**CZECH UNIVERSITY OF LIFE SCIENCES PRAGUE**

**Faculty of Tropical AgriSciences**


# Evaluation of antibacterial activity of Indian essential oils by new broth macrodilution volatilization method

MASTER'S THESIS

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# Declaration

I hereby declare that I have done this thesis entitled Evaluation of antibacterial activity of Indian essential oils by new broth macrodilution volatilization method independently, all texts in this thesis are original, and all the sources have been quoted and acknowledged by means of complete references and according to Citation rules of the FTA.

In Prague 20.04.2022

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## Abstract

Since the resistance to antibiotics still increases, there is a need to discover new anti-infective agents. Plants provide rich source of secondary metabolites including essential oils possessing antimicrobial activity.

In this study, a new broth macrodilution volatilization method for the simple and rapid determination of the antibacterial effect of volatile agents simultaneously in the liquid and vapor phase was used with the aim to assess their therapeutic potential for the development of new pharmaceutical preparations that are based on volatile antimicrobials. The antibacterial activity of essential oils was evaluated against bacteria associated with respiratory infections, namely *Haemophilus influenzae*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, and *Streptococcus pyogenes*. The highest antibacterial activity produced *T. ammi* against *H. influenzae*, with MIC 128 μg/mL in liquid and vapor phase. Moderate effect was produced by *C. citratus* EO which inhibited growth of *H. influenzae* with MIC 256 μg/mL in liquid and vapour phase. *C. scariosus* and *T. ammi* showed only weak inhibitory effect (MIC = 512 μg/mL) in both phases against *H. influenzae* and *S. aureus*, respectively. Similarly, *C. citratus* and *T. ammi* produced weak activity against *S. pneumoniae* and *S. pyogenes* in liquid phase (MIC 512 μg/mL). All other bacteria were resistant to EOs tested (MIC ≥ 1024 μg/mL).

In addition, broth macrodilution volatilization method was evaluated as a suitable method for evaluation of EOs antimicrobial effect, simultaneously in the liquid and the vapor phases at variable concentrations

Since *T. ammi* possessed strongest antimicrobial effect, its chemical composition was subsequently analysed using gas chromatography-mass spectrometry (GC-MS). Total, eight compounds were identified. The main are thymol, followed by γ-terpinene and ρ-cymene.



**Key words**: antimicrobial; macrodilution method; respiratory infections; vapor phase; volatile compound, Indian medicinal plants

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## List of the abbreviations used in the thesis

| | |
|---|---|
| ARI | Acute respiratory infections |
| ATCC | American Type Culture Collection |
| BC | Before Christ |
| CLSI | Clinical and Laboratory Standards Institute |
| DMSO | Dimethyl sulfoxide |
| DNA | Deoxyribonucleic acid |
| EENT | Eyes, ears, nose and throat |
| EO | Essential oil |
| GC-MS | Gas chromatography-mass spectrometry |
| MIC | Minimum inhibitory concentration |
| WHO | World Health Organisation |

## 1.1. Introduction and Literature Review

## 1.2. Bacterial respiratory diseases

### Epidemiology

Despite of the advance progress in human medicine combating the infectious diseases, they are still the major threat of mortality, especially in developing countries (Bloom & Cadarette). Respiratory diseases are one of the top 10 causes of death, and they are one of the main reasons of high mortality in low-income countries. Range of these diseases is from mild, such as the common cold to life-threatening disease such as pulmonary embolism, acute asthma, and lung cancer. Lower respiratory infections include bronchitis, pneumonia, and tuberculosis, remained the deadliest communicable disease, causing 3.0 million deaths worldwide, 8.7 million people develop tuberculosis annually and it kills 1.3 million people every year (World Health Organization 2018). Infectious diseases are caused by viruses, bacteria or other pathogenic microbes and they are leading to many different diseases as a cystic fibrosis, varicella, rubella, tuberculosis, measles, influenza and more. It also includes coronavirus infections SARS-CoV, SARS-CoV-2 and MERS-CoV. SARS-CoV-2 as a new virus from Wuhan, China causes COVID-19 disease. Since December 2019 there are more than 5,000,000 deaths worldwide to January 2022. Another very common disease is pneumonia estimating annually 450 million episodes and 4 million deaths worldwide. As the largest infectious cause of death children worldwide, it killed more than 800 000 children under the age of 5, that is more than 2,000 every day (WHO 2018). The most common locations are attacking Sub-Saharan Africa and South Asia, but get infected, is possible everywhere. For example, in India was reported that in 2019 there were 41,996,260 cases and 3,740 deaths from respiratory infections in 2018. Acute respiratory infections (ARI) accounted for 69% of the total cases of communicable diseases, and this scenario is before the era of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). After the coronavirus disease 2019 (COVID-19) pandemic, the total number of infected numbers rose to millions and cases are still getting added. The spread of the virus was so fast and wide that the World Health Organization (WHO) had to declare this infection as a global pandemic on March 11, 2020. The United States has been reporting the largest number of cases with 35,283,729 and over 626,668

deaths, followed by India with 31,341,507 and over 420,196 deaths as per data until July 24, 2021 (Worldometer 2021).

One of the most common infectious diseases in tropical areas is pneumonia, a form of acute respiratory infection that affects the lungs, when alveoli are filled with pus and fluid. That makes breathing painful and limits oxygen intake. It is primarily caused by bacteria species as a *Haemophilus influenzae*, *Moraxella catarrhalis*, *Mycoplasma pneumoniae*, *Mycobacterium tuberculosis*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*. Pneumonia can be spread in a few ways. The viruses and bacteria that are commonly found in a child's nose or throat can infect the lungs if they are inhaled. They may also spread via air and in addition, may be spread through blood, especially during and shortly after birth. Patients are typically affected with fever, cough, chills, fatigue and difficult breathing and pleuritic chest pain and headache. Treatment should be conducted with antibiotics and specifically with oral antibiotics in most cases. Hospitalization is usually not needed, but in some cases could be necessarily. In India 14% of child died due to pneumonia in 2017, and it was the second biggest killer of children under-five in 2017 (WHO 2017).

### 1.2.1. Microbial pathogens

***Haemophilus influenzae***

It is a gram-negative, coccobacillary, facultatively anaerobic pathogenic bacterium that belongs to the Pasteurellaceae family. It was first described by Richard Pfeiffer in 1892, who recovered it from the sputa of several patients suffering from influenza virus infections. This bacteria specie has high nutritional requirements and grow in the laboratory only when provided with complex, nutrient-rich media (Kuhnert 2008). *H. influenzae* can be commonly find in upper respiratory tracts, where it can cause invasive infections, as a pericarditis, pneumonia, septic arthritis, and it was major cause of bacterial epiglottitis and meningitis in children under five years old. The major virulence factor of *H. influenzae* strains causing invasive infections is the polysaccharide capsule. *H. influenzae* produces beta-lactamases, and it is also able to modify its penicillin-binding proteins, so it has gained resistance to the penicillin family of antibiotics (James et al. 1996). The important antibiotics applied in the treatment of less severe infections include amoxicillin, cephalosporin, azithromycin, doxycycline, and fluoroquinolone. However,

serious infections are managed by broad-spectrum cephalosporins and carbapenems (Murray & Rosenthal 2015). Nonetheless, WHO has recently placed ampicillin-resistant *H. influenzae* strains, in the list of antibiotic-resistant bacteria and medium priority category, in terms of the emergency of developing new antibiotics (WHO 2017).

***Staphylococcus aureus***

It is a gram-positive bacterium, occurring singly or in grape-like cluster. *S. aureus* is non-motile, non-capsular, and non-sporulating and it is facultative anaerobes. It produces a carotenoid pigment resulting in a golden-coloured colony, hence the name aureus (golden). Along with many other staphylococci, it is natural present in the nose, throat, skin and hair of healthy humans and animals (Ray & Bhunia 2014). *S. aureus* very often causes various infections ranging from mild skin and soft tissue inflammation to life-threatening sepsis such as blood poisoning, leading to septic shock associated with organ failure, toxic shock syndrome and necrotizing pneumonia. The pathogenicity of this bacterium results from the production of toxins, namely exfoliative toxins, enterotoxins and other, that cause destroying tissues of infected organism. Also, production of coagulase is an important indicator of pathogenicity (Murray et al. 1999). *S. aureus* resistance to penicillin was reported in the 1940s, and infections with antibiotic resistant remain a problem today. It was found that 60% of hospital-acquired isolates in 2003 were methicillin-resistant *S. aureus* (MRSA). Hospitalized patients with this type of infection have five times the risk of in-hospital mortality compared with inpatients without this infection. Antimicrobial therapy should be guided by the susceptibility profile of the organism. Methicillin-susceptible *S. aureus* (MSSA) is preferably treated with a semi-synthetic penicillin as a nafcillin, oxacillin or dicloxacillin. In case of MRSA, more advanced antibiotics should be given, for example daptomycin or telavancin (Rungelrath & DeLeo 2021).

***Streptococcus pyogenes***

It is a gram-positive, aerotolerant bacterium from genus Streptococcus. These bacteria are extracellular and made up of non-motile and non-sporing cocci. It is clinically important for humans. It is an infrequent, but usually pathogenic, part of the skin microbiota (Cunningham 2008). Generally, the first description of streptococcal infection is attributed to the Austrian surgeon, Theodor Billroth when he described the organism in cases of erysipelas and wound infections (Billroth 1877). *S. pyogenes* typically colonizes the throat, genital mucosa, rectum, and skin. There are four methods for the transmission

of this bacterium: inhalation of respiratory droplets, skin contact, contact with objects, surface, or dust that is contaminated with bacteria or, less commonly, transmission through food. The numerous virulence factors of *S. pyogenes* allow it to cause a wide array of serious infections including pharyngitis, respiratory infection as a pneumonia, necrotizing fasciitis, scarlet fever, myonecrosis and Streptococcal Toxic Shock Syndrome (StrepTSS), skin and soft tissue infections and more (Murray et al. 1999). Patients with invasive infection form have a relatively low mortality rate unless they meet the established criteria for StrepTSS (Hoge et al. 1993). Once the etymology of *S. pyogenes* is confirmed, high-dose of penicillin and clindamycin should be given (Stevens et al. 2014).

***Streptococcus pneumoniae***

This gram-positive, facultative, anaerobic bacterium occurs in a shape of a slightly pointed cocci. They are usually found in pairs (diplococci) but are also found singly and in short chains. In 1881, *S. pneumoniae* was first isolated simultaneously and independently by the U.S. Army physician George Sternberg and the French chemist Louis Pasteur. It is a highly infectious bacterial microorganism that can cause several serious illnesses, some of which can be life-threatening (Sheposh 2019). The bacterium is most commonly found in the human respiratory tract and can be transmitted from person to person through droplets dispersed in the air. It is a leading cause of bacterial pneumonia and invasive pneumococcal disease, including meningitis, worldwide. These diseases especially affect the young, the elderly, and the immunocompromised (Örtqvist et al. 2005). More advanced beta-lactam antibiotics are commonly used in combination with other antibiotics to treat meningitis and pneumonia. In adults recently developed fluoroquinolones such as levofloxacin and moxifloxacin are often used to provide empiric coverage for patients with pneumonia, but in parts of the world where these medications are used to treat tuberculosis, resistance has been described (Von Gottberg et al. 2008).

### 1.2.2. Treatment

In general, lower respiratory tract infections are difficult to treat due to the sequestration of microorganisms deep within the airways, where only limited portions of drug gain access after traditional systemic treatment (Wenzler 2016). Nevertheless, timely antibiotic therapy can considerably reduce fatal cases, for example in case of pneumonia

(Sazawal & Black 2003). Combination of β-lactams and macrolides have become the standard care for patients with pneumonia (Restrepo et al. 2017). Co-trimoxazole and amoxicillin are recommended as the first line drugs for treatment of non-severe pneumonia (Singh & Aneja 2011). Benzylpenicillin, ampicillin, gentamicin, ceftriaxone, and oxacillin are used as agent for the cure of severe pneumonia (Hale & Isaacs 2006). Nevertheless, it is well understood that effective antimicrobial therapy requires drug concentrations at the target site of infection. There is a risk of degradation of active components in the gastrointestinal tract as well. To reach the deep airways in sufficient concentrations, often toxic doses of drugs would need to be given systemically (Ambrose et al. 2009).

