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Antibacterial activity of essential oils from Indian medicinal plants in vapor phase

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Declaration

I, Karolína Hvězdová, hereby declare that I have done this thesis entitled “Antibacterial activity of essential oils from Indian medicinal plants in vapor phase” 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, 15th April 2022

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Karolína Hvězdová

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Abstract

Pathogenic bacteria, such as *Haemophilus influenzae, Staphylococcus aureus*, *Streptococcus pneumoniae,* and *Streptococcus pyogenes*, can cause severe respiratory diseases. Inhalation therapy based on the administration of antibacterial agents directly to the site of infection might be one of the possible treatments for respiratory tract infections. The antibacterial activity can be exhibited by essential oils (EOs), which are produced by the plants as secondary metabolites. The objective of this thesis was to evaluate the antimicrobial activity of EOs obtained from the Indian medicinal plants, namely *Cinnamomum camphora, Cymbopogon citratus, Cyperus scariosus, Eucalyptus globulus, Ocimum gratissimum, Piper longum, Psidium guajava, Trachyspermum ammi*, and *Syzygium cumini* against potentially pathogenic microorganisms of the respiratory tract (*H. influenzae, S. aureus, S. pneumoniae, S. pyogenes*). The EOs were obtained by hydrodistillation, then the antimicrobial activity was assayed using the broth microdilution volatilization method. Minimum inhibitory concentrations (MICs) were determined for both liquid and vapor phases. The results showed that out of the 9 EOs tested only 3 produced positive effects against at least one of the bacteria strains in liquid or vapor phases. *Trachyspermum ammi* showed the highest growth-inhibitory effect against *H. influenzae* with MICs from 128 µg/mL to 256 µg/mL in the liquid and vapor phase, respectively. *Cymbopogon citratus* was the second most effective EO with MIC 256 µg/mL in both phases. *S. aureus* was slightly susceptible to all 3 EOs with MICs ≥ 256 µg/mL. *S. pneumoniae* and *S. pyogenes* were resistant to EOs tested with MIC values > 512 µg/mL. EOs have relatively low efficacy and therefore their application in practice is quite limited. However, these findings might be improved with further investigation. The plant volatile agents could also be tested for other microorganisms.

Keywords: Antibacterial activity, essential oil, Indian medicinal plants, vapor

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List of the abbreviations used in the thesis

ATCC	American Type Culture Collection
BC	Before Christ
COPD	Chronic Obstructive Pulmonary Disease
DMSO	Dimethyl sulfoxide
EO	Essential Oil
HD	Hydrodistillation
MIC	Minimum Inhibited Concentration
MRSA	Methicillin-Resistant *Streptococcus Aureus*
MTT	Thiazolyl Blue Tetrazolium Bromide Dye
WHO	World Health Organization

1. Introduction

Infections of the respiratory tract caused by pathogenic bacteria are one of the most serious diseases causing death. In addition, lower respiratory tract infections affect millions of people annually, especially in low-income countries (Horváth & Ács 2015). For example, pneumonia is considered a major infectious cause of morbidity and mortality worldwide (Loebinger & Wilson 2012). Synthetic drugs are the most common treatment for respiratory infections. However, the negative effects of these drugs are becoming more widely known and therefore the treatment with natural remedies could be an option to avoid side effects. This is well-known in Indian traditional medicine, where medicinal plants have been used to treat infectious diseases since ancient times (Verma & Singh 2008).

Plants are producers of a wide spectrum of secondary metabolites that have the ability to protect plants against predators and pathogenic microorganisms through biocidal properties (Bassolé & Juliani 2012). Essential oils, as secondary metabolites, release volatile agents that contain a wide range of properties, including antibacterial effects (Burt 2004). In addition, EO vapors are never in direct contact with the microorganisms, thus their efficacy is influenced by the volatility of the chemical components contained in the EO (Reyes-Jurado et al. 2020). The antimicrobial activity of EOs is mostly found in oxygenated terpenoids and hydrocarbons. Furthermore, the interaction between the EOs can cause antagonistic, additive, or synergistic effects (Bassolé & Juliani 2012). Besides, the activity of a volatile compound depends on several factors such as the extraction method of EOs and the susceptibility of the bacteria (Reyes-Jurado et al. 2015; Pandey et al. 2017).

For simple and rapid determination of the antibacterial activity of volatile plant compounds in the liquid and vapor phase, a new screening method based on broth microdilution volatilization was proposed. This method could be used in the development of new medications for inhalation therapy to treat respiratory diseases (Houdkova et al. 2021). However, evaluation of the antimicrobial activity of EOs and volatile compounds in the vapor phase is still challenging because there is not a standardized method yet.

2. Literature Review

2.1. Essential oils

Essential oils are natural plant products extracted from various aromatic plants generally localized from temperate to tropical area. They represent an essential part of traditional pharmacology. Aromatic plants, which contain EOs, have been used since ancient times for disease prevention and treatment (Shaaban et al. 2012). Furthermore, it is the main medicine used in developing countries and its usage has increased there.

Nowadays it is estimated that over 3,000 essential oils are known and around 300 are commercially important (Bassolé & Juliani 2012). They have therapeutic uses in human medicine due to their antiviral, antioxidant, anticancerogenic, and antinociceptive qualities (Baser & Buchbauer 2010). Moreover, the EOs are known for their antifungal, antibacterial or insecticidal activities and they also participate a role in plant defence. In medical practice in Asia, they are widely applied, but in Western medicine are currently used in order to improve the taste of drugs and as household preparations such as disinfectants, against colds, and in liniments against muscle pains (Kokoska 2003). Furthermore, they are used in many sectors such as aromatherapy, cosmetics, and perfume industries where they are used for their specific aroma (Bakkali et al. 2008). Pharmaceutical and agronomic industries use them for antibacterial activity. In general, essential oils are important also for foodstuff, sanitary, and dentistry as additives or natural remedies (Bakkali et al. 2008). For example, lemongrass oil is an important aroma product, whereas the leaves and leaf bases have a culinary use in Indonesia and elsewhere, citrus oils are indispensable for perfumery. We can also use them for flavouring soft drinks alongside rose petals which are the source of one of the most important and costly EOs (Kokoska 2003). In this bachelor thesis, the EOs have been researched for human purposes, but they can also be used for animals.

2.1.1. Botany

At present, it is known about 60 families of plants producing EOs. The most famous and economically important families are Myrtaceae (e.g., *Eucalyptus* and *Corymbia* genera), Oleaceae (e.g., *Jasminum* spp.), Pinaceae (e.g., *Cedrus* and *Pinus* genera), Rosaceae (e.g., *Rosa* spp.), Rutaceae (e.g., *Citrus* spp.), Lauraceae (e.g., *Litsea* spp.), Poaceae (e.g., *Cymbopogon* spp.), Lamiaceae (e.g., *Mentha* spp.), also Apiaceae (e.g., *Anethum* spp.), Santalaceae (e.g., *Santalum* spp.) and Zingiberaceae (e.g., *Zingiber* spp.) (Oyen & Dung 1999). One family famous for its scented flowers, but hardly mentioned here, is Orchidaceae. Furthermore, a few cryptogams yield EO, and the most important ones are the lichen *Evernia prunastri* (L.) and the seaweed *Fucus vesicilosus* (Kokoska 2003).

