties of the oil, in part as a response to increasing resistance of microorganisms to conventional pharmaceuticals (Moon et al., 2007). Another plant from *Lamiaceae* family, *Satureja hortensis* has a strong aromatic flavour, which could be compared to thyme (particularly, thyme harvested in summer). Essential oils, obtained by the distillation of plant foliage and even the foliage itself of certain aromatic plants have traditionally been utilized to protect stored grains and legumes against different pests (Maedeh et al., 2012). Furthermore,

2.2 Chemistry

EOs are defined as any volatile oils that have strong aromatic components that give characteristic odour, flavour, or smell to a plant. These are the by-products of plant metabolism and are frequently referred to as plant secondary metabolites (Memar et al., 2017). In nature, EOs play an important role in the protection of the plants as antibacterial agents, antivirals, antifungals, insecticides and against herbivores by reducing their appetite (Bakkali et al., 2008). EOs can be extracted from plants by steam distillation, hydro diffusion, or pressure although most common way to extract EO is by hydro distillation (Horváth & Ács, 2015). They are soluble in alcohol, ether, and fixed oils, but insoluble in water. EOs are generally composed of a mixture of terpenes, terpiniods, aldehydes and alcohols, many of which are as mentioned volatile and their antimicrobial effects are well known (Romeo et al., 2011). The gas

chromatographic methods are used almost exclusively for the analysis of plant volatiles. Gas chromatography (GC) with flame ionization detection is the traditional method for quantification of EOs and extracts containing volatile compounds while GC coupled with mass spectrometry (MS) is the most common analytical method for qualitative analysis (Stahl-Biskup & Sáez 2003).For extraction of the volatile fraction of aromatic compounds found in EOs can be used a headspace solid-phase microextraction also HS-SPME (Moghaddam & Mehdizadeh, 2017). It is a simple, rapid, and solventless technique that requires a small amount of sample and allows the analysis of highly volatile compounds (Antih et al., 2021).

2.3 Antimicrobial effect

Phenolic components present in the oils have been known to possess antimicrobial activity and some are classified as Generally Recognized as Safe (GRAS) substances and therefore could be used to prevent post-harvest growth of native and contaminant bacteria (Romeo et al., 2011). Recently new methods of treating pathogens of respiratory diseases were tested as strains of bacteria are becoming antibiotic resistant (Jones, 2003). Natural treatments have been proven to be effective, such as EOs derived from herbal plants. Many studies confirm that vapour phases of EOs are more effective than their liquid phases in antimicrobial matter (Laird & Phillips, 2012). Furthermore, new findings show that interaction of EOs and other compounds can work together and create synergistic antimicrobial effect (Netopilova et al., 2021). As mentioned above certain bacterial pathogens are antibiotic resistant and volatile compounds found in EOs are applicable for inhalation therapy as a treatment (Houdkova et al., 2018). Other studies showed that EOs had antimicrobial properties on bacteria and yeast. An important feature of those oils is their hydrophobicity, which allows them to partition into lipids of the cell membrane of bacteria by disrupting the structure and making it more permeable (Dhifi et al., 2016). However, a combination of compounds can lead to synergism, which occurs when the combined effect of sub-stances is higher than the sum of the individual effects (Shaaban et al., 2012).

3 Thymus vulgaris

3.1 Botany

T. vulgaris from the family *Lamiaceae* that belongs to tribe *Mentheae* within the subfamily *Nepetoideae*., it is perennial subshrub with a height of 40 cm and is commonly known as thyme, however the word thyme is a general name for more than three hundred thymus species from different hybrids, varieties, and ecotypes (Figueiredo et al., 2008). Stem and branches are stiff and woody, obscurely quadrangular, faintly furrowed in the upper part. It has very short internodes. Its branchlets are densely coated with grey hairs. Leaves are opposite, sometimes seemingly whorled by the presence of axillary leaves (de Guzman and Siemonsma, 1999). Inflorescence spiciform and interrupted. Flowers (pedicellate or not) usually with little bracteoles. It has a two-lipped calyx which sometimes is nearly regular and cylindrical. The upper lip has three triangular teeth which is sometimes reduced to one and the lower lip has two long triangular teeth curved upwards or widespread with four stamens, sometimes reduced or not present at all (gynodioecy). Anthers have two parallel thecae with branched apex and ovoid, smooth nutlets (Stahl-Biskup & Sáez 2003).

3.2 Uses

Along with being a culinary additive and medicinal remedy it also became widely used in cosmetics products such as soaps, oral hygiene products or perfumes (Adeyemi Sherif Babatunde, 2017). *T. vulgaris* also has antispasmodic properties, which make it an effective remedy for the sore throats, irritable coughs, and bronchitis (Figueiredo et al., 2008). It also can be used to treat diabetes and digestive upset (Ziyat et al., 2021). However, most of the studies have reported the antimicrobial activity of *T. vulgaris* extracts (ethanol and water) as well as its use in the form of essential oil against foodborne pathogens (Patil et al., 2021).