Other options of treatment are inhalation therapy. Since systemically administered antibiotics used to treat respiratory infections often have poor penetration into the lung parenchyma and narrow therapeutic windows between efficacy and toxicity, direct access of antibiotics to the site of infection in the lung parenchyma via inhalation could overcome these obstacles (Wenzler 2016). Inhaled antimicrobial agents have the capability of directly targeting the airways, creating increased and more sustained local concentrations, and thereby increasing the therapeutic index, improving efficacy, minimizing toxicity, and decreasing the time of onset for the administered drug without the systemic exposures (Flume & VanDevanter 2015). Optimal characteristic of inhaled antimicrobial are lipophilicity, positive charge and high molecular mass, these properties should be balanced against and appropriate particle size (Wenzler 2016). There are several types of devices for effective delivery of inhaled medications to the lungs: soft-mist nebulizers (jet, vibrating mesh, or ultrasonic), pressurized metered-dose inhalers, and dry-powder inhalers (Dolovich et al. 2011). However, there are several problems with the use of inhaler devices, such as the deposition of aerosolized particles in the oropharyngeal region and upper airways while the deposition of medication in the lungs is reduced due to patient-specific respiratory tract physiology, especially in children and elderly (Ibrahim et al 2015). As well as the distribution of antibiotic agent can be limited because of lung morphology or clearance mechanisms (Merchant et al. 2016).

Due to the inappropriate use of antibiotics increase antibiotic resistance, occurrence of side effects and falling eradication rates, there is a need to develop novel agents. For example, in India, especially in hospitals, rate of antimicrobial prescribing is high

compared to many other countries, which is a concern that requires addressing urgently. The most frequently used antibiotics used for pneumonia were piperacillin–tazobactam (18%) followed by meropenem (16%) and azithromycin (9%) (Singh & Aneja. 2011).

## 1.3. Essential oils

Essential oils are aromatic, volatile liquids obtained by distillation from plant material. They can be defined as mixtures of fragrant and odourless substances. These fragrant substances are chemically pure compounds that are volatile under normal conditions. EOs vary greatly, sometimes due to genetic causes, but also because of climate, rainfall, or geographic origin. They are composed principally of lipophilic and highly volatile secondary plant metabolites (Rios 2016).

Chemical constituents found in plants are often classified as primary and secondary metabolites. Primary metabolites as sugars, amino acids, common fatty acids, nucleotides, and polymers from them are essential for the survival and well-being of the organism. In plants such compounds are responsible for the primary life processes of respiration, photosynthesis, growth, development, and other essential roles. Plants also use metabolic pathways to produce compounds that often have no readily apparent function, and these are secondary metabolites which includes essential oils (EOs). These secondary compounds appear to play no direct role in a plant´s primary metabolism, but apparently have an ecological function. They may attract pollinators, help the plant adapt to environmental stressors, or serve as chemical defences against microorganism, insect and other predators, or even other plants (Hoffman 2003). They are natural substances products, composed from mixture of fragrant, volatile compounds. Aromatic plants produce fragrant essences in the secretory cells and settle them in intercellular spaces, channels, special trichomes and in other places in the plant. Today we know about 3000 types of EOs, of which approximately 10% are commercially important (Van de Braak 1999). They are used in many fields for various uses, for example pharmaceutical and agronomical industries use them because of antimicrobial effects, while cosmetics, perfumery, and aromatherapy profits from their aromatic components (Buchbauer 2000). EOs are extracted by steam distillation, hydrodistillation or solvent extractions from various aromatic plants generally localized in temperate and warm countries like Mediterranean and tropical countries. They can be produced in all plant organs (buds,

flowers, seeds, fruits, leaves, stems, bark, wood and roots) where are stored in secretory cells, canals, epidermic cells or glandular trichomes (Baser & Buchbauer 2015). The amount of produced EO depends on several extrinsic factors such as climatic conditions, altitude, air humidity and soil composition and intrinsic factors especially season and genetic variations (Procházka 1957).

Recently, the attention has been paid to the medicinal plants, which are considered as valuable sources of wide spectrum of secondary metabolites possessing biological activities that may contribute to effective therapeutic treatment and human health (Essawi & Srour 2000). Medicinal plants have played important role for curing various diseases in folk medicine for thousands of years. In the developing countries, about 80% of the people still rely on plant therapy and the tendency of using ethnomedicine has been gradually increasing in the developed countries. Over 21,000 plant species were recorded by WHO for their medicinal uses throughout the world, especially the flora of the tropical areas by virtue of its diversity plays a significant role in being able to provide rich source of phyto-medications (Hossen et al. 2016) . The natural products derived from medicinal plants have proven to be an abundant source of biologically active compounds and many of them have been the basis for the development of new pharmaceuticals (Palombo 2011). Furthermore, it is estimated that approximately 25% of modern drugs and as many as 60% of antitumor drugs are derived from natural products (Newman & Cragg 2012). The development of new products from natural sources is also encouraged because it is estimated that of the 500,000 plant species occurring worldwide, only 1% has been phytochemically investigated, therefore there is a great potential for discovering novel bioactive substances (Palombo 2011). The multi-component plant extracts help to prevent the bacterial resistance as well as it can lead to pleiotropic, synergistic, or additive effects in the organism (Ayrle 2016). The primary benefits of using plant derived medicines are relative safety and affordable cure, thus demands of use of herbal remedies is increasing nowadays. In many regions of the world, folk medicine using healing effects of plants has a long tradition. Chinese, Ayurvedic, and Unani medicine are among the most important followed by other indigenous traditional medicine practices developed by various cultures all around the world (Rehman et al. 2016).

The plant EOs are of great potential for the development of novel antimicrobial preparations. They have been widely used for their diverse biological effects since the

Middle Ages (Bakkali et al. 2008). Since the presence of volatile compounds is characteristic for some plant taxa, chemotaxonomic research is a frequent approach to their exploration. Due to the volatility of EOs, they are suitable for inhalation therapy, which is an effective way for the healing of respiratory ailments such as pneumonia. Their vapours can act directly on the site of infection in the respiratory system and simultaneously restrict systemic exposure, degradation of active components in the gastrointestinal tract and associated toxicity (Kuzmov & Minko 2015). In addition, EOs contain a broad spectrum of chemically diverse substances with antimicrobial effect: thus, it is more difficult for bacterial pathogens to develop resistance to these multi component mixtures than to single ingredient conventional antibiotics (Yang et al. 2015). During the last few years, several inhalation devices and suitable delivery systems for essential oils in the treatment of respiratory infections (e.g. pocket inhaler, aromatherapy patch, decongestant on a foraminous carrier, and encapsulated essential oils) have been developed and patented (Horvath & Acs 2015).

### 1.3.1. Taxonomical distribution

There are about 60 families of plants producing EOs. Most known are Apiaceae (genera *Anethum, Coriandrum,* and *Foeniculum*) typically annual, biennial, and also perennial plants, many of them have economic importance and firm place in human nutrition; Asteraceae *(Artemisia and Echinacea)* biggest family of angiosperm plants including herbs, shrubs, trees; Cupresaceae *(Cupressus, Juniperus)* a group of conifers producing EOs in their wood and needles; Lamiaceae *(Levandula, Thymus, Rosemarine)* aromatic herbs and bushes with volatile compounds stored in trichomes; Lauraceae (*Cinnamomum, Laurus, Litsea*) mostly tree or shrubs containing large amount of EOs in cells within the bark, wood and leaves; Myrtaceae (*Eucalyptus, Myrtus, Syzigium, Xanthostemon)* group of woody plants including also fruits species; Pinaceae *(Cedrus, Picea)* are trees or shrub including of the well-known conifers of commercial importance; Pipereceae (*Piper)* pantropical shrubs, herbs or small trees, incorporates famous spices; Rutaceae (*Citrus, Murraya)* belongs to the most economically important EOs; Zingiberaceae *(Etlingera, Zingiber)* perennial aromatic herbs, that are known for their ability to produce substances in very high quality for industrial and medicinal use (Raut & Karuppavil 2014).

### 1.3.2. Chemistry

Chemically, the EOs are volatile aromatic liquids usually colourless substances, generally of lower density than water. They are lipophilic and soluble in organic solvents (Burt 2004). As each plant species has evolved to protect itself against predator and stress condition, each plant produces its own specific mixture of EO chemical constituents. This can contain 20–60 constituents at varying concentrations with two or three major compounds representing 20–70 % of all content that usually define the biological properties of EOs (War et al. 2012). Based on chemical composition of EOs, they are broadly classified into oxygenated compounds and hydrocarbons. Oxygenated compounds include esters, aldehydes, ketones, alcohols, phenols, and oxides, whereas hydrocarbons are composed of components with an isoprene structure, which are called terpenes (Cowan 1999). Major classes of terpenes include monoterpenes and sesquiterpenes, but monoterpenes contribute to 90 % of EOs overall. They are volatile, aromatic, colourless, oily substances, although a few (such as camphor) are crystalline. In some plants, there may be considerable chemical variation within the specie. Different plant populations contain different mixture of monoterpenes. Plants displaying these chemical variations are called chemotypes (Hoffman 2003). When these substances contain additional elements (e.g. oxygen), they are termed terpenoids. Terpenoids are synthetized from acetate units, and as such they share their origins with fatty acids, but they are extensive branched and cyclized (Blowman 2018). In fact, composition of EOs is largely affected by extraction method. It has been found that traditional techniques used for their extraction can cause losses of some volatiles and degradation of unsaturated or ester compounds through thermal or hydrolytic effects, while the use of solvent extraction can cause presence of toxic solvent residue. Moreover, obtained EO are susceptible to degradation by several factors, such as light, heat, oxidation, and hydration (Reyes-Jurado et al. 2015). The antimicrobial properties of EOs are determined by effects of their constituents. 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 method for qualitative analysis (Baser & Buchbauer 2015).

### 1.3.3. Antimicrobial activity

The biological activities of EOs have been known since ancient times. They were used for prevention and treatment of diseases, food flavouring and preservation, and aromatherapy (Zink 1997). EOs possess a range of biological properties including antimicrobial effect contributing to human health (Raut & Karuppavil 2014). Nowadays, many studies are researching biologically active substances, because of the increasing resistance of microorganisms against many anti-microbial agents. Antimicrobial activity is determined by their active compounds such as terpenes, phenols or hydrocarbons. In general, phenols have the highest antimicrobial activity, followed by aldehydes, ketones, alcohols, esters and hydrocarbons (Berger 2007). As we know from some studies, phenolic substances such as thymol or carvacrol are the most effective and they possess active defence spectrum of microorganisms. One of the first systematic *in vitro* examinations of the antimicrobial activity dates back to late 19$^{th}$ century when Buchholtz studied the growth inhibitory properties of caraway oil, thyme oil, phenol, and thymol on bacteria having been cultivated in a tobacco decoction. In this examination, thymol turned out to be 10-fold stronger than phenol (Buchholtz 1875). During late 20$^{th}$ century, the use of EO diminished as a result of development of organic chemistry, however, the demand for safe and natural alternative medicine had risen again with the concern about toxicity of synthetic compounds (Gaysinsky & Weiss 2007). The susceptibility of a microorganism to EOs depends on the properties of both, therefore antimicrobial activity can be classified according to the microorganisms against which it acts primarily (Chouhan et al. 2017).

### 1.3.4. Methods of susceptibility testing in vapour phase

*In vitro* screening is typically the first step in the process of discovery of new antimicrobial drugs, including those derived from plant volatiles. However, the susceptibility testing of microorganisms to volatile agents using standard methods, such as broth dilution and disk diffusion assays, is a challenging task because of their specific physico-chemical properties, including high volatility, hydrophobicity, and viscosity (Reyes-Jurado 2015). The main problem is that their hydrophobic nature worsens the solubility of these compounds in water-based media as an agar or broth and their volatility increases the risk of loss of active substances via evaporation during sample handling,

experiment preparation, and incubation. This is even more complicated in the case of antimicrobial vapours testing. In contrast to well-established methods for antimicrobial susceptibility testing on solid (agar disc diffusion) and liquid (broth dilution) media (CLSI 2012; CLSI 2015) there are lack of standardized methods for the determination of microbial sensitivity to volatile compounds in the vapor phase. In recent years, several methods for the testing of the antimicrobial effects of volatile plant-derived products in the vapor phase have been developed. However, most of them have some specific limitations, such not being designed for high-throughput screening, and some of them need special equipment that is not commonly available. According to the literature, 11 varied techniques and their modifications were developed to test the inhibitory effect of vapours on microbial growth. Classification of these methods can be based on the form of matrices to which the volatile agents to be tested are applied because the carrier medium selection is crucial for the volatilization of the tested agents. As an example, here are 6 of the assays categorized into two groups: solid and liquid matrix volatilization methods (Houdkova & Kokoska 2020).