EOs can be synthesized by all plant organs such as leaves (e.g., *Cinnamomum camphora, Laurus nobilis*, *Pogostemon cablin*), seeds (e.g., *Elettaria cardamomum*, *Piper nigrum*, *Anethum graveolens*), fruits (e.g., *Citrus limon*, *Citrus reticulata*), roots (e.g., *Acorus calamus*, *Iris germanica*), wood (e.g., *Santalum album*, *Cedrus libani*, *Dalbergia nigra*), buds or flowers (e.g., *Lavandula angustifolia*, *Magnolia officinalis*, *Rosa chinensis*), twigs (e.g., *Eucalyptus globulus*, *Pinus nigra*), stems (e.g., *Etlingera elatior*, *Ruta graveolens*), bark (e.g., *Betula pendula, Cinnamomum cassia*) and are stored in secretory cavities, canals, secretory and epidermis cells or trichomes (glandular hairs) (Oyen & Dung 1999). The EO can be found in the space between the cell wall and the cuticula. For example, in Rutaceae and Myrtaceae, it is concentrated in large subepidermal glands arising from the specialized mother cells. The mother cell splits into daughter cells that separate from each other and disintegrate to leave a central cavity. The cells that surround the cavity produce EO to some amount, and the cavity enlarges as the surrounding cells walls break down (Kokoska 2003).

Plants produce essential oils for many purposes, for example in flowers, the fragrance may attract insect pollinators, in fruits animals distribute the seed, while in leaves the essential oil may have function as an insect repellent and in wood as a preservative (Oyen & Dung 1999). Also, different parts of the same plant can contain volatile oils of different chemical compositions (Oyen & Dung 1999; Baser & Buchbauer 2010). In most cases, it is because of the phytochemical polymorphism (Baser & Buchbauer 2010).

2.1.2. Chemistry

EOs are volatile aromatic liquids that are obtained from plants as secondary metabolites, generally of lower density than water. They are usually odorous, hydrophobic, and colourless or derive from bright to dark yellow. EOs are lipophilic, soluble in organic solvents, and immiscible with water from which can be separated by decantation (Bakkali et al. 2008).

Plants produce their specific mixture of chemical constituents. The mixture can contain 20-60 constituents at fluctuating concentrations with two or three main compounds which are representing 20-70 % of the whole content (Bakkali et al. 2008). The main compounds occur in relatively high concentrations (20-95%) and other components are present in trace amounts (Shaaban et al. 2012). The constituents with high concentrations in EOs are for example methyl salicylate (90%) in *Gaultheria procumbens* oil, l-borneol (80.6%) in *Blumea balsamifera* leaf oil, citronellal (80.1%) in *Corymbia citriodora* oil, d-limonene (80%) in citrus peel oils, 1,8-cineole (50%) and borneol (81.8%) in *Cinnamomum camphora* oil, menthol (59%) in *Mentha piperita* oil, carvone (58%) in *Anethum graveolens* seed oil, α-/β-thujone (57%) and camphor (24%) in *Artemisia herba-alba* oil, α-phellandrene (36%) and limonene (31%) in *Anethum graveolens* leaf oil, carvacrol (30%) and thymol (27%) in *Origanum compactum* oil (Oyen & Dung 1999; Shaaban et al. 2012).

We can classify chemical compounds of EOs into two groups such as oxygenated derivates and hydrocarbons. Oxygenated derivates include alcohols, aldehydes, esters, ketones, and phenols. Hydrocarbon compounds can be also called terpenes because of their isoprene structure. Thus, terpenes are composed of several C_5 (5-carbon) base units known as isoprene and the number of carbons reaches from C_{10} to C_{40}. The main classes of terpenes are monoterpenes C_{10} and sesquiterpenes C_{15}. Monoterpenes can be acyclic (e.g., myrcene and ocimene), monocyclic (e.g., α-terpinene, γ-terpinene, limonene, α-phellandrene), bicyclic or there are even a few tricyclic monoterpenes (cyclopentene, tricyclene) (Bakkali et al. 2008). Technologically the most important bicyclic terpenes are α-pinene and β-pinene. Sesquiterpenes are compounds where it is impossible to generalize about their molecular structure (Kokoska 2003). There is another group called terpenoids which is generated by different biochemical modifications like rearrangement and oxidation of terpenes (Wani et al. 2021). In general, it means that they have a more

complex structure. Many EO constituents are derived from the isoprene molecule, which is also involved in the synthesis of important biological compounds like chlorophylls, gibberellins, carotenoids, and steroids. (Oyen & Dung 1999).

Extraction methods

EOs are obtained from plant material by several distinct extraction methods. The main processes used to produce EOs are distillation, expression, and solvent extraction (Kokoska 2003). It has been determined that some traditional techniques used for their extraction could cause losses of some volatiles, and degradation of unsaturated or ester compounds due to thermal or hydrolytic effects. Furthermore, extracted EOs are susceptible to degradation by several factors such as oxidation, hydration, light or heat (Kalemba & Kunicka 2003; Reyes-Jurado et al. 2015).

The use of solvent extraction may cause presence of toxic solvent residue. Moreover, it is generally more expensive and requires large factories than small-scale processing units. Expression is applied to obtain citrus peel oils. Many components of EOs from citrus fruits are delicate and significantly suffer from heat degradation when exposed to steam distillation, so 'cold expression' process is used. In addition, hydrodistillation (HD), steam distillation, and hydrodiffusion are the most important forms of distillation in order to produce EOs (Kokoska 2003). Steam distillation is considered to have less susceptibility to hydrolysis and presents higher yields than HD and hydrodiffusion. HD is faster but produces less oil and also has a high susceptibility to hydrolysis than the other two methods (Reyes-Jurado et al. 2015).

Analytical methods

For studying EOs, it is important to evaluate their chemical composition and identification of individual constituents, which may also be challenging task. There are many compounds present in each EO, most of them are present in minor quantities, thus requiring methods with low detection limits. The chemical analysis of the compounds can be achieved by gas chromatography, high-performance liquid chromatography, mass spectrometry, and nuclear magnetic resonance (Ribeiro-Santos et al. 2018). The most used technique for identifying and quantifying EO compounds is gas chromatography connected with mass spectrometry. Gas chromatography-mass spectrometry is one of the most suitable methods for volatile compounds analysis because it can achieve the highest

resolution of EOs. There are also some studies using liquid chromatography for EOs analysis (Nakatsu et al. 2000).

2.1.3. Antimicrobial activity

Nowadays, there are many studies published focusing on biologically active substances because the resistance of microorganisms against antimicrobial agents has rapidly increased. The antimicrobial activity primarily depends on the quality and quantity of EO and its components, which could be affected by several factors such as origin, cultivation, and extraction method. Dilution and diffusion methods are the most common methods for evaluating the antimicrobial activity of EOs *in vitro* (Reyes-Jurado et al. 2015).

It has been determined that EOs containing aldehydes or phenols, such as cinnamaldehyde, citral, carvacrol, eugenol or thymol as major components showed the highest antibacterial activity, followed by EOs containing terpene alcohols. Other EOs, containing ketones or esters (e.g., β-myrcene, α-thujone or geranyl acetate) have much weaker activity (Bassolé & Juliani 2012). Moreover, volatile oils containing terpene hydrocarbons are usually inactive (Dorman & Deans 2000; Inouye et al. 2001; Bassolé & Juliani 2012).