3.3 Chemical composition

Chemically, EOs are a complex mixture of constituents that belong to two groups: terpenoids and aromatic compounds (Dhifi et al., 2016), They must be volatile since they are removed by distillation, usually hydrophobic and generally from colourless

to deep yellow or orange colour. Each plant produces its own mixture of 20-60 chemical constituents at with two or three major compounds representing 20–70 % of all content that usually define the biological activity of EOs (Bakkali 2008). Volatile compounds that can be generally found in thyme EO are thymol, carvacrol, linalool, geraniol, α-terpineol and thuyanol-4 which of thymol for example can also be applied as an alternative antimicrobial agent against antibiotic-resistant pathogenic bacteria (Memar et al., 2017). Thymol is a phenolic compound present EOs and it's a natural monoterpene (Memar et al., 2017) and a compound that is able to inhibit both gram-positive and gram-negative bacteria, including the potential pathogenic strains of *S. aureus.* (Dorman & Deans, 2000). (György et al., 2020). Interestingly, first person to extract thymol for the first time was Caspar Neumann in 1719 (Salehi et al., 2018). In addition, another monoterpene found in thyme EO is α-pinene. With its volatile and hydrophobic properties, it has a fresh pine scent and woody flavour. α-Pinene was tested against several gram-negative and gram-positive bacterial strains and activity against methicillin-resistant *S. aureus* was observed (Utegenova et al., 2018). The percentage of those chemotypes within plants can be affected by the environment that they grow in. Studies have shown that the highest amount of thymol can be found inside of plants that grow at elevations not higher than 400 m close to the Mediterranean Sea, in shallow grounds with stony soil in areas where temperatures in winter are not so cold. On the other hand, the highest amount of chemotypes such as α-terpineol, geraniol and linalool are found in plants that grow at elevations above 400 m, in deeper humid soils where temperatures can get below -10 °C. Composition of EOs derived from *T. vulgaris* can depend on several factors which can affect its biological activity. The quality (and quantity) of EO obtained from *T. vulgaris* plant can be affected by geographical environment, harvesting season and other agronomical factors such as fertilizers, pesticides, or insecticides (Alizadeh et al., 2011). Antimicrobial activity can be classified according to the microorganisms against which it acts primarily (Soylu et al., 2006). These factors include environmental conditions, type of soil mixes, chemotype of plant species, isolation processes, etc (Horváth & Ács, 2015).

3.4 Biological activities

Some monoterpenes that a commonly found in EOs of the genus of Thymus, are monocylic *p*-cymene. It is used in the synthesis of fungicides, pesticides, perfumes,

fragrances mainly because of its aromatic character. *p*-cymene has shown a variety of pharmacological properties, including antimicrobial, antioxidant anti-inflammatory, antiparasitic and antiviral activities (Balahbib et al., 2021). Thymol is a phenolic compound present EOs and it's a natural monoterpene (Memar et al., 2017) and a compound that is able to inhibit both gram-positive and gram-negative bacteria, including the potential pathogenic strains of *S. aureus*. (György et al., 2020). For very long time T. vulgaris has been studied for its antiseptic, antibiotic and antifungal properties which are related to thymol (Ziyat et al., 2021). Studies have shown that thymol is used to treat cough and respiratory infections, because it tends to reduce inflammation and infection (Boukhatem et al., 2020). Cervitec Plus (Ivoclar Vivadent, Schaan, Liechtenstein) and Listerine can be given as an example of commercially used products containing thymol. Those products are used for oral infections and dental cariesoral infections and dental caries. Clinical studies are also investigating the effect of plant-derived products in the treatment of respiratory and pulmonary infections are for example Bronchipret Saft, which is used traditional herbal medicinal product used in productive cough. It contains combination of *Hedera helix* and *T. vulgaris* extracts and Bronchipret TP (Kokoska et al. 2019). Moreover, another study showed that combinatory activity of thymol and carvacrol produced the additive antimicrobial effect against S. aureus. Joined activity of thymol and carvacrol showed additive antimicrobial effect that have been tested against twelve strains of *S. aureus*. However, there are no standardized methods for evaluation of interactions between volatile compounds in vapour phase (Netopilova et al. 2018)

4 Aims of the Thesis

The main aim of the study is to analyse chemical composition and antibacterial activity of *T. vulgaris* EO vapours against pathogens causing pneumonia

Specific objectives are:

- Determination of *in vitro* growth-inhibitory effect of *T. vulgaris* EO against *H. influenzae*, *S. aureus* and *S. pyogenes* in vapour and liquid phase using broth microdilution volatilization method
- Analysis of chemical composition of *T. vulgaris* EO and its vapours using GC-MS and HS-SPME sampling technique.

5 Materials and methods

5.1 Plant Material

The plant materials were obtained from dried aerial parts of *T. vulgaris* from a supplier C (Lbros s.r.o., Vrchlabí, Czech Republic). Plant materials were ground and homogenised using a Grindomix apparatus (GM 100 Retsch, Haan, Germany). We evaluated the residual moisture content at 130◦C for 1 h by Scaltec SMO 01 Analyzer (ScaltecInstruments, Gottingen, Germany) in triplicates and results were expressed as arithmetic averages (13.13%).

5.2 Distillation of EOs

For obtaining EOs, hydrodistillation was used as it is one of the common methods for not only commercial production. 100 g of ground plant material placed in 1 l of distilled water was distilled for 3 h using Clevenger-type apparatus (Merci, Brno, Czech Republic). The extracted EOs were stored in sealed glass vials at 4◦C until further handling.