**Solid matrix volatilization methods**

Disc volatilization assay or inverted petri plate method is based on a very simple modification of the standardized disc diffusion method, and it is the most frequently used to evaluate the antimicrobial effects of volatiles in the vapour phase. Generally, antimicrobial agents diffuse from the disc to the atmosphere inside the petri dishes and then to the agar, which inhibits the growth of the test microorganism (Lopez et al. 2005). Although methods based on the disc volatilization assay method performed in petri dishes are a useful tool in the simple and low-cost assessment of the growth-inhibitory potential of EOs in the vapour phase, they are not designed for high throughput screening. The relatively high consumption of material and labour are the main disadvantages.

Dressing model volatilization test is designed as a more specified alteration of the disc volatilization method, modifying the matrix from which the compound tested is evaporated. This model can be used in the development of new wound healing preparations in medicine. Experiment is performed in a petri dish covered with various layers composed of different materials commonly used in the treatment of skin infections, however, its weakness is possibly the high level of interference of the tested agents with dressing models (Edward-Jones et al. 2004).

Airtight apparatus disc volatilization methods are improvement of the disc volatilization assay method by using an airtight box, which are placed into the petri dishes. The inside part of the box is covered with aluminium foil to prevent plastic absorption of the volatile agent and to protect the wall of the container from its direct contamination (Inouye et al. 2001). It is expected that this method could reduce the time required for the evaluation of the antimicrobial effect of volatile agent in the gaseous phase, because it is possible to test several concentrations simultaneously. Nevertheless, this assay method requires special equipment that is not commonly available (Houdkova & Kokoska 2020).

**Liquid matrix volatilization methods**

Broth microdilution volatilization was designed, based on the principles of broth microdilution and disc volatilization methods, which is suitable for high-throughput screening of volatile compounds simultaneously in the liquid and vapor phase. This method can also be easily used for determination of the antibacterial effects of essential oil vapours. The experiments are performed on standard 96-well immune plates, covered by tight-fitting lids with flanges designed to reduce evaporation. Although the broth microdilution volatilization method is fast, simple and labour-effective, it has several weaknesses. For example, clamps and wooden pads are required for a better sealing and fixing the microtiter plate and its lid together. Moreover, the limited volume of agar that is applied on the lid can affect the growth of the microorganisms tested (Houdkova et al. 2017).

Microplate patch volatilization assay introduced an assay method under the original name of vapour-phase-mediated patch assay for detecting vapour phase antimicrobial activity of the volatile agents, which uses U-shaped, 96- wells microtitre plates, where a patch is defined as the set of wells in an area (square) surrounding one or more test wells. This microtitre plate setup can be used to easily unmask false-positive results caused by the vapours. However, since it is not a quantitative method, it does not determine the exact values needed to assess the level of antimicrobial potential of the vapour phase (Feyaerts et al. 2017).

Agar plug-based vapour phase assay was developed as a method providing both qualitative and quantitative measurements on the vapour phase antimicrobial activities of volatile agents. For this method, two separate agar plates are used. In comparison with the assays performed in petri dishes based on inhibition zone measuring, the agar plug-

based vapour phase assay method, representing viable cell counting methods, provides more accurate data on reduced growth potential (Amat et al. 2017). This model enables to test simultaneously several sample replicates against one bacterium or one volatile compound against different bacterial strains on one agar plate, while both options are applicable for a range of concentrations. However, preparation of the agar plugs may be labour and time consuming (Houdkova & Kokoska 2020).

Airtight apparatus liquid volatilization method is method originally called the vapour-agar contact method. It is performed in a sealed container containing an inoculated agar plate and a petri dish with the volatile compound to be tested (Sekiyama et al. 1996). Similarly, like in case of the above described method using an airtight box, the distribution of the vapours and the final concentrations of the active compounds affecting bacterial growth on the surface of the agar plate is debatable.

Although the broth microdilution volatilization method is fast, simple, and labour-effective, it has several weaknesses. For example, clamps and wooden pads are required for a better sealing and fixing the microtiter plate and its lid together. Moreover, the limited volume of agar that is applied on the lid can affect the growth of the microorganisms tested. With the aim to overcome the above-mentioned drawbacks of previously developed methods used for testing of volatile antimicrobial agents in the vapor phase, Laboratory of ethnobotany and ethnopharmacology in Prague designed a novel macrodilution volatilization assay that combines the principles of broth microdilution volatilization (Houdkova et al. 2017) and standard broth macrodilution methods (CLSI 2012).

### 1.3.1. Herbal medicine

Approximately 90% of ayurvedic preparations are plant based. Such actions enable the plant to reverse pathophysiological processes and stabilize the doshas. Classical ayurvedic preparations, made from such plants, are known as “yoga” in Sanskrit. Yogas have developed from years of practical experience combining plants to get the optimal effect. Polyherbal combinations have also proven lastingly effective than single herbs. In Ayurveda, most of the classical preparations are polyherbal, with a combination of 3 to 30 plants involved. These constituents are combined accurately, in such a way that the formula is balanced and reproducible. One or two of the plants in these combinations will

be active and the others will play a supporting role. The supporting herbs will each have different actions, acting as catalysts to help proper absorption, transportation, and to reduce toxicity (Kumar et al. 2017). Herbal medicines are commonly available at pharmacies and substances which are not requiring prescription can be sold also at other outlets, and by licensed practitioners. In 2010, 13 000 herbal medicines in India were registered (WHO 2019). Here are some examples of medicinal plant producing EOs with medicinal properties cultivated in India and traditionally used in Ayurveda.

Ashwagandha (*Withnania somnifera*) is one of the most important herbs, mainly used as an antidepressant and it improves chronic insomnia and nervous agitation. Generally, it is applied in psychological disorders and to improve cardiac function (Kashyap et al. 2022). Its roots are utilized for the preparation of decoction and extracts used to treat asthma and bronchitis. Its use in a commercial product for these diseases indicates its significance (Afzal 2021). *Azadirachta indica*, known also as a neem, is one of the most versatile, multitudinous trees having immense potentials. It has been known as the multi-directional therapeutic uses in India and its oil possesses great medicinal properties. Different parts of neem have been used for treating various diseases such as cancer, dental diseases, stress, ulcers, heart diseases, malaria, and skin diseases (Oli & Gautam 2021). Commercially important specie is *Centella asiatica* and products from this herb are available under the name gotu kola. It is valued for its anti-inflammatory effects, slows skin aging, improves skin elasticity and heals wounds has soothing, antibacterial, antioxidant, regenerative and moisturizing effects on the skin cosmetic products, such as face creams and serums. It has been used as in Indian medicine as a drug for enhancing cognitive function by revitalizing the nerve and brain cells by support the vascular and lymphatic system. Nowadays it is still used for these purposes in a fresh form or in a form of tablets (Sudhakaran 2017). *Momordica charantia* is suggested as one of the most promising plants for diabetes. It helps lower blood sugar levels and promote the secretion of insulin, the hormone responsible for keeping blood sugar levels stable (Joseph & Jini 2013). Herb known for its positive effect in oral hygiene is Licorice root, which comes from the *Glycyrrhiza glabra* plant. It appears to offer relief from a sore throat and promote oral health by protecting against dental cavities. Test-tube and human studies also suggest that licorice root may help reduce inflammation and fight viruses and bacteria (Messier et al. 2012). Known for their distinctive earthy, nutty, and spicy flavour is *Cuminum cyminum* which is added into the meals also for improving digestion. Research shows that

cumin may boost the activity of digestive enzymes and facilitate the release of bile from the liver, speeding digestion and easing the digestion of fats. This spice can also reduce symptoms of irritable bowel syndrome, such as abdominal pain and bloating (Muthamma et al. 2008). From long list of plants used in Ayurveda medicine, last plant here to mention is *Curcuma longa*, commonly called turmeric. Turmeric has been proposed as a treatment for variety of digestive problems, such as stomach discomfort, bloating, appetite loss, and nausea. It helps to keep muscles and joints flexible and supports the immune system and mental balance. Curcuminoids present in turmeric have been identified as bioactive compounds that impart it pharmaceutical properties (Gupta et al. 2013).

There are many species which are mainly focused for treatment of respiratory diseases. For example, *Piper longum* is used to treat bronchitis, asthma, or cough (Kumar et al. 2017). A combination of *Thymus* and *Hedera helix* leaf has shown to reduce cough significantly in adults with acute bronchitis. Very popular for treating cold, cough, throat infection, and influenza is *Zingiber officinale* where an underground stem is used to make juice and a paste is made by grinding, infusion, and extraction. *Mentha piperita* is a common plant in which leaves, and the stem are used to make mainly hot beverages or decoction, mainly helpful to treat cough, sore throat, and influenza. *Mangifera indica* is a perennial tree whose bark and leaves are used to treat the sore throat by making juice and paste by crushing the leaves, infusion and bark extract is also made for crude preparation. *Glycyrrhiza glabra* is a small herb plant where the plant's roots are used to make the decoction, paste, and extract helpful in curing asthma, cough, and pneumonia. *Viola odorata* is an herb commonly known as Banafsha, used in traditional remedies for cough and other respiratory disorders by making decoction and extract of stem and root. Decoction from the seeds of *Foeniculum vulgare*, is used to treat cold and cough, while extract and paste of the stem and leaves are consumed for respiratory disorders. *Justicia adhatoda* is a shrub plant that is considered important to cure asthma and cough by preparing extract, paste, powder, and ash, which is also prepared for tuberculosis and sore throat (Afzal 2021). Consumption of *Camelia sinensis* has been proven to be beneficial to patients with pulmonary fibrosis and lung cancer. In addition, green tea possesses strong antioxidant and anti-inflammatory effects, especially due to contain of polyphenol epigallocatechin gallate (EGCG). EGCG has significant potential in inhibiting chemoattractants and regulating inflammatory responses in multiple fibrotic diseases. In addition, in vivo studies have confirmed that EGCG is beneficial in ameliorating the lung

injuries exposed to cigarette smoke, therefore, EGCG has a high potential in improving the well-being of patients with respiratory diseases (Hwang & Ho 2018). Another important group of plants possessing anti-inflammatory effect, are trees from families Pinaceae and Cupressaceae. *Pinus* spp., *Abies* spp., *Picea* spp., *Thuja* spp., and *Juniperus* spp. resin and branch tips have immunopharmacological potential and they are used as a phytotherapy for acute and chronic infections of the upper respiratory tract (Yarnell 2018). Common herbs used in steam inhalation are *Eucalyptus globules*, *Vitex negundo*, *Justicia adhatoda* (Gowrishankar et al. 2021). *Salvia apiana*, *Salvia officinalis*, *Thymus vulgaris*, *Rosmarinus officinalis*, and *Prunella vulgaris* are received well by patients based on taste and smell. Although a number of these plants are used in Ayurveda for inhalation therapy of respiratory diseases, their antimicrobial activity in the vapour phase has not been sufficiently investigated so far. Examples of these plants are given below.

***Cymbopogon citratus***

This is the most important specie from family *Poaceae*. It is a perennial, tropical grass (see Figure 2 and 3; Appendix 1) which comes from south of India (Huxley et al. 1992). Lemongrass is a favourite ingredient of Asian cuisine for its slightly expanding, bulbous juicy base. The rest of the plant can be cooked also for its lemon flavour present in all parts of the plant, but it is usually removed before consumption. Outside cultivation as a culinary plant, it is grown for EOs production through the tropics, mainly in southeast Asia as China, India, and Sri Lanka. About 0,2-0,4 % of fresh plant are EOs. Main compound is citral (65-85 % ) which carriers lemon odour and flavour and it is also used for synthetic violet fragrances in cosmetic industry. Next is geraniol, which can make up 40 %, known to exhibit insecticidal and used as a natural pest control agent exhibiting low toxicity (Duke & duCellier 1993). It is widely served in treatment of many problems as an asthma, cough, gastric disorders, or fever in a form of decoction (Duke & Ayensu 1985).