Many studies showed that EOs have antibacterial properties against a wide range of bacterial strains, such as *Bacillus cereus, Escherichia coli, Listeria monocytogenes*, and *Salmonella typhimurium, Shigella dysenteria, Staphylococcus aureus* (Shaaban et al. 2012). EOs obtained from *Cymbopogon citratus, Nigella sativa*, and *Pulicaria undulata* inhibit the growth of *Bacillus subtilis, Pseudomonas aeruginosa* and *S. aureus* (Khan 1999; Pandey et al. 2017). Moreover, it was indicated that 30 out of 60 EOs exhibited strong inhibitory activity against *Helicobacter pylori,* which is associated with severe gastritis and increased incidence of peptic ulcers (Kelly 1998; Shaaban et al. 2012). In addition, other bacterial pathogens such as *H. influenzae*, *S. pneumonia*, and *S. pyogenes* were inhibited by *Eucalyptus odorata* EO under *in vitro* conditions (Pandey et al. 2017). The antimicrobial activity of a volatile compound depends mainly on the susceptibility of the bacteria. Generally, in most of the studies is reported, EOs are a bit more active against Gram-positive (e.g., *B. subtilis, S. aureus, Staphylococcus epidermis* A) than Gram-negative (e.g., *E. coli, P. aeruginosa, Klebsiella pneumoniae*) bacteria (Shelef et

al. 1984; Burt 2004; Pandey et al. 2017; Reyes-Jurado et al. 2020). Gram-negative bacteria's outer membrane contains hydrophilic lipopolysaccharides, which act as a barrier to macromolecules and hydrophobic compounds (Pandey et al. 2017; Reyes-Jurado et al. 2020). The antimicrobial effect of EO is usually quantified using minimum inhibitory concentration (MIC) values (Reyes-Jurado et al. 2020). The definition of MIC indicates that the lowest concentration of an EO prevents the visible growth of microorganisms under well-defined conditions (Mann & Markham 1998; Reyes-Jurado et al. 2020).

Vapor phase

In contrast to other antimicrobial agents, EOs have different physical and chemical properties such as poor water solubility and volatility. These properties can be well exploited in the gaseous phase. In many studies, it has been found that the antimicrobial activity of EOs in the vapor phase could be useful for treating diseases of the respiratory tract. The advantage of essential oils' volatility is that they can treat large areas or products without requiring direct contact with the surface (Tyagi & Malik 2010). Generally, in the vapor phase, EOs are never in direct contact with the microorganism being evaluated, therefore the effectiveness of these methods depends on the volatility of the chemical compounds present in EOs (Reyes-Jurado et al. 2020). For example, EOs of *Sinapis* spp. and *Syzygium aromaticum* showed clear differences in the amount of activity in the vapor phase compared to the same oils produced by the direct contact method. Furthermore, a combination of *S. aromaticum* and *Cinnamomum* spp. EOs showed higher antimicrobial activity in the vapor phase than in the liquid phase (Goñi et al. 2009; Shaaban et al. 2012).

Before we can practically use a new preparation based on the volatile agents, we need to get a detailed evaluation of their efficiency and safety. In general, *in vitro* screening is the first step in the process (Houdkova & Kokoska 2020). Disc-diffusion, broth microdilution, and drop-agar-diffusion are the most common methods used for screening, and contact techniques in agar (Tyagi & Malik 2010). In the vapor phase is not a standardised method for studying the antimicrobial activity of EOs (Oyen & Dung 1999). However, among *in vitro* methods, the most appropriate way how to test the vapor activity of EOs is the vapor phase test (Horváth & Ács 2015). The test respects the high volatility, hydrophobicity, and viscosity of vapor agents (Houdkova & Kokoska 2020).

Volatility causes a risk of substance losses due to evaporation during sample handling, experiment preparation, and time and temperature associated with incubation (Kalemba & Kunicka 2003; Rondevaldova et al. 2017; Houdkova & Kokoska 2020). One of the factors influencing how quickly and intensely the vapours evaporate into the atmosphere is the type of matrix onto which they are applied (e.g., paper disc, cultivation broth). For example, when the compound was mixed into the broth, less evaporation was observed (Orchard & van Vuuren 2017).

2.2. Bacterial respiratory diseases

These diseases are pathological conditions affecting organs and tissues that worsen breathing. The respiratory system is divided into upper and lower respiratory tracts where the upper tract includes the epiglottis and surrounding tissues, larynx, nasal cavity, throat, and the lower tract includes the trachea, bronchi, and bronchioles. Moreover, infections of the upper respiratory tract may spread and become more serious because the mucosa is continuous with the lining of the sinuses, middle ear, Eustachian tube, and lower tract. Most of these infections are primarily caused by viruses, but bacteria can be involved as well (Horváth & Ács 2015). Infections of the lower tract are more frequent and more severe in indigenous people and there is a higher incidence of pneumonia, bronchiolitis and Chronic Obstructive Pulmonary Disease (COPD) (Basnayake et al. 2017). The occurrence of these illnesses is often associated with lack of health care, poverty, behavioural factors, and the environment. The risks of these diseases are increased and influenced for example by malnutrition, lack of breastfeeding, HIV infection, use of poor cooking fuels in a household, bad hygiene habits, and air pollution (Schuchat & Dowell 2004). Respiratory diseases can be mild such as common cold and influenza or life-threatening such as pneumonia, acute asthma, lung cancer, pulmonary embolism, cystic fibrosis and much more (Horváth & Ács 2015).

2.2.1. Epidemiology

Respiratory diseases are one of the most substantial diseases causing death. Children under 5 years of age, immune-compromised people, the elderly and generally people living in low-income countries are the most at risk of respiratory diseases. The World Health Organization (WHO) reports that up to 3 million people worldwide die

from lower respiratory tract infections (Horváth & Ács 2015). Bacterial respiratory diseases such as pneumonia and bronchitis are the leading causes of morbidity and mortality in low-income countries. WHO also estimates that there are 450 million cases of pneumonia worldwide each year (Loebinger & Wilson 2012). Moreover, pneumonia is the major infectious cause of death in children around the world and is responsible for over two million child deaths annually. This disease affects children and families globally, but the most in South Asia and sub-Saharan Africa (World Health Organization 2021a). In addition, the most common cause of childhood pneumonia is pneumococcal pneumonia, especially in children under five years of age, and it also causes community-acquired pneumonia in adults, accounting for 10-30% of all cases (CDC 2021). Another serious disease is COPD which caused 3.2 million deaths in 2019 and over 80 % of the deaths occur in low- and middle-income countries. Furthermore, it was estimated that 235 million people suffer from asthma and more than 50 million people fight occupational lung diseases (World Health Organization 2021b).

2.2.2. Types of respiratory diseases

The form of illnesses can be infectious or chronic. Most of the pulmonary diseases are bacterial and viral, but they can be in a small percentage caused by another organism. In the viral type, a pathogen replicates inside a cell and this is lethal for the cell, thus causing disease (i.e., diarrhoea or respiratory tract diseases) (Payne 2017). Bacteria cause diseases by secreting or excreting toxins by producing toxins, which are released when the bacteria disintegrate, or by inducing sensitivity to their antigenic properties (Britannica & The Editors of Encyclopaedia 2018).

One of the most common infectious diseases in tropical areas is pneumonia which is a form of acute respiratory infection. It affects the lungs, where the target is the alveoli, basic structure unit of the lungs. If someone has pneumonia, the alveoli become clogged with fluid or pus, causing painful breathing and limits of oxygen intake to the lungs (World Health Organization 2021a). It can be caused by viruses or fungi, but mainly it is inflicted by pathogenic bacteria (e.g., *H. influenzae, Mycobacterium tuberculosis*, *P. aeruginosa, S. aureus, S. pneumoniae, S. pyogenes)* (van der Poll & Opal 2009; Weers 2015). Pneumonia can be spread in several ways. The bacteria and viruses are commonly found in a child's nose or throat and if they are inhaled, they can infect the lungs. They

can also spread through the air as droplets from a cough or sneeze. Furthermore, pneumonia may be spread in the bloodstream, especially during and shortly after birth. The main symptoms of this disease include cough, fever, chills, fatigue, fast and difficult breathing, and chest pain. Patients with this disease are treated with antibiotics, in most of the cases with oral antibiotics. Only for acute cases of pneumonia, hospitalization is needed (Basnayake et al. 2017).