5.3 Bacterial Strains and Culture Media

The following standard strains of the American Type Culture Collection (ATCC) were used: *H. influenzae* ATCC 49247, *S. aureus* ATCC 29213, and *S. pyogenes* ATCC 19615. The cultivation and assay media (broth/ agar) were Mueller-Hinton broth (MHB) complemented by Haemophilus Test Medium and defibrinated horse blood for *H. influenzae*, MH only for *S. aureus*, and Brain Heart Infusion when working with *S. pyogenes*. The pH of broths is equilibrated to final value of 7.6 using Trizma® base (Sigma-Aldrich, Prague, Czech Republic). All microbial strains and cultivation media were purchased from Oxoid (Basingstoke, UK). Stock cultures of bacterial strains were being cultivated in appropriate medium at 37 °C for 24 h prior the testing, and then the turbidity of the bacterial suspension was adjusted to 0.5 McFarland standard using Densi- La-Meter II (Lachema, Brno, Czech Republic). The susceptibilities of *H. influenzae*, *S. aureus* and *S. pyogenes* to amoxicillin, ampicillin and oxacillin were

checked as positive antibiotic controls. Ampicillin, oxacillin, and amoxicillin were purchased from Sigma-Aldrich (Prague, Czech Republic).

5.4 Antimicrobial Assay

Antibacterial potential of plant volatile compounds in liquid and vapour phase was determined using broth microdilution volatilization method. The experiments were performed in standard Nunclon 96-well microtiter plates (well volume = 400 µl), covered by tight-fitting lids with flanges designed to reduce evaporation (SPL Life Sciences, Naechon-Myeon, Korea). Initially, 30 µl of agar was pipetted into every flange on the lid except the outermost flanges and inoculated with 5 µl of bacterial suspension after agar solidification. In the second part of this method, each sample of volatile compounds were dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Prague, Czech Republic) at maximum concentration 1% and diluted in appropriate broth medium. Seven two-fold serially diluted concentrations of samples starting from 1024 µg/ml are prepared for all EOs. The final volume in each well was 100 µl. The plates were then inoculated with bacterial suspensions using a 96-pin multi-blot replicator (National Institute of Public Health, Prague, Czech Republic) then the wells containing inoculated and non-inoculated broth was prepared as growth and purity controls simultaneously. The outer most wells were left empty to prevent edge effect. Finally, the clamps (Lux Tool, Prague, Czech Republic) were used for fastening plate and lid together, with the handmade wooden pads (size 8.5 × 13 × 2 mm) for better fixing and the microtiter plates were incubated at 37 °C for 24 h. The minimum inhibitory concentrations (MICs) were evaluated by visual assessment of bacterial growth after colouring of metabolically active bacterial colony with thiazolyl blue tetrazolium bromide dye (MTT) at a concentration of 600 µg/ml (Sigma-Aldrich, Prague, Czech Republic), the interface of colour changed from yellow and purple (relative to that of colours in control wells) were recorded in broth and agar.