***Cyperus scariosus***

This specie is member of Cyperaceae family. Its native to Australia and New Guinea and it was introduced to other parts of south-east Asia, including India. It is hardy grass-like perennial herb (See Figure 4; Appendix 1). EO is extracted from roots (See Figure 5; Appendix 1) and rhizomes. Rotundone was found to have the highest odour-activity value of compounds and, together with the other ketones, contributes to the woody–amber

character of cypriol oil, which is commercially produced from wild collected plants that grow abundantly in certain parts of India. The plant regenerates and spreads rapidly by means of its rhizomes and is thus considered a weed when it invades cultivated land. It is used in perfumery for its warm and woody character with facets of cedarwood (Clery et al. 2016). It is widely used in Ayurveda to treat several ailments for its ability to produce tonic and exhilarant effect on the heart (Nafees et al. 2020) and for treatment of cough and bronchitis. Herbal syrup used for colds has also been made by using *C. scariosus*, which had no side effects like drowsiness and other harmful cardiac effects (Utreja & Ekta 2015).

***Trachyspermum ammi***

*T. ammi* is a member of family Apiaceae native to Egypt and is cultivated in Middle East and in India as a highly valued medicinally important seed spice, known as ajwain (See Figure 6; Appendix 1). The roots are diuretic in nature and the seeds possess excellent aphrodisiac properties. The seeds contain 2–4.4% brown coloured oil known as ajwain oil. The main component of this oil is thymol from up to 50%, which is used in the treatment of gastro-intestinal ailments, lack of appetite and bronchial problems. The oil exhibits fungicidal, antimicrobial, and anti-aggregatory effects on humans. Ajwain is a traditional potential herb and is widely used for curing various diseases in humans and animals. The seed of ajwain (see Figure 7; Appendix 1) is bitter, pungent and it acts as antimicrobial and anti-inflammatory agent use to treat many diseases including respiratory as a caught, cold, and asthma (Bairwa et al. 2012). Antitussive effect of Ajwain has been reported in traditional medical manuscripts. Decoction from the seeds revealed significant reduction of cough number which may be a result of its potent antitussive effect and bronchodilatory effect of decocted extract of Ajwain on the asthmatic patients' airways was examined (Srivastava 1988). Also results of another study showed that *T. ammi* has a relatively bronchodilatory effect (Boskabady et al. 2007).

As far as antimicrobial activity in vapour phase of above mentioned species is considered, EO vapours of *C. citratus* have been recorded against *Pseudomonas fluorescens* and *Candida albicans* (Tyagi & Malik 2010), *Aspergillus flavus*, *Aspergillus niger*, *Fusarium proliferatum*, and *Curvularia lunata* (Sawangsri et al. 2020). Antifungal effect of *T. ammi* in vapour phase was detected against *C. albicans* (Sharifzadeh et al. 2015) and *Aspergillus*

species (Kim et al. 2016). However, there is lack of research on their effects in vapour phase against bacteria causing respiratory diseases. According to our knowledge, studies on *C. scariosus* antimicrobial properties in vapour phase has not been exanimated yet.

## 1.4. Traditional Indian medicine

Ayurveda, the ancient healing system of India, is one of the great healing traditions of the world. It is a medical programme grounded in a comprehensive spiritual view of life with holistic approach to health and personalized medicine. It is one of the oldest medical systems, which comprises thousands of medical concepts and hypothesis. Interestingly, it has ability to treat many chronic diseases such as cancer, diabetes, arthritis, and asthma, which are untreatable in modern medicine. Unfortunately, due to lack of scientific validation in various concepts, this precious gift from our ancestors is trailing. Hence, evidence-based research is highly needed for global recognition and acceptance, which needs further advancements in the research methodology (EBSCO CAM Review board 2022).

Ayurvedic treatment is highly individualized and incorporates a wide range of methods, including dietary changes, herbal therapy, exercise, massage, meditation, and numerous special procedures such as cleansing of the nasal passages. Although the scientific base is not strong, some of its methods have undergone meaningful scientific evaluation, and worldwide interest continues to increase (EBSCO CAM Review board 2022). In this holistic system, each cell is considered to be inherently an essential expression of pure intelligence hence called self-healing science. In addition, to the self-healing concept, the use of herbal treatment is equally important in this Indian traditional system of medicine (Lad 1987).

According to the World Health Organization, about 70–80% of the world populations rely on nonconventional medicines mainly of herbal sources in their healthcare (WHO 2019). Public interest for the treatment with complementary and alternative medicine is mainly due to increased side effects in synthetic drugs, lack of curative treatment for several chronic diseases, high cost of new drugs, microbial resistance, or emerging diseases (Humber 2002).

### 1.4.1. History

Ayurvedic knowledge originated in India more than 5,000 years ago and is often called the “Mother of All Healing.” It stems from the ancient Vedic culture and was taught for many thousands of years in an oral tradition from accomplished masters to their disciples.

Some of this knowledge was set to print a few thousand years ago, but much of it is inaccessible (EBSCO CAM Review board 2022). The golden age of Indian medicine, from 800 BCE until about 1000 CE, was marked especially by the production of the medical treatises known as the Caraka-samhita and Susruta-samhita, attributed respectively to Caraka, a physician, and Susruta, a surgeon. Estimates place the Caraka-samhita in its present form as dating from the 1st century CE, although there were earlier versions. The Susruta-samhita probably originated in the last centuries BCE and had become fixed in its present form by the 7th century CE. Of somewhat lesser importance are the treatises attributed to Vagbhata. All later writings on Indian medicine were based on these works, which analyse the human body in terms of earth, water, fire, air, and ether as well as the three bodily humours called vata, pitta, and kappa (Britannica 2019).

The earliest classical Sanskrit works on Ayurveda describe medicine as being divided into eight components. This characterization of the physician's art, "the medicine that has eight components" is first found in the Sanskrit epic the Mahabharata, in 4th century BCE (Wujastyk 2003). The components are Kaya Chikitsa, which means internal medicine and deals with general ailments, like fever, diarrhoea, cough, or skin disorders. The imbalance in the body is cause by ‘Agni’ principle and the discipline of Kaya Chikitsa brings Agni back in balance. Baala Chikitsa means paediatrics and it works with children and infants. Dosages are different from that of the adults and the medicines need to be tolerable for their bodies. It also discusses the art of nursing, infertility, mental health of the mother and its influence on the infant’s well-being. Psychology is called Graha Chikitsa in Ayurveda. It works with diseases and illnesses of the mind or diseases with psychosomatic roots. This field talks about herbs and their applications as disinfectants and their abilities to bring positivity in the atmosphere, as well as about diet, use of specific mantras, breathing techniques, meditation techniques and yoga practice. Shalakya Chikitsa  deals with treating diseases and imbalances in body parts above the shoulders through holistic treatments, cleanses and herbal formulations. Shalakya Tantra is a field equivalent to otorhinolaryngology and ophthalmology field works with conditions of the eyes, ears, nose, lips, brain, central nervous system, skull, and throat. Surgery, called Shalya Chikitsa has long history and operations have been found to be performed 3000 to 5000 years ago. The ancient Indians were the pioneers in many complicated operations like perforation of intestine, obstructed labour, how to perform prosthetic surgery to replace limbs, cosmetic surgery on the nose and even cranial

surgeries. Most of these instruments were made of stone, wood, bark, or thick leaves. Samstra Chikitsa , known as a toxicology in western world, is involved in the removal of toxins in the body. It solves problems with poisoning from animals, plants, vegetables, or metals or man-made poison. But more importantly, this branch of Ayurveda also considered air and water pollution as a form of poisoning that needed to be purified for health and well-being. Rasayan Chikitsa is an area that deals with diseases and illnesses related to aging, as well as science of longevity and rejuvenation. It deals with preventive healthcare, tips, treatments, and herbal medicines to enhance quality of life. Vajikarana Chikitsa promote sexual health of men and women and improvement in fertility. Herbs and medicines administered under the Vajikarna sciences are for example specific type of meats, milk, fruit pulps, fruit from *Phyllantus emblica*, *Terminalia chebula* and use of *Myristica fragrans* (Meulenbeld 1999).

Ayurveda became a part of the Indian National health care system, with state hospitals established across the country for this purpose. However, the treatments of traditional medicines were not always integrated with others. Nowadays it is heavily practiced in India and Nepal, where around 80% of the population report using this unique healing system (WHO 2014). The principles of many of the natural healing systems now familiar in the West have their roots in Ayurveda, including Homeopathy and Polarity Therapy (EBSCO CAM Review board 2022).

### 1.4.2. Principles and praxis

Ayurveda is a Sanskrit word derived from ayuh (life) and veda (knowledge) and is also known as the “science of life”. Rigveda (2000 BC) is the oldest recorded document regarding use of plants as medicine in India, and this tradition continued in another ancient text, Atharvaveda, which described more plants and introduced basic concepts. It describes the beneficial, nonbeneficial, happy and unhappy aspects of life. Health is defined as the state of equilibrium of dosha (humours), agni (digestive juices, enzymes and hormones), dhatu (tissues) and the normal excretion of mala (waste materials), along with a happy state of atma (soul), indriya (sensory and motor organs), and manas (mind) (WHO 2010).

All life is made out of the five great elements called the Panchamahabhutas that form the three fundamental bio energies governing multiple life functions. These three bio energies

are called Doshas.Pitta dosha (fire and water), vata dosha (air and ether) and kapha dosha (earth and water). In an individual usually any one or two of them predominate and form the basis of our unique physio mental constitution called Prakruti. The doshas are present in every cell and move through every channel of the body (Lad 1987).

The fundamental theories and principles are derived from experience. These are ascertained by a fourfold examination, which means authoritative statement, perception, inference, and rationale. Ayurveda is based on the principle that the entire universe and the human body are one, and that the same principles govern both. The changes that occur in the universe with the passage of time also occur in the human body. Hence, substances of natural origin are congenial to the human body and help to maintain the balance of its constituents. Both the universe and the human body are made up of five elements. A balanced state of these elements in the body brings health, and an imbalance brings disease. The elemental composition of the body is explained in the theory of dosha, dhatu and mala, which is the foundation of this healing system. The aim is to achieve equilibrium between doshas and dhatus, which is believed to bring health and longevity(WHO 2010).

**Inhalation therapy**

Pranayama is traditional technique practicing from ancient time of Indian civilization. Prana, life force as nerve energy, enters the body through the breath taken in through the nose. This practise includes complex treatment of respiratory diseases, as a several breathing techniques, and inhalation therapy (Sengupta 2012). Before the healing itself begins, there is a need to prepare the body with prescribed methods to encourage it to let go of the toxins. These two procedures are snehan and svedana. Snehan is the oil massage. Oil is applied to the entire body with a particular type of massage that helps the toxins to move towards the gastrointestinal tract and it is given daily for three to seven days, as indicated. Svedana is sudation or sweating and is given every day immediately following the snehan. Herbal products have a great beneficial effect when are added into steam inhalation. In case of common cold, mild flu, sinusitis, and upper respiratory tract infections it has a very beneficial role. Important is the duration of steam inhalation, which typically should not last for more than five minutes at the most. Steam inhalation has a preventive, curative as well as health promoting effect on the upper respiratory tract (Singh et al. 2009). Inhaling steam is one of the major treatments for respiratory

complications and is recommended also for bronchitis, sinusitis, asthma, and allergies. It moistens dry air passages and mucus is loosened and eliminated easier by coughing or by blowing the nose. Another respiratory healing techniques including also nose massage and therapeutic vomiting - vamana (Sengupta 2012).

# 2. Aims of the Thesis

The main aim of this work is to determine chemical composition and antibacterial effect of EOs from Indian medicinal plants against bacteria causing respiratory diseases using the broth macrodilution volatilization method.

The specific objectives are:

1. Isolation of EOs from *Cymbopogon citrus*, *Cyperus scariosus* and *Trachyspermum ammi* and evaluation of their physical properties.

2. Optimization of broth macrodilution volatilization method for evaluation of antimicrobial effect of EOs.

3. Determination of MICs of isolated EOs in liquid and vapour phase against *Haemophilus influenzae*, *Staphylococcus aureus*, *Streptococcus pneumoniae* and *Streptococcus pyogenes.*

4. Characterisation of the chemical composition of most antimicrobially active EO using gas chromatography-mass spectrometry (GC-MS).