2.2.3. Bacterial species

Haemophilus influenzae

This Gram-negative, facultatively anaerobic coccobacillus belongs to the Pesteurellaceae family. It was discovered in the sputum of several patients suffering from influenza virus infections by Richard Pfeiffer in 1892 (Kuhnert & Christensen 2008). Moreover, it is a common part of the upper respiratory tract, where the nasopharynx is considered a potential reservoir of infection from which this bacterium can spread to the lower respiratory tract. Although it has been recognized that this bacterium plays a role in lower respiratory tract inflammation, the interaction between *H. influenzae* and the lung is still not well defined (King 2012). The main virulence factor influencing strains of *H. influenzae* that cause invasive infections is the polysaccharide capsule (Shenoy 2016). Based on the polysaccharide capsule presence it can be classified as encapsulated form, which is divided into types a-f, with type b being the most dangerous and frequent. The less invasive is a non-encapsulated form that can create an inflammatory response thus causing a variety of symptoms in humans (St. Geme 2000).

H. influenzae spreads via airborne droplets and direct contact with secretions. In addition to most often caused pneumonia, it can also cause illnesses such as epiglottitis, meningitis, bloodstream infections, and arthritis (St. Geme 2000). Non-encapsulated strains are a major cause of morbidity and mortality in developed and low-income countries (Foxwell et al. 1998), and it is considered a major cause of chronic respiratory infections and pneumonia in adults. Pneumonia can be caused by both strains of *H. influenzae* (Slack 2015). Since *H. influenzae* produces β-lactamases and can modify its penicillin-binding proteins, it has developed resistance to the penicillin family of antibiotics (James et al. 1996). However, the implementation of *H. influenzae* b conjugate vaccines has reduced the incidence of invasive diseases, including pneumonia (Slack

2015; Shenoy 2016). People who are diagnosed with this bacterium are therefore treated with antibiotics, and in the case of severe infection, hospital care is necessary (NCIRD & DBD 2020).

Staphylococcus aureus

This is a Gram-positive, coccal bacterium with an aerobic to facultative anaerobic lifestyle, belonging to the Staphylococcaceae family (Reddy et al. 2017). *S. aureus* was discovered in 1880 by surgeon Alexander Ogston, who found it in pus from a surgical abscess (Fitzgerald et al. 2001). It is a common part of the human microflora and can be found on the skin, microbiome and in the upper respiratory tract, especially mucosal membranes in the nose (Kluytmans et al. 1997; Smith & Jarvis 1999). *S. aureus* can be also found inhabiting in most human environments. Moreover, recent studies suggested that the most common source of *S. aureus* is the pus, and it could be isolated even from sterile body fluids or urine specimens (Shrestha et al. 2021). In general, *S. aureus* acts as a commensal in its host, but if it enters host tissues through injuries, syringe inoculation, or direct implantation with medical devices, it reverts to a pathogenic lifestyle (Reddy et al. 2017).

This pathogen is a leading cause of bacterial infections worldwide. Moreover, *S. aureus* is considered one of the most serious human pathogens throughout history due to its potential for rapid acquisition of drug resistance (French 2010). It can infect probably any tissue in the human body (Daum 2008). For example, life-threatening infections include endocarditis, osteomyelitis, pneumonia, and meningitis (Smith & Jarvis 1999; Bhattacharyya et al. 2012; Reddy et al. 2017). *S. aureus* is considered an important cause of community-acquired pneumonia with the ability to infect otherwise healthy adults as well as children (Ragle et al. 2010). The severity of infection generally depends on the virulence factor and the individual's immunity. In addition, staphylococci can develop antimicrobial resistance (Casey et al. 2007). *S. aureus* can become resistant by mutation, conjugation, transduction, or transformation. However, for staphylococci, transduction and transformation are very rare methods for resistance acquisition (Sendi & Proctor 2009). For the treatment of infections have been used β-lactamases such as penicillin and methicillin. Although penicillin was the first antibiotic used to treat infections caused by *S. aureus*, later the resistance of the bacterium had spread around the world (Unni et al. 2021). Furthermore, *S. aureus* is also one of the leading pathogens for deaths associated

with antimicrobial resistance and the emergence of antibiotic-resistant strains such as methicillin-resistant *S. aureus* (MRSA) is a worldwide problem in clinical medicine (Kluytmans et al. 1997). In the beginning, the bacterial resistance to antimicrobial drugs was solved by the discovery of new classes of antibiotics, such as aminoglycosides, macrolides, and glycopeptides, and by the chemical modification of previously existing drugs (Gold & Moellering 1996). However, then it was discovered *S. aureus* can become resistant to all useful antibiotics and become multidrug-resistant (Gibbons 2004).

Streptococcus pneumoniae

This is a Gram-positive, facultatively anaerobic, and lancet-shaped bacterium from the genus *Streptococcus*. It was first isolated by Luis Pasteur in 1881 from the saliva of a patient with rabies (CDC 2021). Later, in the late 19th century it was indicated as a major cause of pneumonia (Ryan et al. 2004). Although these bacteria are mostly found in pairs called diplococci, they can also occur singly or in short chains (CDC 2021). The main virulence factor that influences *S. pneumoniae* strains is the polysaccharide capsule (Brown et al. 2015).

It spreads by direct contact between persons through droplets and by autoinoculation in persons that are carriers of the bacteria. It can become pathogenic, especially in elders and young children because of the weaker immune systems (CDC 2021). Moreover, it is the most identified bacterial pathogen in children and adults with community-acquired pneumonia (Slack 2015). Most pneumococcal infections are caused by a few serotypes of *S. pneumoniae*, with the range and prevalence of serotypes varying depending on the patient's age as well as geographical location. Pneumococcal infections can be invasive (e.g., pneumonia with bacteraemia and meningitis) or non-invasive, such as pneumonia without bacteraemia. Furthermore, pneumococcal pneumonia is estimated to cause over 150.000 hospitalizations in the United States each year, and it has been shown to complicate influenza infection (CDC 2021). One of the most powerful selection pressures supporting the pathogen’s evolution is the colonisation of the upper respiratory tract and subsequent transfer to a new host. Besides, HIV and influenza increase susceptibility to colonization by *S. pneumoniae*, increasing the severity of the disease and facilitating transmission of this pathogen to the host (Brown et al. 2015). The bacterium is characterized by pleuritic chest pain, acute onset, and high fever symptoms. Furthermore, by changes in penicillin-binding proteins, it has developed resistance to

penicillin. Therefore, for the treatment of diseases caused by *S. pneumoniae*, the most suitable and widely used antibiotic is amoxicillin (Loebinger & Wilson 2012). There is also a vaccine that can reduce the severity of this disease (Slack 2015).