Figure 1 Schematic design of the experiment: flat-bottom wells

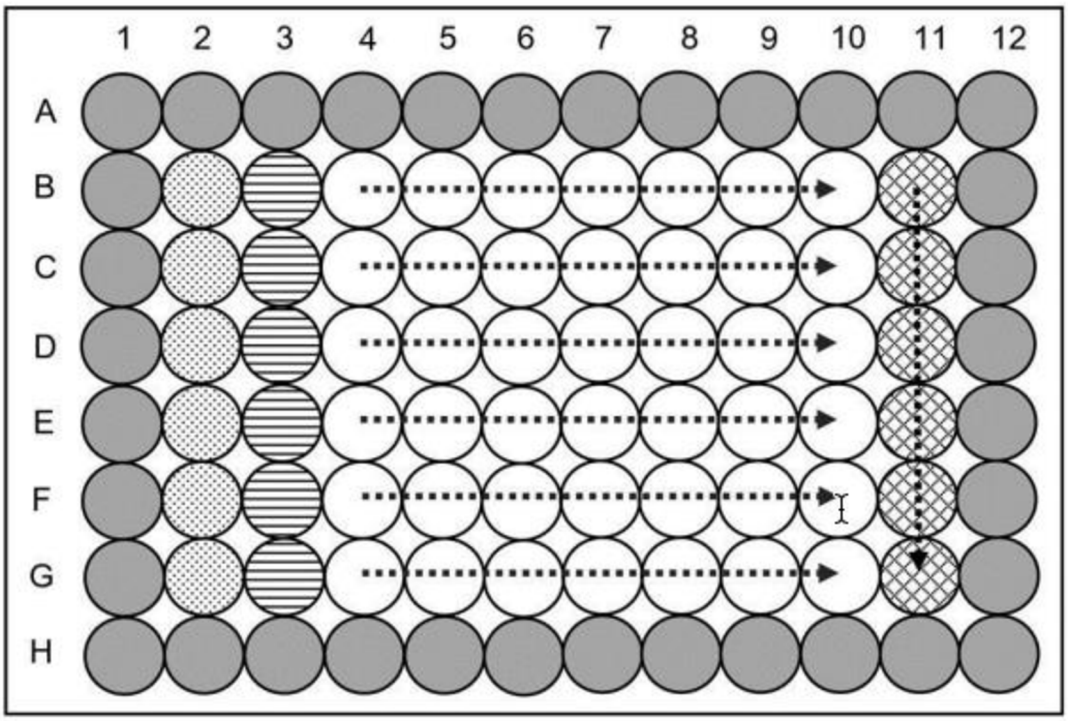
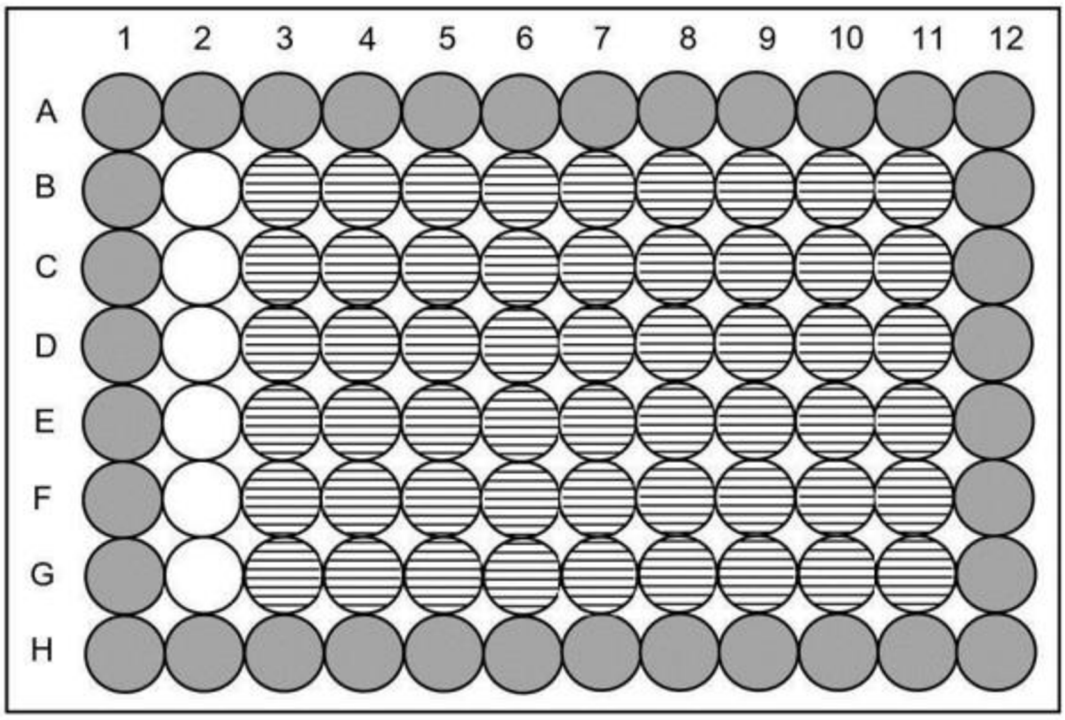


Figure 2 Schematic design of experiment: flanged lids



5.5 Chemical Analysis of EOs

To evaluate the chemical composition of EOs, GC/ MS analysis was performed using gas chromatograph Agilent GC-7890B system (Agilent Technologies, Santa Clara, CA, USA) equipped with autosampler Agilent 7693, a fused-silica HP-5MS column (30 m × 0.25 mm, film thickness 0.25 μm, Agilent 19091s-433 and a flame-ionisation detector (FID) coupled with single quadrupole mass selective detector AgilentMSD-5977B. The operational parameters were the following: helium as carrier gas at 1 ml/min, injector temperature 250°C for both columns. The oven temperature was raised for both columns after 3 min from 50 to 280°C. Initially, the heating velocity was 3°C/min until the system reached a temperature of 120°C. The velocity was raised afterwards to 5°C/min until a temperature of 250°C, and after 5 min holding time, the heating speed reached 15°C/ min until achieving a temperature of 280°C. Heating was followed for 20 min. The EO samples were diluted in n-hexane for GC/ MS (Merck KGaA, Darmstadt, Germany) at the concentration 20 μl/ ml. One microliter of the solution was injected in split mode in a split ratio 1:30. For the mass detector, following conditions were set: ionisation energy 70 eV, ion source temperature 230°C, scan time 1 s, mass range 40–600 m/z. Constituents were identified based on comparison of their retention indices (RIs), retention times (RT),

and spectra. National Institute of Standards and Technology Library ver. 2.0.f and the available literature (Kloucek et al., 2012) were used for this purpose. HP-5MS column separated the RIs were calculated for compounds. The final number of compounds was calculated as the sum of components that were identified using both columns and the remaining constituents identified by individual columns only. Quantitative data are shown as a percentage content of constituents determined by the FID.