# 3. Material and methods

## 3.1. Plant material

Plant material was selected based on chemotaxonomic criteria, whereas four plant species were chosen as a less explored in vapour phase. Air dried samples of *C. citratus*, *C. scariosus* and *T. ammi* were ordered from Bhagyashree Herbal Farms, which is located close to the city Raipur, India. It is a registered company, which is collecting the pure certified organic herbs from the forest of Chhattisgarh. This part of India is situated inland in the west of the country.

## 3.2. Distillation of essential oils

Dried plant material was ground into powder using an electric mill Grindomix (GM100 Retsch, Haan, Germany). Required amount of powdered sample was subjected to hydrodistillation in distilled 1 L of distilled water for 3 h using Clevenger-type apparatus (Merci, Prague, Czech Republic), according to the c (European Pharmacopoeia 2013) (See figure 8; Appendix 2). The EO was then collected and stored in sealed glass vials at 4°C.

## 3.3. Microorganism and media

The following four bacterial standard strains from the American Type Culture Collection (ATCC, Manassas, VA, USA) were used: *Haemophilus influenzae* ATCC 49247, *Staphylococcus aureus* ATCC 29213, *Streptococcus pneumoniae* ATCC 49619, and *Streptococcus pyogenes* ATCC 19615. Cultivation and assay media (broth/agar) were Mueller–Hinton (MH) complemented by Haemophilus Tested Medium (*H. influenzae*), MH (*S. aureus*), and Brain Heart Infusion (*S. pneumoniae* and *S. pyogenes*). The pH of the broths was equilibrated to a final value of 7.6 using Trizma base (Sigma-Aldrich, Praha, Czech Republic). All microbial strains and cultivation media were purchased from Oxoid (Basingstoke, UK). Stock cultures of bacterial strains were cultivated in broth medium at 37°C for 24 h prior to testing. For the preparation of inoculum, the turbidity of the bacterial suspension was adjusted to 0.5 McFarland standard using a Densi-La-

Meter II (Lachema, Brno, Czech Republic) to obtain a final concentration of 108 CFU/mL. Amoxicillin (90 %, CAS 26787-78-0), ampicillin (84.5 %, CAS 69-52-3), oxacillin (86.3 %, CAS 7240-38-2), and tetracycline (98–102 %, CAS 60-54-8) were used as positive antibiotic controls. The chemicals used for antimicrobial susceptibility testing were as follows: dimethyl sulfoxide (DMSO, CAS 67-68-5) and thiazolyl blue tetrazolium bromide dye (MTT, CAS 298-93-1). All antibiotics were obtained from Sigma-Aldrich (Prague, Czech Republic).

## 3.4. Antimicrobial assay

The antibacterial potential of volatile plant-derived compounds in the liquid and vapor phases was determined using a newly developed broth macrodilution volatilization method performed in standard 2 mL microtubes with snap caps (Eppendorf, Hamburg, Germany), which was optimized for testing of EOs. Initially, each sample of compound was dissolved in DMSO at maximum concentration of 1% and diluted in the appropriate broth medium. With the aim to prepare a sufficient amount of stock solutions of the compounds assayed, six two-fold serially diluted concentrations of samples were prepared in 15 mL test tubes closed with plugs to avoid the losses of active compounds by evaporation (Gama Group, Ceske Budejovice, Czech Republic). The concentration of EO´s started from 1024 µg/mL. In the second step, 90 µL of melted agar was pipetted into rims on the caps and inoculated with 5 µL of bacterial suspension after agar solidification. Subsequently, the appropriate concentrations of each sample previously prepared in test tubes were pipetted into microtubes in a final volume of 1500 µL. Then, the microtubes were inoculated with 10 µL of bacterial suspension and closed properly. Microtubes containing inoculated and non-inoculated media were prepared as growth and purity controls simultaneously. After incubation at 37°C for 24 h, the MICs were evaluated by the visual assessment of bacterial growth after colouring metabolically active bacterial colonies with MTT dye (See Figure 10; Appendix 2). The respective volumes of 30 and 375 µL of MTT at a concentration of 600 µg/mL were pipetted into the caps and in the microtubes when the interface of colour change from yellow to purple, relative to that of colours in control wells, was recorded in broth and agar. A black and white scheme of a cross-sectional view of a microtube filled with broth and agar shows the effective flow of sample vapours in the closed testing system (see Figure

1;Appendix 1). The MIC values were determined as the lowest concentrations that inhibited bacterial growth compared with the compound-free control and are expressed in µg/mL. In the case of the vapor phase, the concentration was also expressed in µg/cm3 as the weight of the volatile agent per volume unit of a microtube. DMSO, assayed as the negative control, did not inhibit any of the strains at the tested concentration (≤1%). The respective susceptibilities of *H. influenzae*, *S. aureus*, *S. pneumoniae*, and *S. pyogenes* to ampicillin, oxacillin, amoxicillin, and tetracycline were checked as positive antibiotic controls (CLSI 2015). Testing of *C. citratus*, *C. scariosus* and *T. ammi* were performed as three independent experiments, each carried out in triplicate, and the results were presented as median/modal values. According to the widely accepted norm in MIC testing, the mode and median were used for the final value calculation when triplicate endpoints were within the two- and three-dilution ranges, respectively.

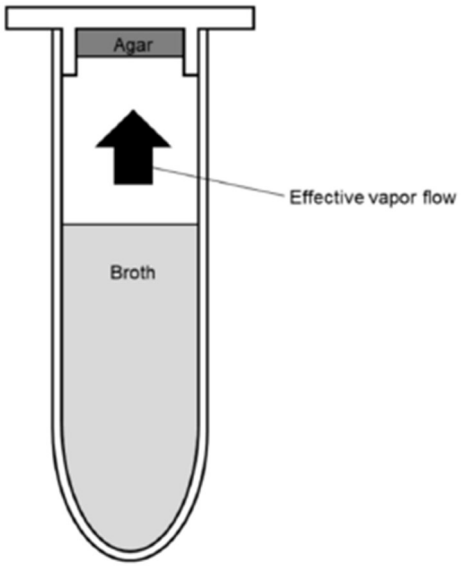


**Figure 1**: Detail of the cross-sectional view of the closed microtube with snap cap containing broth

## 3.5. Chemical analysis using GC-MS

For determination of the main components of *T. ammi*, GC-MS analysis was carried out using the gas chromatograph system Agilent GC-7890B 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 ionization detector (FID) coupled with single quadrupole mass selective detector Agilent MSD-5977B (Agilent Technologies, Santa

Clara, CA, USA). The operational parameters were as follows: helium as carrier gas at 1 mL/min, injector temperature 250 °C. The oven temperature was raised from 50 to 300°C. Samples of EOs diluted in n-hexane for GC/MS (Merck KGaA, Darmstadt, DE) at concentration 1 μg/mL and 1 μL of solution was injected in splitless mode. The mass detector was set to following conditions: ionization energy 70 eV, ion source temperature 200°C, scan time 1 s, mass range 30–600 m/z.

The identification of constituents was based on comparison of their retention indices (RI) and retention times (RT) with the National Institute of Standards and Technology Library ver. 2.0.f (NIST, USA), and literature (Adams 2007). The RI were calculated for compounds using the retention times of n-alkanes series ranging from C9 to C29 (Sigma-Aldrich, Prague, CZ). The relative percentage contents of EO components were determined by FID.

# 4. Results and Discussion

### Physical characteristics of EOs

In total, 4 EOs from different plant parts of 4 species were distilled. The highest yields of EO were obtained from *T. ammi* with 1% yield, second was *C. citratus* with 0.7%, and *C. scariosus* yield was 0.2%. These oils are commonly distilled for commercial uses and scientific research. In correspondence with our results *C. scariosus* oil yield varied from 0.2% to 0.58% (Kumar et al. 2016) and *C. citratus* yield was found to be 0.62% (Zhang et al. 2022). Higher amounts of EO usually possessing *T. ammi* which possess yield between 1.6 and 3.7% (Mazzara et al. 2021).

All oils have less density compared to water and very nice odour. In general, all the EOs were limpid and colourless after 20 minutes of distillation, however with a prolonged time the colour changed to some shade of yellow. *C. citratus* EO had very powerful, citrus scent and yellow colour, which is confirmed by another studies (Vázquez-Briones et al. 2015; Majewska et al. 2019). *C. scariosus* oil had woody-like smell with spicy tonnes of pepper, resembling cedarwood and pale-yellow colour (see Figure 9; Appendix 2). These results were also obtained by Kasana et al. and Clery et al. (Kasana et al. 2013; Clery et al. 2016). Fragrance of *T. ammi* was aromatic, spicy herbaceous scent resembling thyme. Unlike the others, the oil was transparent. This contradicts with other studies, which are reporting yellow-brownish colour (Zarshenas et al. 2013; Bhadra 2020).

**Table 1.** Characteristics of plant material

| Plant species | Family | Plant part | Essential oil yield % (v/w) | Essential oil colour |
|---|---|---|---|---|
| *Cymbopogon citratus* | Poaceae | leaves | 0.7 | yellow |
| *Cyperus scariosus* | Cyperaceae | roots | 0.2 | pale yellow |
| *Trachyspermum ammi* | Apiaceae | seeds | 1.0 | transparent |

### Evaluation of broth microdilution volatilisation method for EOs testing

Results of in vitro growth-inhibitory effect of plant EOs against *H. influenzae*, *S. aureus*, *S. pneumoniae*, *S.pyogenes* in liquid and vapour phase using the broth macrodilution volatilization method showed that differences in sample testing were always within the

range of three concentrations which corresponds to the CLSI standards of the MICs determination (CLSI 2015). The results were in agreement with the microdilution method (Hvězdová 2022). According to our results this method was confirmed to be suitable for rapid simultaneous determination of antibacterial potential of EOs in the liquid and the vapour phase at different concentrations.

**Antimicrobial effect of isolated EOs in liquid and vapour phase**

The detailed results on antibacterial activity of EOs from Indian medicinal plants in liquid and vapour phase are displayed in the Table 2. All three tested EOs possessed certain degree of antibacterial activity in broth and in agar as well. *H. influenzae* was the most susceptible strain among all bacteria tested. The highest antibacterial activity produced *T. ammi* against *H. influenzae*, with MIC 128 μg/mL in liquid and vapor phase. Moderate effect was produced by *C. citratus* EO which inhibited growth of *H. influenzae* with MIC 256 μg/mL in liquid and vapour phase. *C. scariosus* and *T. ammi* showed only weak inhibitory effect (MIC = 512 μg/mL) in both phases against *H. influenzae* and*S. aureus*, respectively. Similarly, *C. citratus* and *T. ammi* produced weak activity against *S. pneumoniae* and *S. pyogenes* in liquid phase (MIC 512 μg/mL). All other bacteria were resistant to EOs tested (MIC ≥ 1024 μg/mL).

Antibacterial activity of EOs tested in this study was previously described in several research papers. For example, *C. citratus* was reported to inhibit the growth of *S. aureus* (Lin et al. 2021), *S. pyogenes* (Sfeir et al. 2013), *S. pneumoniae* (Ntulume et al. 2019) and *H. influenzae* (Inouye et al. 2001). Moreover, its antimicrobial effect in vapour phase was reported against *Pseudomonas fluorescens* (Tyagi & Malik 2010), *Candida albicans* (Tyagi & Malik 2010). *H. influenzae, S. pyogenes, S. pneumoniae* and *S. aureus* (Inouye et al. 2001). Similarly, *T. ammi* was found to inhibit growth of *S. aureus* (Goudarzi et al. 2011, Hassanshahian et al. 2014, Hashemi et al. 2021) and *S. pneumonia*e (Gradinaru et al. 2018). In addition, its antibacterial effect in vapour phase was also proved against *S. pneumoniae S. pyogenes* (Srivastava *et al.* 1999).

Antimicrobial activity of *C. scariosus* was previously proved against certain bacterial species as as *Bacillus cereus* and *Pseudomonas aeruginosa* (Kasana et al. 2013). According to our best knowledge, this is the first report on in vitro growth-inhibitory effects C. scariosus EO against pneumonia causing bacteria in vapour phase.