Streptococcus pyogenes

S. pyogenes, also known as group A streptococci, is a Gram-positive bacterium from the genus *Streptococcus*. These pathogens are extracellular and aerotolerant. In general, they can colonize the throat, rectum, genital mucosa, and skin (Cunningham 2000). *S. pyogenes* spreads in several ways such as airborne droplets, skin contact, contact with objects or surfaces which are contaminated with bacteria, and contaminated food sources (Efstratiou & Lamagni 2016). Due to a wide scale of virulence factors, it can cause a various number of serious infections such as bacteraemia, meningitis, pneumonia, pharyngitis, scarlet fever, streptococcal toxic shock syndrome, and necrotizing fasciitis (Cunningham 2000). Furthermore, because of the new virulent strains, invasive infections have increased worldwide (Al-Khadidi et al. 2017). The infections can be caused in people of all age groups, but children are far more susceptible to it. Pneumonia caused by this pathogen is rare in adults, but more frequent in children, where it can cause lung parenchymal damage and, in some cases, purulent pleural effusion (Saldías et al. 2008). Acute viral respiratory infections, such as influenza, have been identified as a risk factor for *S. pyogenes* invasive infection. For the treatment of respiratory diseases, antibiotics are mainly used to reduce the transmission of this pathogen (Efstratiou & Lamagni 2016).

2.2.4. Treatment

Antibiotics

This type of treatment is generally considered as effective against most infective strains. However, there are several potential problems such as drug allergies, pathogen resistance, lack of penetration in lung tissues, and other undesirable effects. Moreover, this type of treatment is expensive for people living in low-income countries. Conventional recommended antibiotics for the treatment of lower respiratory infections are for example amoxicillin and clarithromycin against *S. pneumoniae*, fluoroquinolone and cephalosporin against *H. influenzae*, linezolid and teicoplanin for inhibiting *S. aureus* and MRSA (Loebinger & Wilson 2012). Furthermore, for treating non-severe pneumonia amoxicillin and co-trimoxazole are recommended. Ampicillin, benzylpenicillin,

ceftriaxone, gentamicin, and oxacillin are used for curing severe pneumonia (Hale & Isaacs 2006; Singh & Aneja 2011). To reduce fatal cases of pneumonia, antibiotics should be given as soon as possible after diagnosis (Loebinger & Wilson 2012).

Inhalation therapy

Inhalation therapy has been used for thousands of years and was practised mainly by ancient civilizations in Greece, Egypt, India, and China (McCaughey et al. 2012). One of the possible treatments could be the use of antibiotics by inhalation and moreover, it was found that the combination of two or more antibiotics agents can increase efficiency against respiratory diseases. It is for example a combination of fosfomycin and tobramycin, where the fosfomycin increases the activity of tobramycin against a wide range of bacteria (MacLeod et al. 2012).

This type of treatment is based on delivering antibacterial agents directly to the site of infection in the respiratory system. It maximises efficacy and at the same time restricts systemic exposure and associated toxicity (Weers 2015). There are several types of devices for delivering inhaled medicaments to the lungs (e.g., dry-powder inhalers, pressurized metered-dose, nebulizers), but they all have a few limitations, such as a short life due to poor bioavailability of bioactive agents at the target site, pulmonary clearance, enzymatic degradation, and fast systemic absorption (Li et al. 2010). Furthermore, the efficiency of the inhalation therapy may be affected by the deposition of the autolyzed particles in the oropharyngeal part and upper airways. In addition, deposition of medications in the lungs can be reduced because of the inappropriate size of its droplets, due to specific respiratory tract anatomy and proper operation, or because of the incorrect use of the device, mainly in children and elderly patients (Cole 2001; Garcia-Contreras et al. 2015). It is, therefore, necessary to look for new antimicrobial agents that inhibit bacteria affecting the respiratory tract and make it easier for these agents to reach the lower respiratory tract. Recently, because of the increasing resistance to conventional antibiotics, medicinal plants have become more considered a treatment option. The EOs have already been shown to be effective in overcoming the problems of microbial resistance (Chouhan et al. 2017).

There are many known components of EOs exhibiting antimicrobial activities that are already used in inhalation therapy. For example, linalool, one of the main components of *Lavandula hybrida* EO, was used for the treatment of menopausal disorders through

inhalation (Yamada et al. 1996). Thymoquinone and benzoquinone, which can be found in the seeds of *Nigella sativa*, have been noticed to produce relatively high antimicrobial activity in the vapor phase against pathogens causing pneumonia (Houdkova et al. 2017). Moreover, for example, β-thujaplicin isolated from the wood of the Cupressaceae species, and thymohydroquinone occurring in the Ranunculaceae and Lamiaceae families, have been observed antimicrobial effects, against bacteria that cause infections of the respiratory tract (Houdkova et al. 2021). Additionally, substances such as 1,8-cineole and α-pinene isolated from the leaves of *E. globulus* have positive effects on treating cough, cold and bronchitis. Likewise, the usage of *Melaleuca alternifolia* includes the treatment of respiratory infections, such as cold, influenza or bronchitis, and the therapeutic application of *Thymus vulgaris* oil includes the treatment of respiratory illnesses in general (Horváth & Ács 2015).

Traditional medicine

This medicine is also known as folk or indigenous medicine and has a long tradition in many parts of the world. Various traditional systems are used worldwide, such as Chinese, Korean, and Indian Ayurvedic medicines, Unani Medicine of Arab cultures and other indigenous medicines have been also developed in other continents by different cultures. The theory and application of these traditional therapy ways differ significantly from those well-developed allopathic drugs (Rehman et al. 2016; Yuan et al. 2016). Recently, because of the pathogen resistance against antibiotics, attention has been paid to the medicinal plants that are considered a source of secondary metabolites exhibiting biological activity, which can be effectively used for therapeutic treatment (Essawi & Srour 2000). For example, practices of traditional Chinese medicine are acupuncture, herbal remedies, moxibustion and tai chi (Manheimer et al. 2009). The herbs that are used most in this medicine are *Artemisia annua*, *Ephedra sinica*, *Panax ginseng* and *Rehum palmatum* (Gurib-Fakim 2006). Traditional Korean medicine has its roots in traditional Chinese medicine, but today it is an independent medical system (Cha et al. 2007). It has developed a type of acupuncture, named herbal acupuncture, where the practitioners use herbal extracts or bee venom, at specific acupoints (Patwardhan et al. 2015). However, the most ancient of all medicinal traditions is probably Indian traditional medicine which is considered to be the origin of systemized medicine (Gurib-Fakim 2006).

2.3. Indian traditional medicine

Traditional medicine in India is divided into six basic systems, of which Ayurveda is the most widespread and widely applied. The other systems include homoeopathy, naturopathy, Unani, Siddha, and yoga (Jaiswal et al. 2016). Although the origins of homoeopathy, naturopathy, and Unani medicine came from different parts of the earth, they were all introduced to the country centuries ago and thus incorporated into Indian culture, specifically Indian traditional medicine (Kang et al. 2017). In addition, out of a total 18,000 flowering plant species that grow in India, about 7,000 are used for traditional medicine purposes by all traditional systems (Jaiswal et al. 2016). Ayurveda is considered to be the oldest medicinal system which has developed around 1500 BC and over the centuries the three major schools have evolved, namely *Athreya sampradaya* (school of medicine), *Dhanwantari* or *Sushruta sampradaya* (school of surgery), and *Kashyapa sampradaya* (school of paediatrics and obstetrics) (Pole 2006; Kang et al. 2017). Furthermore, the *Charaka*, *Sushruta*, and *Ashtanga Hridayam* are the three most important ancient Ayurvedic texts which contain the original and complete knowledge of Ayurvedic world medicine. Although they are thought to be 1,200 years old, they are still used today (Tirtha 1998; Pole 2006).