5.6 Chemical Analysis of EO's Vapour Phase

To analyze the chemical composition of the headspace above, a mixture of MH broth and *T. vulgaris* EO at a concentration of 512 µg/ ml (i.e., the lowest MIC value obtained from the BMV assay). HS-SPME sampling technique was performed when a set of five samples was prepared for each experiment. a volume of 2 ml of the mixture (MHB and *T. vulgaris* EO) was introduced into a 4 ml glass vial. EO samples, except for the first one (t = 0 h) were placed into an oven set at a temperature of 37◦C for incubation until analysis, which was at 3, 6, 9, and 12 h. To analyse chemical compounds, a SPME fibre assembly coated with 50/30 µm layer of divinylbenzene/ carboxen /polydimethylsiloxane (DVB/CAR/PDMS—SUPELCO, Bellefonte, PA, USA) was used. As mentioned above, the sample solutions were prepared into a 4 ml glass vial and incubated at a temperature of 37 °C until its analysis. The needle of the HS-SPME holder was inserted into the vial when equilibrium was reached in between the headspace and the mixture. For adsorption of the volatile compounds, the coated fibre was exposed to the headspace for 15 minutes. Afterwards the needle was removed and inserted into the GC injector port, and set in splitless mode, where the desorption of analytes happened. The temperature was set at 250◦C, and the fibre is left into the injector for the entire analysis and removed only before the next measurement. EO samples is being measured repeatedly every three hours during a 12-hour incubation.

6 Results

Antimicrobial Assay

As a result of antibacterial susceptibility testing, *T.vulgaris* L. EO produced *in vitro* growth-inhibitory effect against all bacterial strains tested (*H. influenzae, S. aureus* and *S.pyogenes*) with MIC value 512 µg/ml in both liquid and vapour phases. Detailed results are shown in the Table 1.

Chemical Analysis of EOs

T. vulgaris EO was extracted with respective yield value of 1.25%. In Table 2, 3 and 4, the complete chemical analyses are provided. 62 components were identified in EO using the HP-5MS column, representing 99.62% of their total constituents. Two dominant groups of volatile agents were identified. Monoterpenoids represented by thymol (48.09%) which is phenolic monoterpene and that sesquiterpenoids were represented by *β*-caryophyllene (2.33%). After thymol, *p*-cymene was the second most abundant constituent together carvacrol (12.75,10.92% respectively).

Chemical Analysis of EOs Vapour Phase

Using the HP-MS column, headspace chemical compositions was measured every 3 h during a 12h experiment. Tables 5, 6 and 7 provides complete analysis. A total of 40, 38, and 43 volatile compounds were identified in the samples of suppliers A, B, and C, respectively using HS-SPME extraction and represented 99.63% of their respective total contents at time 12 hrs. Monoterpenoids and sesquiterpene hydrocarbons were the most frequent volatile compounds. First most common chemical compound, using HS-GTS extraction, found is *p*-cymene (44.80 to 49.28%). Second abundant compound is γ-terpinene (9.54 to 11.85%). Moreover, third and most important monoterpenoid; thymol was found in the sample during the entire 12h period with values varying from 3.72 to 5.27%. Lastly the headspace analysis of the sampling method showed no significant changes in the chemical composition in the vapour phase of the EO sample over time.

Table 1 In vitro growth inhibitory effect of Thymus vulgaris EO

Essential oils	**Bacteria/Growth medium/Minimum inhibitory concentration (µg/ml)**					
	Staphylococcus aureus		*Streptococcus pyogenes*		*Haemophilus Influenzae*	
	Broth	Agar	Broth	Agar	Broth	Agar
Thymus vulgaris L.	512	512	512	512	512	512
Positive antibiotic control						
Oxacilin	0.25	>2	NT	NT	NT	NT
Amoxicilin	NT	NT	0.06	>2	NT	NT
Ampicilin	NT	NT	NT	NT	0.5	>16

NT: Not tested

Table 2 Chemical composition of *T.vulgaris*

Ri[a]		Compound			HP-5MS	**Identification e**
Obs.	Lit		C[b]	RF[c]	[%]	HP-5MS
761	783[d]	Methyl *α*-methylbutanoate	E	1.197	0,05 ± 0,01	GC-MS
923	924	*α*-Thujene	MH	0.765	0,26 ± 0,02	RI, GC-MS
929	939	*α*-Pinene	MH	0.765	0,43 ± 0,03	RI, GC-MS
944	945	Camphene	MH	0.765	0,19 ± 0,01	RI, GC-MS
972	969	Sabinene	MH	0.765	0,10 ± 0,01	RI, GC-MS
977	979	1-octen-3-ol	MO	0.893	0,35 ± 0,02	RI, GC-MS
989	988	*β*-Myrcene	MH	0.765	0,58 ± 0,04	RI, GC-MS
994	996	3-Octanol	MO	0.871	tr.	RI, GC-MS
1002	1002	*α*-Phellandrene	MH	0.765	0,10 ± 0,01	RI, GC-MS
1007	1008	3-Carene	MH	0.765	tr.	RI, GC-MS
1014	1014	*α*-Terpinene	MH	0.765	1,07 ± 0,07	RI, GC-MS
1025	1020	*p*-Cymene	MH	0.698	12,75 ± 0,84	RI, GC-MS
1027	1031	D-Limonene	MH	0.765	0,41 ± 0,03	RI, GC-MS
1028	1023	*m*-Cymene	MH	0.698	-	RI, GC-MS
1030	1032	1,8-Cineole	MO	0.869	0,39 ± 0,03	RI, GC-MS
1058	1054	γ-Terpinene	MH	0.765	5,22 ± 0,33	RI, GC-MS
1065	1068	cis-Sabinene hydrate	E	0.869	0,37 ± 0,03	RI, GC-MS
1071	1078	cis-Linalool oxide	MO	0.996	-	RI, GC-MS
1087	1086	Terpinolene	MH	0.765	0,11 ± 0,01	RI, GC-MS
1096	1102	trans-Sabinene hydrate	E	0.869	tr.	RI, GC-MS
1100	1095	Linalool	MO	0.869	1,53 ± 0,07	RI, GC-MS

Table 3 Cont.