**Table 2**. Antibacterial activity of essential oils from Indian medicinal plants in liquid and vapour phase

| Plant specie | Plant part | Bacteria/growth medium/MIC (µg/mL) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | ***Haemophilus influenzae ATCC 49247*** | | ***Staphylococcus aureus ATCC 29213*** | | ***Streptococcus pneumonie ATCC 49619*** | | ***Streptococcus pyogenes ATCC 19615*** | |
| | | **broth** | **agar** | **broth** | **agar** | **broth** | **agar** | **broth** | **agar** |
| *Cymbopogon citratus* | leaves | 256 | 256 | 256 | 1024 | 512 | 1024 | 512 | 1024 |
| *Cyperus scariosus* | roots | 512 | 512 | 1024 | 1024 | 1024 | >1024 | 1024 | >1024 |
| *Trachyspermum ammi* | seeds | 128 | 128 | 512 | 512 | 512 | 1024 | 512 | 1024 |
| **Positive antibiotic control** | | | | | | | | | |
| Amoxycilin | | - | - | - | - | 0.25 | >4 | - | - |
| Ampicilin | | 1 | >4 | - | - | - | - | - | - |
| Oxacilin | | - | - | 0.5 | >4 | - | - | - | - |
| Tetralicin | | - | - | - | - | - | - | 0.25 | >4 |

MIC: minimum inhibitory concentration; - : not tested

**GC-MC Analysis of *T. ammi***

Since EO of *T. ammi* produced strongest antimicrobial effect, its chemical composition was subsequently analysed using GC-MS. The detailed results of chemical analysis are shown in Table 3 and chromatogram in Figure 11. Total, 8 compounds were identified which represents 99.98 % of their total content. Thymol was the main component (55.07 %), followed by γ-terpinene (21.92 %), ρ-cymene (19.05 %), and β-pinene (2.64 %). Remaining four components (α- and β-thujene, β-myrcene and isoterpinolene) were present in less than 1 %. The chemical composition of EO from *T. ammi* has been previously described in various studies. Although previous studies are reporting higher numbers of components present in the EO [e.g. 20 (Jain et al. 2018) and 26 compounds (Gaba et al. 2018)], main compounds (thymol, γ-terpinene, ρ-cymene and β-pinene) reported in the literature (Delgado et al 2004) are also majority constituents of EO analysed in this study. In addition, many compounds reported in previous studies are accounting for less than 1 % of the total volume of *T. ammi* EO. According to literature data, antimicrobial activities are mostly attributable to the presence of phenolic compounds such as thymol (Dolovich et al. 2011). The differences in qualitative and quantitative compositions of our, and previously analysed, EOs can be caused by genetic and environmental factors including geographical origin, as well as by various methods used for their isolation and chemical characterization.

**Table 3.** Chemical composition of *T. ammi*

| Compound | RI | | Column/Content % | Identification |
|---|---|---|---|---|
| | Obs | Lit | HP-5MS | |
| α-Thujene | 914 | 931 | 0,35 | RI, GC/MS |
| β-Thujene | 920 | 920 | 0,24 | RI, GC/MS |
| β-Pinene | 964 | 980 | 2,64 | RI, GC/MS |
| β-Myrcene | 980 | 991 | 0,46 | RI, GC/MS |
| Isoterpinolene | 1006 | 1088 | 0,25 | RI, GC/MS |
| ρ-Cymene | 1016 | 1022 | 19,05 | RI, GC/MS |
| γ-Terpinene | 1050 | 1062 | 21,92 | RI, GC/MS |
| Thymol | 1315 | 1290 | 55,07 | RI, GC/MS |
| Total identified (%) | | | 99,98 | |

RI = retention indices. Obs. = retention indices determined relative to a homologous series of n-alkanes (C8–C40) on an HP-5MS column. Lit. = literature RI values (Adams 2007; Ghasem et al. 2007 ); Identification method: GC/MS = mass spectrum was identical to that of National Institute of Standards and Technology Library (ver. 2.0.f).

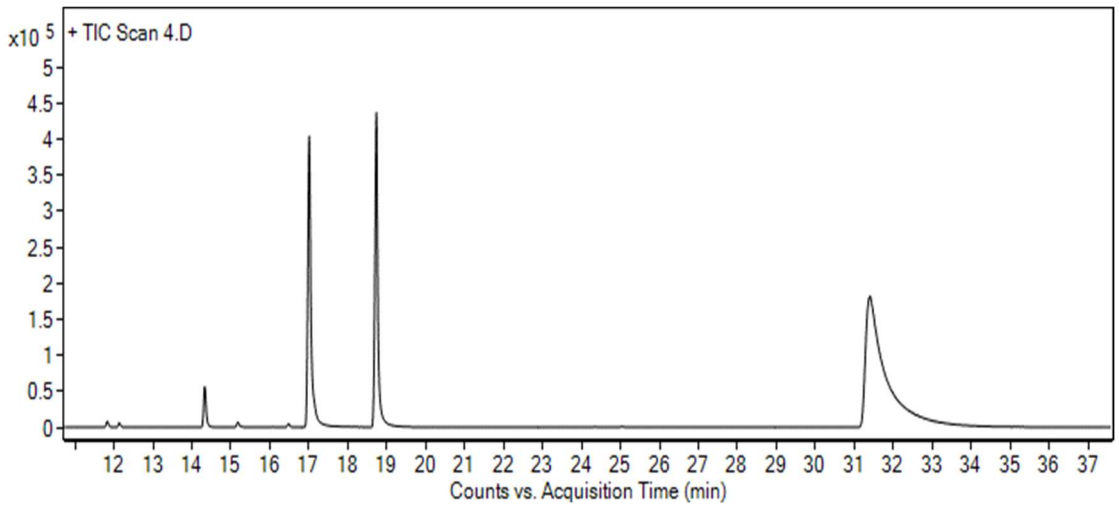


**Figure 11**. Chromatogram of *T. ammi* essential oil

# 5. Conclusions

In this study, antibacterial effects of 4 EOs from different plant parts of 4 species of Indian plants, namely *C. citratus*, *C. scariosus* and *T. ammi*, were distilled and tested against *H. influenze*, *S. aureus*, *S.pneumonie* and *S. pyogenes* using new broth macrodilution volatilization method. Among all samples assayed, *T. ammi* was found to be most effective antibacterial agent, whereas moderate or weak effects were observed for *C. citratus* and *C. scariosus*. To our best knowledge, EO from *C. scariosus* was tested in vapour phase for the first time. The results of GC-MS analysis showed that thymol was the main component of the most active EO from *T. ammi*. Our findings could serve as a theoretical background for future development of new pharmaceutical preparations that are based on volatile antimicrobials. However, further research focused on evaluation of their toxicity and antibacterial effects *in vivo* will be needed prior to its possible pharmacological application. In addition, broth macrodilution volatilization method was evaluated as a suitable method for evaluation of EOs antimicrobial effect, simultaneously in the liquid and the vapor phases at variable concentrations. This rapid, simple, cost- and labour- effective technique, which combines the principles of broth microdilution volatilization and standard broth macrodilution methods, is performed in commercially available microtubes and, therefore, does not require specialized equipment.

# 6. References


Adams RP. 2007. Identification of essential oil components by gas chromatography/mass spectrometry. Allured Publishing Corporation, Carol Stream.

Afzal S, Ahmad HI, Jabbar A, Tolba MM, Abouzid S, Irm N, Zulfiqar F, Iqbal MZ, Ahmad S, Aslam Z. 2021. Use of Medicinal Plants for Respiratory Diseases in Bahawalpur, Pakistan. BioMed Research International **2021**:1-10.

Amat S, Baines D, Alexander TW. 2017. A vapour phase assay for evaluating the antimicrobial activities of essential oils against bovine respiratory bacterial pathogens. Letters in Applied Microbiology **65**:489-495.

Ambrose PG, Bhavnani SM, Grosse EJE, Drusano GL. 2010. Pharmacokinetic-pharmacodynamic considerations in the design of hospital-acquired or ventilator-associated bacterial pneumonia studies: Look before you leap! Clinical Infectious Diseases **51**:103-110.

Ayrle H, Mevissen M, Kaske M, Nathues H, Gruetzner N, Melzig M, Walkenhorst M. 2016. Medicinal plants – prophylactic and therapeutic options for gastrointestinal and respiratory diseases in calves and piglets? A systematic review. BMC Veterinary Research **12**:89-119.

Bairwa R, Sodha RS, Rajawat BS. 2012. *Trachyspermum ammi*. Pharmacognosy Reviews **6**:56-60.

Bakkali F, Averbeck S, Averbeck D, Idaomar M. 2008. Biological effect of essential oils – A review. Food and Chemical Toxicology **46**:446-475.

Baser KHC, Buchbauer G. 2015. Handbook of EOs: science, technology, and applications. CRC Press, Boca Raton.

Berger R. 2007. Flavours and Fragrances Chemistry, Bioprocessing and Sustainability. Springer, Berlin.

Bhadra P. 2020. An Overview of Ajwain (*Trachyspermum ammi*). Indian Journal of Natural Sciences **10**:18466-2474.

Billroth T. 1877. Lectures on surgical pathology and therapeutics. The New Sydenham Society, London.

Bloom DE, Cadarette D. 2019. Infectious Disease Threats in the Twenty-First Century: Strengthening the Global Response. Frontiers in Immunology **10**:549.

Blowman K, Magalhaes M, Lemos MFL, Cabral C,Pires IM. 2018. Anticancer properties of essential oils and other natural products. Evidence-Based Complementary and Alternative Medicine **2018**:1-12.

Boskabady MH, Alizadeh M, Jahanbin B. 2007. Bronchodilatory Effect of *Carum copticum* in Airways of Asthmatic Patients. Therapies **62**:23-29.

Britannica. 2019. Ayurveda. Encyclopedia Britannica. Available from https://www.britannica.com/science/Ayurveda (accessed March 2022).

Buchholtz L. 1875. Antiseptika und Bakterien. Archiv experimental Pathologie und Pharmakologie **4**:1–82.

Burt S. 2004. Essential oils: their antibacterial properties and potential applications in foods - a review. International Journal of Food Microbiology **94:**223-253.

Chouhan S, Sharma K, Guleria S. 2017 Antimicrobial Activity of Some Essential Oils-Present Status and Future Perspectives. Medicines **4**:58.

CLSI. 2012. Performance Standards for Antimicrobial Disk Susceptibility Tests, Approved Standard, CLSI Document M02-A11. CLSI. Wayne.

CLSI. 2015. Performance Standards for Antimicrobial Susceptibility Testing; 25th Informational Supplement M100-S25. CLSI. Wayne.

Clery RA, Cason JRL, Zelenay V. 2016. Constituents of Cypriol Oil (*Cyperus scariosus* R.Br.): N-Containing Molecules and Key Aroma Components. Journal of Agricultural and Food Chemistry **64**:4566–4573.

Cowan MM. 1999. Plant products as antimicrobial agents. Clinical Microbiology Reviews **12**:564-582.

Davidse G. 2018. *Cyperus scariosus*. Missouri Botanical Garden. Available from http://www.worldfloraonline.org/image/120882 (accessed April 2022).

Delgado B, Fernández PS, Palop A, Periago PM. 2004. Effect of thymol and cymene on *Bacillus cereus* vegetative cells evaluated through the use of frequency distributions. Food Microbiology **21**:327-34.

Dolovich MB, Myrna B, Dhand R. 2011. Aerosol drug delivery: developments in device design and clinical use. Lancet **377**:1032–1045.

Duke JA, Ayensu ES. 1985. Medicinal plants of China. Reference Publications, Algonac.

Duke JA, duCellier JL. 1993. Page 536 in CRC handbook of alternative cash crops. CRC Press, Boca Raton.

EBSCO CAM Review Board. 2022. Ayurveda. Salem Press Encyclopedia of Health.

Ecorral. 2021. Cymbopogon citratus (DC.) Stapf. iNaturalist, California Academy of Science. Available from https://www.inaturalist.org/photos/137705388 (accessed April 2022).

Edwards-Jones V, Buck R, Shawcross SG, Dawson MM, Dunn K. 2004. The effect of essential oils on methicillin-resistant *Staphylococcus aureus* using a dressing model. Burns **30**:772–777.

Essawi T, Srour M. 2000. Screening of some Palestinian medicinal plants for antibacterial activity. Journal of Ethnopharmacology **70**:343-349.