The word Ayurveda is a combination of two Sanskrit words, namely 'Ayus' (life) and 'Veda' (knowledge or science), therefore it literally means the ‘science of life’. It is a holistic approach to life, health, and disease management based on medicinal herbs, minerals, diet, lifestyle, and spirituality (Mukherjee et al. 2012). Moreover, Ayurveda cures diseases, maintains human health, and promotes longevity, but most importantly it prevents diseases in general. In addition, it treats every single person as a combination of body, mind and soul and is thus widely respected for its uniqueness (Ravishankar & Shukla 2008; Mukherjee et al. 2012). Ayurvedic medicine uses mainly herbal and herbal-mineral preparations to treat all diseases, where the plant material can be used for different conditions and can acquire different activities. This activity depends also on the type of processing such as heating, cooking, or cooling (Patwardhan 2000).

2.3.1. Principles

Ayurveda believes that the universe is made of five elements, which include air, earth, fire, water, and space or ether. The elements are categorized into three basic humors of the human body known as *Doshas* or *Tridoshas*. These energies are called *Vata, Pitta*, and *Kapha* and they are considered to regulate the basic physiological and psychological processes of the body (Patwardhan et al. 2015; Jaiswal & Williams 2017). In general, *Vata* describes all biological processes controlled by the central and autonomous nervous system, *Pitta* helps the function of most thermogenic and metabolic processes, such as assimilation, blood pigmentation, digestion, endocrine gland activity and more. *Kapha* is involved in heat regulation and the production of various preservative fluids, and its main function is to provide nutrition to the body tissues (Mukherjee 2001). Each of the *Doshas* is divided into one or two more elements (Patwardhan et al. 2015). Besides, in case of any imbalance between them, the disease can occur, therefore practitioners maintain balance through meditation, diet, yoga, or herbal medications. Ayurvedic medicine also believes that the body produces three types of waste products named *Tri Malas*, including *Purisa* (faeces), *Mutra* (urine) and *Sveda* (sweat), and they have metabolic and digestion functions. According to Ayurveda, if the *Tridoshas* are not balanced, these waste products are not adequately removed which leads to further problems such as diarrhoea, asthma, arthritis, and others (Jaiswal & Williams 2017). Furthermore, Ayurvedic medicine recognizes six tastes, and each of them plays an important role in our physiology and health. Astringent, bitter, pungent, salty, sour, and sweet tastes can be combined in numerous ways, thus creating an incredible variety of flavours. Each of them has a predominant element, for example, the sweet taste is made from earth and water element (Pole 2006).

2.3.2. Herbal medicines

As people become more aware of the potency and side effects of synthetic drugs, there is a growing interest in natural product medicine with a basic approach to nature in the Western world. Over 1.5 million people practice traditional treatment, where they use medicinal plants in preventive, promotional, and curative applications (Verma & Singh 2008). Herbal medicines help to protect and increase the biodiversity of an ecosystem and they are an important part of the relationship between humans and nature. In India, the

majority of herbs come from the wild, therefore 90 % of herbal material used in Ayurveda comes from the local forests, mountains, and plains (Pole 2006). In addition, most of the Ayurvedic medicines are derived from natural sources such as plant roots, leaves, fruits, bark, and seeds. The advantage of Ayurvedic medicines compared to allopathic or other alternative medicine systems is that they have very few or no adverse effects and side effects. However, in the case of ayurvedic medicine, problems such as the slow onset of action and prolonged therapy time can occur (Suryawanshi et al. 2019).

The main forms in which herbs are administered are, for example, fresh juice from leaves of *Aloe vera*, herbal paste, where the dry material is usually mixed with water (e.g., *Eclipta alba*), herbal jams and jellies (e.g., *Withania somnifera*), decoction (*Psidium guajava*), and more (Pole 2006). For the treatment of steam inhalation, the species such as *T. vulgaris*, *Mentha* sp., *E. globulus,* and *S. aromaticum*, are often used. Among the Ayurvedic medicinal plants, the most famous is *Azadirachta indica, Centella asiatica, Cinnamomum camphora, Elettaria cardamomum, Rauwolfia serpentina, Terminalia* spp., and *Santalum album* (Jaiswal et al. 2016). In a recent study, it was indicated that for the treatment of respiratory diseases several plant species are traditionally used in the Uttarakhand state of India. For example, *Viola canescens* and *Zingiber officinale* are used for treating cough, cold and bronchitis. For treatment of pneumonia, *Ficus racemose, Nepeta glutinosa, Ricinus communis*, *T. chebula*, and *Vitex negundo* are recommended, whereas *Allium cepa, Ficus religiosa, Myrica esculenta,* and *Terminalia bellrica* are used as a treatment of asthma. *Origanum vulgare* and *V. negundo* are administered in case of influenza (Kala 2020). *Ocimum gratissimum* has been used in religious ceremonies and rituals in India and its EO is utilized for treating fever, inflammations of the throat, ears or eyes, and stomach pain (Oyen & Dung 1999). Furthermore, the whole plant of *Solanum xanthocarpum* showed an effect against asthma and related respiratory disorders (Ravishankar & Shukla 2008). However, in Ayurveda, as was mentioned before, different parts of the plants can show different effects. In the case of *Curcuma longa*, the whole plant can be used. For example, rhizomes can be useful for treating cold, inflammation, rheumatism, and respiratory diseases. In contrast, the leaves of *C. longa* are considered to have an antimicrobial effect and also reduce gas and bloating (Jaiswal et al. 2016).

2.3.3. Antimicrobial activity of Ayurvedic essential oils bearing plants

A number of EOs have antimicrobial properties due to the presence of components such as thymol, eugenol, 1,8 cineole, pinenes, linalool, and terpineol. However, the antimicrobial activity may vary because the relative concentration of the compounds differs from oil to oil (Sasidharan & Menon 2010). For example, EOs from *C. longa, Cinnamomum* sp., *Cymbopogon* sp., *Ocimum* sp., *Syzygium* sp., and *Vitex* sp. have well-known antimicrobial effects (Pandey et al. 2017). *C. citratus* which is originated probably in Malaysia is notorious for its very strong, fresh-grassy, herbaceous odour and wide usage in the kitchen. The major compound is citral which is a mixture of the geranial and neral, and other components contained in the EO are myrcene, limonene, and geraniol (Oyen & Dung 1999). It is a traditional remedy for coughs, malaria, and pneumonia, and the leaves exhibited antimicrobial activity against *S. aureus* (Ekpenyong et al. 2014). G-terpinene is one of the main compounds found in the volatile oil of *T. ammi,* which is a very popular aromatic plant in India (Baser & Buchbauer 2010), and the oil showed inhibitory effects against *E. coli, K. pneumoniae*, and *S. aureus* (Hassanshahian et al. 2014). The EO of *Momordica charantia* is primarily composed of benzimidazoles and oxygen derivative monoterpenoids and it demonstrated antimicrobial activity against *E. coli, P. aeruginosa, Klebsiella pneumoniae,* and *S. aureus* respectively (Ramalingam et al. 2020). Likewise, the *P. cablin* showed the highest activity against the same pathogens as *M. charantia* (Munda et al. 2019). Furthermore, the volatile compounds of *E. globulus* EO showed antibacterial activity against *S. aureus*, and the oil of *E. odorata* was evaluated to inhibit respiratory pathogens, including *H. influenzae, S. pneumonia*, and *S. pyogenes* (Pandey et al. 2017).