Ri[a]		Compound			HP-5MS	**Identification e**
Obs.	Lit		C[b]	RF[c]	[%]	HP-5MS
1149	1141	Camphor	MO	0.887	0,06 ± 0,01	RI, GC-MS
1165	1165	Endo-borneol	MO	0.869	0,63 ± 0,03	RI, GC-MS
1176	1174	Terpinen-4-ol	MO	0.869	0,62 ± 0,04	RI, GC-MS
1186	1180	*m*-Cymen-8-ol	MO	0.808	-	RI, GC-MS
1190	1183	*p*-Cymen-8-ol	MO	0.808	-	RI, GC-MS
1196	1189	*α*-Terpineol	MO	0.869	0,16 ± 0,02	RI, GC-MS
1204	1195	cis-Dihydrocarvone	MO	0.887	tr.	RI, GC-MS
1235	1235	Thymol methyl ether	MO	0.798	0,76 ± 0,05	RI, GC-MS
1244	1241	Carvacrol methyl ether	MO	0.798	0,66 ± 0,04	RI, GC-MS
1256	1242	Carvone	MO	0.907	1,01 ± 0,05	RI, GC-MS
1269	1255	Geraniol	MO	0.869	0,05 ± 0,01	RI, GC-MS
1286	1282	Anethole	MO	0.824	0,11 ± 0,06	RI, GC-MS
1304	1290	Thymol	MO	0.808	48,09 ± 2,53	RI, GC-MS
1310	1298	Carvacrol	MO	0.808	10,92 ± 3,50	RI, GC-MS
1361	1357	Eugenol	SO	0.849	tr.	RI, GC-MS
1379	1374	*α*-Copaene	SH	0.751	0,09 ± 0,01	RI, GC-MS
1388	1387	*β*-Bourbonene	SH	0.794	tr.	RI, GC-MS
1424	1418	*β*-Caryophyllene	SH	0.751	2,33 ± 0,13	RI, GC-MS
1433	1446[d]	Isogermacrene D	SH	0.751	tr.	GC-MS
1458	1452	*α*-Humulene	SH	0.751	0,11 ± 0,01	RI, GC-MS
1475	1475	Geranyl propionate	E	0.935	0,10 ± 0,01	RI, GC-MS
1480	1478	γ-Muurolene	SH	0.751	0,21 ± 0,02	RI, GC-MS

Table 4 Cont.

Ri[a]		Compound			HP-5MS	**Identification e**
Obs.	Lit		C[b]	RF[c]	[%]	HP-5MS
1483	1493	α-Amorphene	SH	0.751	tr.	RI, GC-MS
1498	1495	Valencene	SH	0.751	0,11 ± 0,01	RI, GC-MS
1503	1499	α-Muurolene	SH	0.751	0,09 ± 0,01	RI, GC-MS
1510	1509	β-Bisabolene	SH	0.726	0,06 ± 0,01	RI, GC-MS
1518	1513	γ-Cadinene	SH	0.751	0,33 ± 0,02	RI, GC-MS
1527	1524	δ-Cadinene	SH	0.751	0,50 ± 0,03	RI, GC-MS
1561	1564	Nerolidol	SO	0.819	tr.	RI, GC-MS
1585	1578	Spathulenol	SO	0.830	tr.	RI, GC-MS
1591	1581	Caryophyllene oxide	SH	0.830	0,68 ± 0,03	RI, GC-MS
1617	1606	Humulene epoxide II	SO	0.830	tr.	RI, GC-MS
1621	1627	Epicubenol	SO	0.819	tr.	RI, GC-MS
1628	1630	γ-Eudesmol	SO	0.809	0,13 ± 0,01	RI, GC-MS
1634	1642	Cubenol	SO	0.819	tr.	RI, GC-MS
1648	1640	α-epi-Cadinol	SO	0.819	0,36 ± 0,03	RI, GC-MS
1652	1645	δ-Cadinol	SO	0.819	tr.	RI, GC-MS
1661	1653	α-Cadinol	SO	0.819	0,07 ± 0,02	RI, GC-MS
1682	1677	Cadalene	SH	0.673	tr.	RI, GC-MS
1844	1844	Perhydrofarnesyl acetone	K	0.782	tr.	RI, GC-MS
		Total identified (%)			**99.62%**	

RI = retention indices; Obs = retention indices determined relative to a homologous series of n-alkanes (C8-C40) on a HP-5MS column, Lit = literature RI values (NIST 2017 or Adams, 2007)
RI = retention indices; Obs = retention indices determined relative to a homologous series of n-alkanes (C8-C40) on a HP-5MS column, Lit = literature RI values (Adams, 2007)
d (Satyal et. al., 2016), NA=RI values not available in the literature
C = Class; E-Esters, K - Ketones, MH - Monoterpene hydrocarbons, MO - Oxygenated monoterpenes, SH - Sesquiterpene hydrocarbons, SO - Oxygenated sesquiterpenes.
RF = Response factor
Identification method: GC-MS = Mass spectrum was identical to that of National Institute of Standards and Technology Library (ver. 2.0.f), RI = the retention index was matching literature database
tr. = traces, relative peak area < 0.05%.
- = not detected