European Pharmacopoeia. 2013. Publ. in accordance with the Convention on the Elaboration of a European Pharmacopoeia. European treaty series No. 50. Strasbourg.

Feyaerts AF, Mathe L, Luyten W, Tournu H, van Dyck K, Broekx L, van Dijck P. 2017. Assay and recommendations for the detection of vapour-phase mediated antimicrobial activities. Flavour and Fragrance Journal **32**:347-353.

Flume PA, VanDevanter DR. 2015. Clinical applications of pulmonary delivery of antibiotics. Advanced Drug Delivery Reviews **85**:1-6.

Oli B, Gautam D. 2021. Medicinal value of *Azadirachta indica*: A review. Modern Phytomorphology **15**:161-167.

Gaba J, Sharma S, Joshi S, Gill P. 2018. Gas Chromatography-Mass Spectrometric Analysis of Essential Oil, Nutritional and Phytochemical Composition of Ajwain Seeds (*Trachyspermum ammi*. L.). Journal of Essential Oil-Bearing Plants **21**:1128-1137.

Gaysinsky S, Weiss J. 2007. Aromatic and spice plants: Uses in food safety. Stewart Postharvest Review **4**:1-9.

Gandomi H, Abbaszadeh S, Jebellijavan A, Sharifzadeh A. 2013. Chemical Constituents, Antimicrobial and Antioxidative Effects of *Trachyspermum ammi* Essential Oil. Journal of food processing and preservation **38**:1690-1695.

Goudarzi GR, Saharkhiz MJ, Sattari M, Zomorodian K. 2011. Antibacterial Activity and Chemical Composition of Ajowan (*Carum copticum* Benth. & Hook) Essential Oil. Journal of Agriculture Science and Technology **13**:203-208.

Gowrishankar S, Muthumanickam S, Kamaladevi A, Karthika C, Jothi R, Boomi P, Maniazhagu D, Pandian SK. 2021. Promising phytochemicals of traditional Indian herbal steam inhalation therapy to combat COVID-19 – An *in-silico* study. Food and Chemical Toxicology 148: 0278-6915.

Gradinaru AC, Trifan A, Şpac A, Brebu M, Miron A. Aprotosoaie AC. 2018. Antibacterial activity of traditional spices against lower respiratory tract pathogens: combinatorial effects of *Trachyspermum ammi* essential oil with conventional antibiotics

Gupta SC, Kismali G, Aggarwal BB. 2013. Curcumin, a component of turmeric: from farm to pharmacy. Biofactors **39**:2–13.

Hale KA, Isaacs D. 2006. Antibiotics in childhood pneumonia. Pediatric Respiratory Reviews **7**:145-151.

Humber JM. 2002. The role of complementary and alternative medicine: Accommodating pluralism. Journal of the American Medicinal Association **288**:1655-6.

Harborne JB, Baxter H. 1993. Phytochemical dictionary: A handbook of Bioactive Compounds from Plants. Taylor & Francis, Washington DC.

Hashemi SMB, Jafarpour D, Gholamhosseinpour A. 2021. Antimicrobial activity of *Carum copticum* and *Satureja khuzestanica* essential oils and acetic acid in vapor phase at different relative humidities and temperatures in peanuts. Journal of food processing and preservation 46 (e16269) DOI: 10.1111/jfpp.16269.

Hassanshahian M, Bayat Z, Saeidi S, Shiri Y. 2014. Antimicrobial activity of *Trachyspermum ammi* essential oil against human bacterial. International Journal of Biomedical and Advance Research **2**:18–24.

Hoffman D. 2003. Medicinal herbalism: The science and practice of herbal medicine. Healing Arts Press, Vermont.

Hoge CW, Schwartz B, Talkington DF, Breiman RF, MacNeill EM, Englender SJ. 1993. The changing epidemiology of invasive group A streptococcal infections and the emergence of streptococcal toxic shock-like syndrome. Journal of the American Medical Association **269**:384–389.

Horvath G, Acs K. 2015. Essential oils in the treatment of respiratory tract diseases highlighting their role in bacterial infections and their anti-inflammatory action: a review. Flavour and Fragrance Journal **30**:331–341.

Hossen MJ, Uddin MB, Ahmed SSU, Yu ZL, Cho JY. 2016. Traditional medicine/plants for the treatment of reproductive disorders in Asia nations. Pakistan Veterinary Journal **36**:127-133.

Houdkova M, Rondevaldova J, Doskocil I, Kokoska, L. 2017. Evaluation of antimicrobial potential and toxicity of plant volatile compounds using new broth microdilution volatilization method and modified MTT assay. Fitoterapia **118**:56–62.

Houdkova M, Urbanova K, Doskocil I, Rondevaldova J, Novy P, Nguon S, Chrun R, Kokoska L. 2018. In vitro growth-inhibitory effect of Cambodian essential oils against pneumonia causing bacteria in liquid and vapour phase and their toxicity to lung fibroblasts. South African Journal of Botany **118**:85–97.

Houdkova M, Kokoska L. 2020. Volatile antimicrobial agents and in vitro methods for evaluating their activity in the vapour phase: A review. Planta Medica **86**:822–857.

Houdkova M, Chaure A, Doskocil I, Havlik J, Kokoska, L. 2021. New Broth Macrodilution Volatilization Method for Antibacterial Susceptibility Testing of Volatile Agents and Evaluation of Their Toxicity Using Modified MTT Assay. Molecules **26**:4179.

Huxley A, Griffiths M, Levy M. 1992. The new Royal Horticulture Society dictionary of gardening. Stockton Press, New York.

Hvězdová K. 2022. Antibacterial activity of essential oils from Indian medicinal plants in vapor phase [BSc. Thesis ]. Czech University of Life Sciences Prague, Prague.

Inouye S, Takizawa T, Yamaguchi H. 2001. Antibacterial activity of essential oils and their major constituents against respiratory tract pathogens by gaseous contact. Journal of Antimicrobial Chemotherapy **47**: 565–573.

Inouye S, Yamaguchi H, Takizawa T. 2001. Screening of the antibacterial effects of a variety of essential oils on respiratory tract pathogens, using a modified dilution assay method. Journal of Antimicrobial Chemotherapy **7**:251–254.

James PA, Lewis DA, Jordens JZ, Cribb J, Dawson SJ, Murray SA. 1996. The incidence and epidemiology of beta-lactam resistance in *Haemophilus influenzae*. Journal of Antimicrobial Chemotherapy **37**:737-746.

Jain N, Sharma M, Joshi SC, Kaushik U. 2018. Chemical Composition, Toxicity and Antidermatophytic Activity of Essential Oil of *Trachyspermum ammi*. Indian Journal of Pharmaceutical Sciences **80**:135-142.

Joseph B, Jini D. 2013. Antidiabetic effects of *Momordica charantia* (bitter melon) and its medicinal potency. Asian Pacific Journal of Tropical Disease **3**:93–102.

Kasana B, Sharma SK, Singh L, Sharmistha M, Singh T. 2013. *Cyperus scariosus*: a potential medicinal herb. International research journal of pharmacy **4**:17–20.

Kashyap VK, Peasah-Darkwah G, Dhasmana A, Jaggi M, Yallapu MM, Chauhan SC. *Withania somnifera* : Progress towards a Pharmaceutical Agent for Immunomodulation and Cancer Therapeutics. Pharmaceutics **14**:611.

Kim E, Oh CS, Koh SH, Kim HS, Kang KS, Park PS, Jang MJ, Lee HR, Park IK. 2016. Antifungal activities after vaporization of ajowan (*Trachyspermum ammi*) and allspice (*Pimenta dioica*) essential oils and blends of their constituents against three *Aspergillus* species, Journal of Essential Oil Research **28**:252-259.

Kumar A, Niranjan A, Lehri A, Srivastava RK, Tewari SK. 2016. Effect of Geographical Climatic Conditions on Yield, Chemical Composition and Carbon Isotope Composition of Nagarmotha (*Cyperus scariosus* R. Br.) Essential Oil. Journal of Essential Oil-Bearing Plants **19**:368-373.

Kumar G. 2011. *Trachyspermum ammi* (L.) sprague fruits. Available from https://www.researchgate.net/figure/Figure-1-Trachyspermum-ammi-L-Sprague-fuits_fig1_267860887 (accessed April 2022).

Kumar S, Dobos GJ, Rampp T. 2017. The Significance of Ayurvedic Medicinal Plants. Journal of Evidance - Based Complementary Alternative Medicine **22**:494-501.

Kuhnert P, Christensen H. 2008. Pasteurellaceae: Biology, Genomics and Molecular Aspects. Caister Academic Press, United Kingdom.

Kuzmov, A, Minko T. 2015. Nanotechnology approaches for inhalation treatment of lung diseases. Journal of Controlled Release **219**:500–518.

Lad V. 1987. Ayurveda, the Science of Self-Healing: A Practical Guide. Lotus Press, New Delhi.

Lin X, Rafique A, Fayyaz T, Bashir W, Luqman M, Zahid FM, Zhou K. 2021. Appraisal of *Cymbopogon Citratus* (Lemon Grass) for Antibacterial Activity Against Uropathogens. Pakistan Veterinary Journal **41**: 122–26.

Lopez P, Sanchez C, Batlle R, Nerin C. 2005. Solid- and vapour-phase antimicrobial activities of six essential oils: susceptibility of selected foodborne bacterial and fungal strains. Journal of Agriculture and Food Chemistry **53**:6939–6946.

Majewska E, Kozlowska M, Gruszczynska-Sekowska E, Kowalska D, Tarnowska K. 2019. Lemongrass (*Cymbopogon citratus*) essential oil: extraction, composition, bioactivity and uses for food preservation-a review. Polish Journal of Food and Nutrition Sciences **69**: 327-341.

Mazzara E, Scortichini S, Fiorini D, Maggi F, Petrelli R, Cappellacci L, Morgese G Morshedloo MR, Palmieri GF, Cespi M. Pharmaceuticals **14**:816.

Merchant Z, Buckton G, Taylor KMG, Stapleton P, Saleem IY, Zariwala MG, Somavarapu S. 2016. A new era of pulmonary delivery of nano-antimicrobial 103 therapeutics to treat chronic pulmonary infections. Current Pharmaceutical Design **22**:2577-2598.

Messier C, Epifano F, Genovese S, Grenier D. 2012. Licorice and its potential beneficial effects in common oro-dental diseases. Oral Diseases **18**:32-9.

Meulenbeld GJ. 1999. A History of Indian Medical Literature. Egberg Forsten, Groningen.

Murray PR, et al. 1999. Manual of clinical microbiology. ASM Press, Washington.

Murray PR, Rosenthal KS, Pfaller MA. 2020. Medical microbiology E-book. Elsevier Health Sciences.

Muthamma M, Purnima HD, Vishveshwaraiah KTP. 2008. Enhancement of digestive enzymatic activity by cumin (*Cuminum cyminum* L.) and role of spent cumin as a bionutrient. Food chemistry **110**:678-683

Nafees S, Rahman SZ, Amin KMY. Evaluation of anti-hypertensive activity of ancient Unani cardiovascular drug "Saad Kufi" *(Cyperus scariosus*) in adrenaline-induced hypertensive rats. Future Journal of Pharmaceutical Sciences **6**:1-6.

Naik MI, Fomda BA, Jaykumar E, Bhat JA. 2010. Antibacterial activity of lemongrass (*Cymbopogon citratus*) oil against some selected pathogenic bacteria. Asian Pacific Journal of Tropical Medicine **3**:535-8.

Newman DJ, Cragg GM. 2012. Review Natural products as sources of new drugs over the 30 years from 1981 to 2010. Journal of Natural Products **75**:311-35.

Ntulume I, Victoria N, Adam AS, Aliero AA. 2019. Evaluation of Antibacterial Activity of *Cymbopogon citratus* Ethanolic Leaf Crude Extract against *Streptococcus pneumoniae* isolated from Kampala International University Teaching Hospital Western Campus, Uganda. International Journal of Applied Sciences and Biotechnology **7**:31–38.

Palombo EA. 2011. Traditional medicinal plant extracts and natural products with activity against oral bacteria: Potential application in the prevention and treatment of oral diseases. Evidence-Based Complementary and Alternative Medicine **2011**:1-15.