The "queen of Indian Ayurveda" is considered *Withania somnifera* from family Solanaceae which is commonly called Ashwagandha and is also known as "Indian Ginseng". The most used part is the root which has antioxidant activity. In addition, the presence of a root-dwelling bacterium in this medicinal plant was detected and its isolation was also identified as having antibacterial activity against pathogenic bacteria such as *P. aeruginosa* (Kumbukgolla et al. 2021). In Ayurveda, numerous Indian herbal components are used to cure common respiratory problems, protect the lungs from dangerous toxins and pollutants, enhance general health, and reduce symptoms of respiratory infections such as coughing, sneezing, and swollen lymph (Kanungo 2016;

Binu 2021). However, even though the use of medicinal plants for the treatment of respiratory diseases has a rich history in India, the antibacterial activity of these plants has not been extensively studied (Kala 2020).

3. Aims of the Thesis

The main aim of this work is to evaluate the *in vitro* growth-inhibitory activity of vapors of EOs from Indian medicinal plants against potentially pathogenic microorganisms of the respiratory tract.

The specific objectives were:

a) Hydrodistillation of EOs from 9 species used in traditional Indian medicine.
b) Determination of MICs of EOs against *Haemophilus influenzae, Staphylococcus aureus, Streptococcus pneumoniae,* and *Streptococcus pyogenes* using the broth microdilution volatilization method in the vapor and liquid phase.

4. Material and Methods

4.1. Plant material

Based on the traditional use in Ayurvedic medicine for the treatment of respiratory diseases, nine local plant species (*C. camphora, C. citratus, C. scariosus, E. globulus, O. grattissimum, P. longum, S. cumini, P. guajava*, and *T. ammi*) were selected. The dried plant material was ordered from Bhagyashree Herbal Farms, Raipur, Chhattisgarh, India. Plant family, weight of the dried material, ethnobotanical use, yield, and colour of extracted EOs were identified. A detailed description of collected plant samples is summarized in Table 1.

Table 1. Ethnobotanical data and physical characteristics of EOs from Indian medicinal plants

Plant species	Family	Plant part	Ethnobotanical use	Yield % (v/w) of EOs	Colour of EOs
Cinnamomum camphora	Lauraceae	Leaves	Bronchitis, inflammation (Hamidpour et al. 2013)	1.5	transparent
Cymbopogon citratus (DC.) Stapf	Poaceae	Leaves	Cough, fever, pneumonia, tuberculosis (D'Souza 2019)	0.7	yellow
Cyperus scariosus R.Br.	Cyperaceae	Roots	Bronchitis, inflammation, fever (Pole 2006)	0.2	pale yellow
Eucalyptus globulus	Myrtaceae	Leaves	Bronchitis, cough, cold (Khare 2007; Prashanth 2016)	1.4	yellow/green
Ocimum gratissimum L.	Lamiaceae	Leaves	Inflammations of the throat and ears, influenza (Oyen & Dung 1999)	1.6	pale yellow
Piper longum L.	Piperaceae	Fruits	Asthma, bronchitis, cough, fever (Ravishankar & Shukla 2008)	0.23	pale yellow
Syzygium cumini (L.) Skeels	Myrtaceae	Leaves	Asthma, bronchitis (Jadhav et al. 2009)	0.20	yellow
Psidium guajava L.	Myrtaceae	Leaves	Bronchitis, cough, tuberculosis (Morais-Braga et al. 2016)	3	pale yellow
Trachyspermum ammi (L.) Sprague	Apiaceae	Seeds	Asthma, cough, emphysema, nasal and sinus congestion (Pole 2006)	1	transparent

4.2. Distillation of essential oils

Dried plant material was grounded into powder by using a Grindomix apparatus (GM100 Retsch, Haan, Germany). 100 g of powdered sample was mixed with 1 L of distilled water. The EOs samples were subjected to hydrodistillation for 3 h by using Clevenger-type apparatus (Merci, Prague, Czech Republic) (Figure 9) according to the procedure described in the European Pharmacopeia (2010). After 3 hours, the EOs were collected and stored in sealed glass vials at 4°C (Figure 11).

4.3. Microorganisms 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.

4.4. Chemicals

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), thiazolyl blue tetrazolium bromide dye (MTT, CAS 298-93-1). All the chemicals were purchased from Sigma-Aldrich (Prague, Czech Republic).

4.5. Antimicrobial assay

The antibacterial activity of EOs in the vapor phase was determined by using a broth microdilution volatilization method (Figure 1,2). The experiments were performed in 96-well microtiter plates, covered by tight-fitting lids with flanges designed to reduce evaporation (SPL Life Sciences, Naechon-Myeon, Republic of Korea). At first, 30 µL of agar was pipetted into every flange on the lid, except the outermost flanges, and inoculated with 5 µL of bacterial suspension after agar solidification. In the second part of this method, each sample of EOs was dissolved in DMSO at a maximum concentration of 1% and diluted in an appropriate broth medium. Seven two-fold serially diluted concentrations of samples starting from 1024 µg/mL were prepared for all EOs. The plates were then inoculated with bacterial suspensions. The wells which were containing inoculated and non-inoculated broth were prepared as growth and purity controls simultaneously. The outermost flanges were left empty to prevent the edge effect. Finally, clamps (Lux Tool, Prague, CZ) were used for fastening the plate and lid together with the handmade wooden pads for better fixing (Figure 13). The microtiter plates were incubated at 37 °C for 24 h. The MICs were evaluated by visual assessment of bacterial growth after colouring of a metabolically active bacterial colony with thiazolyl blue tetrazolium bromide dye (MTT) at a concentration of 600 µg/mL, 25 µL in each well, when the interface of colour change from yellow and purple was recorded in broth and agar (Figure 14,15) (Houdkova et al. 2017). The values of MIC were determined as the lowest concentrations that inhibited bacterial growth compared with the compound-free control and expressed in µg/mL. All experiments were carried out in triplicate in three independent experiments and results were expressed as median/modal MICs values.

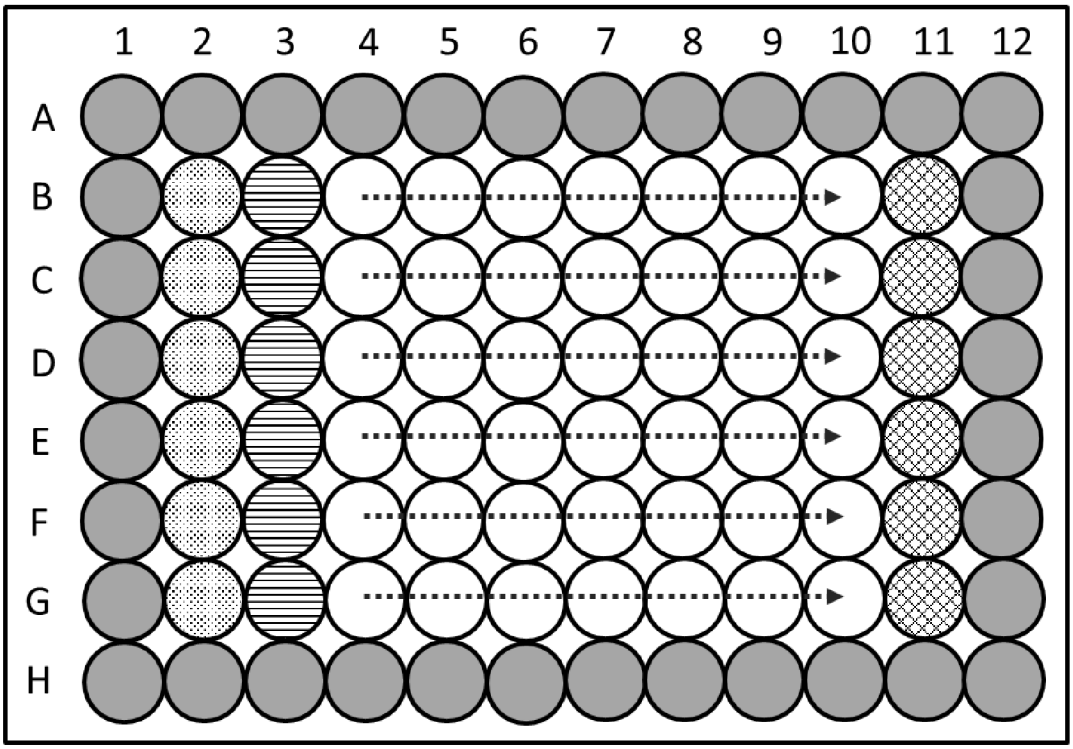

Figure 1. Schematic design of experiment: flat-bottom wells demonstrating: Grey-coloured wells: empty wells, not used (because of evaporation) ; dotted wells: purity control (0% growth of bacteria) ; striped wells: growth control (100% growth of bacteria) ; white-coloured wells: serial two-fold dilution of tested volatile compounds; gridded wells: serial two-fold dilution of positive antibiotic control (Houdkova et al. 2017).