Table 5 Chemical composition (%) of a headpace above *T.vulgaris.* EO at a concentration of 512 µg/mL using Solid-Phase microExtraction (HS-SPME-GC-MS)

RI[a]		Compounds	C[b]	Time (h)/Content (%)				
Obs	Lit			0	3	6	9	12
921	921	Tricyclene	MH	0,08 ± 0,02	0,08 ± 0,01	0,07 ± 0,02	0,10 ± 0,01	0,08 ± 0,02
927	924	α-Thujene	MH	1,01 ± 0,05	1,00 ± 0,03	0,93 ± 0,03	0,94 ± 0,13	0,94 ± 0,05
933	939	α-Pinene	MH	2,73 ± 0,18	2,69 ± 0,13	2,57 ± 0,26	2,82 ± 0,30	2,70 ± 0,26
949	945	Camphene	MH	1,58 ± 0,56	1,56 ± 0,53	1,52 ± 0,58	2,13 ± 0,16	1,82 ± 0,57
976	969	Sabinene	MH	tr.	tr.	tr.	0,07 ± 0,00	0,06 ± 0,02
978	974	β-Pinene	MH	0,45 ± 0,01	0,49 ± 0,06	0,48 ± 0,07	0,56 ± 0,04	0,51 ± 0,06
991	988	3-Octanone	K	-	-	-	-	-
995	988	β-Myrcene	MH	1,62 ± 0,32	1,53 ± 0,18	1,51 ± 0,29	1,83 ± 0,03	1,62 ± 0,26
1009	1002	α-Phellandrene	MH	0,26 ± 0,03	0,26 ± 0,02	0,25 ± 0,03	0,28 ± 0,01	0,24 ± 0,03
1013	1008	3-Carene	MH	0,11 ± 0,04	0,10 ± 0,03	0,10 ± 0,03	0,14 ± 0,01	0,12 ± 0,03
1021	1014	α-Terpinene	MH	2,20 ± 0,66	2,17 ± 0,64	2,14 ± 0,65	2,92 ± 0,05	2,46 ± 0,64
1036	1020	p-Cymene	MH	67,21 ± 7,03	66,38 ± 7,46	65,89 ± 6,38	58,38 ± 0,23	60,62 ± 6,81
1055	1023	m-Cymene	MH	-	-	-	tr.	tr.
1067	1054	γ-Terpinene	MH	12,37 ± 2,86	12,51 ± 2,64	12,67 ± 3,02	16,18 ± 0,16	14,14 ± 2,88
1094	1086	Terpinolene	MH	0,16 ± 0,10	0,16 ± 0,10	0,17 ± 0,10	0,27 ± 0,01	0,21 ± 0,09
1101	1089	p-Cymenene	MH	0,12 ± 0,06	0,10 ± 0,04	0,09 ± 0,02	0,14 ± 0,01	0,11 ± 0,03
1115	1095	Linalool	MO	0,23 ± 0,21	0,16 ± 0,12	0,16 ± 0,13	0,29 ± 0,03	0,19 ± 0,10
1153	1141	Camphor	MO	tr.	tr.	0,06 ± 0,04	0,09 ± 0,01	0,08 ± 0,04
1186	1165	Endo-borneol	MO	0,11 ± 0,02	0,09 ± 0,01	0,08 ± 0,01	0,07 ± 0,01	0,08 ± 0,01
1192	1174	Terpinen-4-ol	MO	tr.	tr.	0,06 ± 0,05	0,10 ± 0,01	0,09 ± 0,04

Table 6 Cont.

Obs	Lit	Compounds	C[b]	Time (h)/Content (%)				
				0	3	6	9	12
1213	1195	Estragole	MO	0,10 ± 0,06	0,13 ± 0,09	0,11 ± 0,04	0,17 ± 0,03	0,18 ± 0,06
1247	1235	Thymol methyl ether	MO	1,83 ± 0,13	2,41 ± 0,17	2,77 ± 0,41	2,61 ± 0,17	2,91 ± 0,34
1257	1241	Carvacrol methyl ether	MO	1,08 ± 0,12	1,37 ± 0,11	1,50 ± 0,14	1,54 ± 0,03	1,69 ± 0,11
1293	1287	Bornyl acetate	E	tr.	0,15 ± 0,08	0,15 ± 0,06	0,25 ± 0,04	0,24 ± 0,11
1306	1284	Anethol	MO	tr.	tr.	0,08 ± 0,00	0,12 ± 0,02	0,12 ± 0,03
1339	1290	Thymol	MO	5,27 ± 1,27	4,57 ± 0,81	4,62 ± 0,84	3,72 ± 0,07	5,05 ± 0,45
1368	1298	Carvacrol	MO	-	tr.	tr.	tr.	tr.
1381	1372	p-Cymen-7-ol	SH	-	-	-	tr.	tr.
1386	1374	α-Copaene	SH	tr.	tr.	0,07 ± 0,08	0,16 ± 0,01	0,19 ± 0,05
1395	1387	β-Bourbonene	SH	tr.	tr.	tr.	0,09 ± 0,01	0,10 ± 0,01
1434	1418	β-Caryophyllene	SH	0,77 ± 0,82	1,19 ± 1,26	1,28 ± 1,29	2,94 ± 0,32	2,40 ± 1,59
1442	1446[d]	isogermacrene D	SH	-	tr.	tr.	0,08 ± 0,06	tr.
1469	1452	α-Humulene	SH	tr.	tr.	tr.	0,07 ± 0,01	0,06 ± 0,03
1475	1465	cis-muurola-4(14),5-diene	SH	-	-	-	tr.	tr.
1482	1475	Geranyl propionate	E	-	-	-	-	tr.
1491	1478	γ-Muurolene	SH	tr. ± tr.	0,07 ± 0,04	0,08 ± 0,05	0,12 ± 0,01	0,11 ± 0,05
1509	1480	Germacrene D	SH	-	-	-	tr.	tr.
1512	1491	Valencene	SH	-	-	-	tr.	tr.