Panda P, Valla S, Lakshmi MU, Harika, Bhadra P. 2020. An Overview of Ajwain (*Trachyspermum ammi*). Indian Journal of Natural Sciences **10**:976-997.

Örtqvist A, Hedlund J, Kalin M. 2005. *Streptococcus pneumoniae*: epidemiology, risk factors, and clinical features. American Journal of Respiratory and Critical Care Medicine **26**:563-74.

Procházka V. 1957. Základy parfumerie. Státní nakladatelství technické literatury, Praha.

Raut JS, Karuppayil SM. 2014. A status review on the medicinal properties of essential oils. Industrial Crops and Products **62**:250-264.

Ray B, Bhunia A. 2014. Fundamental food microbiology. CRC Press, Boca Raton.

Rehman SU, Choe K, Yoo HH. 2016. Review on traditional herbal medicine, *Eurycoma longifolia* Jack (Tongkat Ali): Its traditional uses, chemistry, evidence-based pharmacology and toxicology. Molecules **21**:331-361.

Reyes-Jurado F, Franco-Vega A, Ramirez-Corona N, Palou E, Lopez-Malo A. 2015. Essential oils: Antimicrobial activities, extraction methods, and their modeling. Food Engineering Reviews **7**:275-297.

Restrepo MI, Chalmers JD, Song Y, Mallow Ch, Hewlett J, Maldonado F, Yarmus L. 2017. Year in review 2016: Respiratory infections, acute respiratory distress syndrome, pleural diseases, lung cancer and interventional pulmonology. Respirology **22**:602-611.

Rios JL. 2016. Page 3-10 in Essential Oils in Food Preservation, Flavor and Safety. Academic Press, London.

Rungelrath V, DeLeo FR. 2021. *Staphylococcus aureus*, Antibiotic Resistance, and the Interaction with Human Neutrophils. Antioxidants & redox signaling **34**:452–470.

Sawangsri P, Mongkol R, Ahebwa A, Kanjanamaneesathian M. 2020. Vapour-phase efficacy of selected essential oils individually and in combination against *Aspergillus flavus*, *A. niger*, *Fusarium proliferatum*, and *Curvularia lunata*. New Zealand Plant Protection. **73**:40-48.

Sazawal S, Black RE. 2003. Effect of pneumonia case management on mortality in neonates, infants, and preschool children: meta-analysis of community-based trials. Lancet Infectious Diseases **3**:547-556.

ScanTree SAS. 2022. *Cyperus scariosus.* Available from https://www.scentree.co/en/Cypriol_EO.html (accessed April 2022).

Sekiyama Y, Mizukami Y, Takada A, Oosono M, Nishimura T. 1996. Effect of mustard extract vapour on fungi and spore-forming bacteria. Journal of Antibacterial and Antifungal Agents **24**: 171–178.

Sengupta P. 2012. Health Impacts of Yoga and Pranayama: A State-of-the-Art Review. International journal of preventive medicine, **3**:444-458.

Sfeir J, Lefrançois C, Baudoux D, Derbré S, Licznar P. 2013. *In Vitro* Antibacterial Activity of Essential Oils against *Streptococcus pyogenes*. Evidence-Based Complementary and Alternative Medicine **2013**:269161.

Sharifzadeh A, Khosravi AR, Shokri H, Sharafi G. 2015. Antifungal effect of *Trachyspermum ammi* against susceptible and fluconazole-resistant strains of *Candida albicans*. Journal de Mycologie Médicale **25**:143-150.

Sheposh R. 2019. *Pneumococcus.* Salem Press Encyclopedia of Health. Available from https://search.ebscohost.com/login.aspx?direct=true&db=ers&AN=89408024&lang=cs&site=eds-live (accessed February 2022).

Singh A, Aneja S. 2011. Pneumonia – management in the developing world. Paediatric Respiratory Reviews **12**:52-59.

Singh G. 2022. *Trachyspermum amii*. Available from https://www.flowersofindia.net/catalog/slides/Ajwain.html (accessed April 2022).

Singh R, Wilczyńska-Kwiatek A, Fedacko, JP, Pella D, De Meester F. 2009. Pranayama: The power of breath. International Journal on Disability and Human Development **8**:141-154.

Srivastava KC. 1988. Extract of a spice–omum (*Trachyspermum ammi*) shows antiaggregatory effects and alters arachidonic acid metabolism in human platelets. Prostaglandins, Leukotrienes & Essentiall Fatty Acids **33**:1-6.

Srivastava M, Saxena A, Baby P. 1999. GC-MS investigation and antimicrobial activity of the essential oil of *Carum copticum* Benth & Hook. Acta Alimentaria **28**:291-295.

Starr F, Starr K. 2008. Cymbopogon citratus (leaves). Wikimedia Commons. Available from https://commons.wikimedia.org/wiki/File:Starr_0806087551_Cymbopogon_citratus.jpg (accessed April 2022).

Stevens DL, Bisno AL, Chambers HF, Dellinger EP, Goldstein EJ, Gorbach SL. 2014. Practice guidelines for the diagnosis and management of skin and soft tissue infections: 2014 update by the Infectious Diseases Society of America. Clinical Infectious Diseases **59**:10-52.

Sudhakaran MV. 2017. Botanical Pharmacognosy of *Centella asiatica* (Linn.) Urban. Pharmacognosy Journal **9**:546–58.

Tyagi AK, Malik A. 2010. Antimicrobial action of essential oil vapours and negative air ions against *Pseudomonas fluorescens*. International Journal of Food Microbiology **143**:205-210.

Tyagi AK, Malik A. 2010. Liquid and vapour-phase antifungal activities of selected essential oils against *Candida albicans*: microscopic observations and chemical characterization of *Cymbopogon citratus*. Complementary and alternative medicine **10**:65.

Utreja D, Ekta SP. 2015. Chemistry and Biology of *Cyperus scariosus*: An Overview. Current Chemical Biology **9**:2–9.

Valíček P. 1989. Pages 216-302 in Užitkové rostliny tropů a subtropů. Academia, Praha.

Van de Braak SAAJ, Leijten GCJJ. 1999. Page 116 in Essential oils and oleoresins: A survey in the Netherlands and other major markets in European Union. CBI, Centre for the Promotion of Imports from Developing Countries, Netherlands.

Vázquez-Briones M, Hernández LR, Guerrero-Beltrán JÁ. 2015. Physicochemical and antioxidant properties of *Cymbopogon citratus* essential oil. Journal of food research **4**:36.

Von Gottberg, A,Klugman KP, Cohen C, Wolter N, De Gouveia L, Du Plessis M, Mpembe R, Quan V, Whitelaw A, Hoffmann R, Govender N, Meiring S, Smith AM, Schrag S. 2008. Emergence of levofloxacin-non-susceptible *Streptococcus pneumoniae* and treatment for multidrug-resistant tuberculosis in children in South Africa: a cohort observational surveillance study. The Lancet **371**: 1108–1113.

War AR, Paulraj MG, Ahmad T. 2012. Mechanisms of plant defense against insect herbivores. Plant Signaling & Behavior **7**:1306-1320.

Worldometer. 2021. Available from at: https://www.worldometers.info/coronavirus/ (accessed Januar 2022).

Wujastyk Dominik. 2003. Indian Medicine. Page 394 in Gavin D, editor. The Blackwell Companion to Hinduism. Blackwell, Oxford.

Yang C, Hu DH, Feng Y. 2015. Antibacterial activity and mode of action of the *Artemisia capillaris* essential oil and its constituents against respiratory tract infection-causing pathogens. Molecular Medicine Reports **11**:2852–2860.

Hwang YY, Ho YS. Nutraceutical support for respiratory diseases. 2018. Food Science and Human Wellness **7**:205-208.

Zink DL. 1997. The impact of consumer demands and trends on food processing. Emerging Infectious Diseases **3**:467–469.

Wenzler E, Fraidenburg DR, Scardina T, Danziger LH. 2016. Inhaled antibiotics for Gram-negative respiratory infections. Clinical Microbiology Reviews **29**:581–632.

WHO. 2001. Legal Status of Traditional Medicine and Complementary/Alternative Medicine: A Worldwide Review. Available from https://web.archive.org/web/20090831034622/http://apps.who.int/medicinedocs/en/d/Jh2943e/8.4.html (accessed 2022).

WHO. 2010. Benchmarks for training in traditional /complementary and alternative medicine: benchmarks for training in Ayurveda. WHO Library Cataloguing-in-Publication Data. Available from https://apps.who.int/iris/bitstream/handle/10665/44352/9789241599627_eng.pdf (accessed January 2022).

WHO. 2015. World Health Statistics 2015. WHO Library Cataloguing-in-Publication Data. Available from https://www.who.int/docs/defaultsource/ gho-documents/world-health-statistic-reports/world-health-statistics 2015.pdf?sfvrsn=afb0629f_2 (accessed January 2022).

WHO. 2017. WHO publishes list of bacteria for which new antibiotics are urgently needed. Available from https://www.who.int/news/item/27-02-2017-who-publishes-list-of-bacteria-for-which-new-antibiotics-are-urgently-needed (accessed April 2022).

WHO. 2019. WHO Global report on traditional and complementary medicine. Available from https://www.who.int/traditional-complementary-integrative-medicine/WhoGlobalReportOnTraditionalAndComplementaryMedicine2019.pdf (accessed March 2022).

Yarnell E. 2018. Herbs for Viral Respiratory Infections. Alternative and Complementary Therapies **24**:35-43.

Zarshenas MM, Moein M, Samani SM, Petramfar P. 2013. An overview on ajwain (*Trachyspermum ammi*) pharmacological effects; modern and traditional. Journal of natural Remedies **14**:98-105.

Zhang X, Zhu H, Wang J, Li F, Wang J, Ma X, Li J, Huang Y, Liu Z, Zhang L, Li S. 2022. Anti-microbial activity of citronella (*Cymbopogon citratus*) essential oil separation by ultrasound assisted ohmic heating hydrodistillation. Industrial Crops & Products **176**:114299.

# Appendix 1. Photographic illustrations of selected plant species

**Figure 2**. *C. citratus*
Source: Ecorral 2021

**Figure 3**. *C. citratus* leaves
Source: Starr & Starr 2008

**Figure 4**. *C. scariosus*
Source: ScanTree SAS 2022

**Figure 5**. *C. scariosus* roots
Source: Davidse 2018

**Figure 6**. *T. ammi*
Source: Singh 2022

**Figure 7**. *T. ammi* seeds
Source: Kumar 2011

# Appendix 2: Photographic illustrations of distillation of EO and of antimicrobial assay

**Figure 8**. Clevenger-type apparatus

**Figure 9**. *C. scariosus* EO

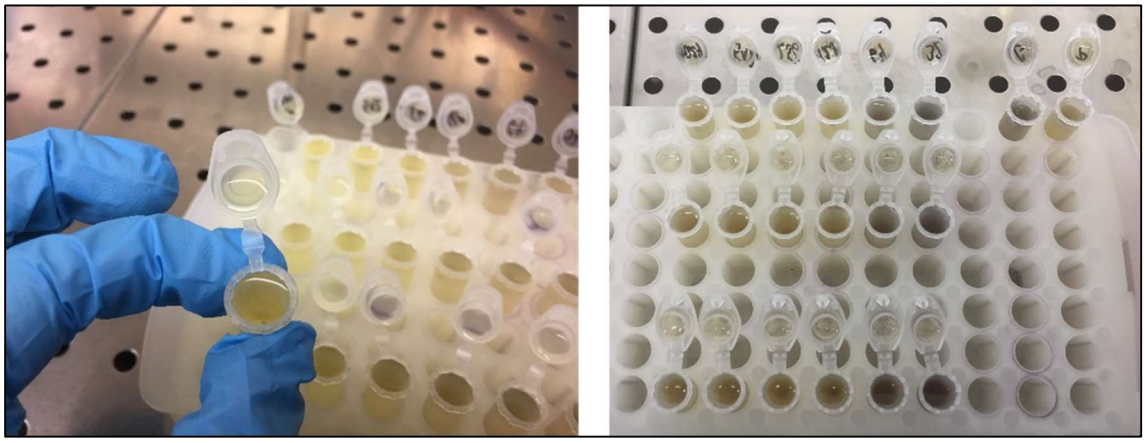

**Figure 10**. Broth macrodilution volatilization method MIC determination: the results are evaluated visually after coloring of living bacterial colonies with MTT dye