Table 7 Cont.

Obs	Lit	Compounds	C[b]	Time (h)/Content (%)				
				0	3	6	9	12
1515	1499	α-Muurolene	SH	-	-	-	tr.	tr.
1520	1509	β-Bisabolene	SH	-	-	-	tr.	tr.
1538	1524	δ-Cadinene	SH	tr.	0,07 ± 0,04	0,08 ± 0,04	0,12 ± 0,02	0,11 ± 0,05
1541	1521	Calamenene	SH	tr.	0,07 ± 0,01	0,06 ± 0,01	0,07 ± 0,01	0,07 ± 0,01
1605	1581	Caryophyllene oxide	SH	-	-	tr.	tr.	tr.
		Total identified (%)		**99.33**	**99.90**	**99.87**	**99.74**	**99.63**

RI = retention indices; Obs = retention indices determined relative to a homologous series of n-alkanes (C8-C40) on a HP-5MS column, Lit = literature RI values (from NIST 2017 or Adams, 2007)
d (Satyal et. al., 2016).
C = Class; E-Esters, K - Ketones, MH - Monoterpene hydrocarbons, MO - Oxygenated monoterpenes, SH - Sesquiterpene hydrocarbons.
Relative peak area percentage as mean of three measurements ± deviation standard
tr. = traces, relative peak area < 0.05%.
- = not detected

7 Discussion

The chemical analysis of the EO samples from supplier C turned out to be the most active EO based on the results of the antimicrobial assay with MICs at 512µg/ml in both broth and agar. Other studies showed that thymol in EOs was active in range from 355 µg/ ml to 1707 µg/ ml against bacterial pathogen *S. aureus*, the initial concentration used for thymol was 2048 µg/ ml (Netopilova et al., 2018). According to these results, the *T. vulgaris* EO significantly reduced the growth of a fungi *A. flavus*. At the highest concentration of 20 µg/ml, the EO vapour inhibited fungal growth (Tian et al., 2019). Antimicrobial action of EOs by gaseous contact is most efficient when exposed at high vapour concentration for a short time. The results of another suggested that a maximal vapour level of 0.1–0.9 mg/L in air may suppress the growth of the bacterial pathogens of respiratory infection (Inouye et al., 2001).

Analysis of volatile compounds of *T. vulgaris* EO vapour was carried out using sampling method: HS-SPME. HS-SPME became the more commonly used method for identifying EOs volatile compounds and it is a method that is being often used in other studies as well. In our study the amount thymol (supplier C samples) during the entire 12h period with values varying from 3.72% to 5.27% which was unusually low in comparison with results of a study that recorded percentage of thymol (using HS-SPME method) high as 34.28% (Chester et al., 2017).

The lack of standardized chemical composition of EO treatment is problematic, even more in vapour phase, which can be difficult to measure its system is depending on conditions and applied environment. Although even that in our study thymol percentage was low it still manifested antimicrobial activity against bacterial pathogens. Results suggest that EO vapours could be used as a method of treating respiratory infections by inhalation. Also, agriculture of EO is more suitable alternative and more natural, also its antimicrobial compounds do not leave toxic residue in products. (El-Mougy, 2009). In this regard, respiratory effects of inhaled *T. vulgaris* were examined in a clinical trial to find an impact of thymol. Urine and plasma samples of the participants after oral administration of *T.vulgaris* pills and did not find free thymol in plasma and urine, only some of the thymol metabolites were found in the urine. However, it was found in respiratory system after administrations inhalation treatment (Ghahremani-Chabok et al., 2021).

8 Conclusion

EO from *T. vulgaris* exhibited an *in vitro* growth-inhibitory effect against three most common pulmonary bacterial pathogens. The results suggest that that *T. vulgaris* is perspective plant material for development of pharmaceutical applications for treatment of acute lower respiratory infections and pneumonia caused by bacterial pathogens such as *S. pneumoniae*, *S. pyogenes,* and *S. aureus* or *H. influenzae* thanks to volatile compound thymol. Not many studies have been done on this subject of vapour phase. Nonetheless, further research on their compositions and determination of active compounds in vapour phase will be needed for its possible further pharmacological application. Aim of future studies should be focused on how to increase chemical composition of thymol and how to incorporate this substance into protentional commercial pharmaceuticals. Potentially, EO vapours could be modified to provide specific dosages of airborne volatiles at specified intervals in hospitals to help decrease symptoms of pneumonia.

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