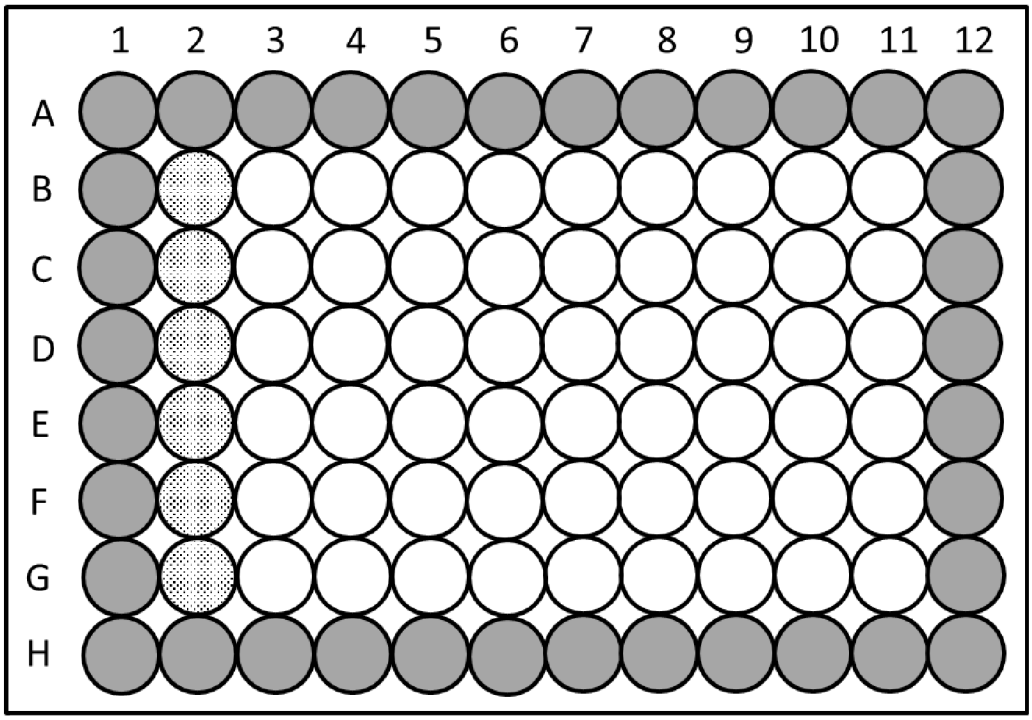

Figure 2. Schematic design of experiment: flanged lid demonstrating: grey-coloured wells: empty wells (not used) ; dotted wells: purity control (agar) ; white-coloured wells: agar and bacteria.

5. Results and Discussion

In this study, 9 EOs derived from different parts (leaves, fruits, roots, and seeds) of 9 species were obtained by hydrodistillation. Initially, all samples were screened for their *in vitro* growth-inhibitory effects against *H. influenzae* and *S. aureus.* Since EOs of *C. camphora* (leaves), *E. globulus* (leaves), *O. gratissimum* (leaves), *P. longum* (fruits), *S. cumini* (leaves), and *P. guajava* (leaves) did not produce significant antibacterial activity in both, the liquid and vapor phases, they were excluded from further research. Among all EOs tested, *C. citratus* (leaves), *C. scariosus* (roots) and *T. ammi* (seeds) exhibited a certain degree of antibacterial activity in the liquid and vapor phase against at least one bacterium associated with respiratory system infections. The detailed results of the *in vitro* growth-inhibitory effect of Indian medicinal plants active against bacterial strains tested are summarised in Table 2.

In contrast to other microorganisms, *H. influenzae* was the most susceptible to all three EOs tested (MICs ranging from 128 µg/mL to 512 µg/mL). EOs hydrodistilled from leaves of *C. citratus* and from seeds of *T. ammi* produced the highest inhibitory effect against *H. influenzae* with MICs 256 µg/mL in the vapor phase. In the liquid phase, *T. ammi* showed the highest activity with MIC 128 µg/mL, followed by *C. citratus* with MIC 256 µg/mL. *C. scariosus* demonstrated a moderate antibacterial effect with MIC 512 µg/mL in both phases. The growth of *S. aureus* was inhibited by all EOs tested within the range of concentrations from 256 µg/mL to 1024 µg/mL. *C. citratus* showed antimicrobial activity with MIC 256 µg/mL in the liquid phase, followed by *T. ammi* with MIC 512 µg/mL. *C. scariosus* produced only low antibacterial activity with MIC 1024 µg/mL in the liquid medium. In the vapor phase, the highest antistaphylococcal activity possessed *T. ammi* (MIC = 512 µg/mL). The low inhibitory effect with MIC 1024 µg/mL was shown by *C. citratus* and *C. scariosus* in the vapor phase. The moderate or weak activity was shown by all EOs tested against *S. pneumoniae* and *S. pyogenes* in liquid (MICs 512-1024 µg/mL) and vapor (MICs ≥ 1024 µg/mL) phase. *C. citratus* and *T. ammi* produced the strongest effect in the liquid and vapor phase with MIC values of 512 µg/mL and 1024 µg/mL, respectively. *C. scariosus* showed low antimicrobial activity against these two bacteria strains with MIC 1024 µg/mL in the liquid phase and no activity in the vapor phase. All bacterial strains tested were susceptible to positive antibiotic controls (amoxicillin, ampicillin, oxacillin, and tetracycline).

Several investigations have recently confirmed that the vapor phases of EOs are more efficient antimicrobials than their liquid phases (Inouye et al. 2001; Tyagi & Malik 2010). One of the reasons that the vapor phase is more effective than the liquid phase is because the lipophilic molecules in the liquid phase form micelles and so inhibit EO adhesion to the organism, whereas the vapor phase allows free attachment. However, in comparison to the amount of data for the efficacy of EOs in the liquid phase, the potential of EO vapors is relatively unexplored (Laird & Phillips 2012).

The antibacterial activity of lemongrass EO is attributed to the interaction between the major oil components such as geranial, neral, and myrcene, and the bacterial cell membrane (Majewska et al. 2019). According to Ács et al. (2018), EOs from leaves of *C. citratus* produced low MIC values against *H. influenzae* (MIC = 50 µg/mL), *S. pneumoniae* (MIC = 50 µg/mL) and *S. pyogenes* (MIC = 125 µg/mL) in the vapor phase and showed certain antibacterial activity in the liquid phase. The different results can be caused due to the use of different solvents for EO dilution or differences in the methodology of antimicrobial assays, where a disc volatilization method was used for activity determination in the vapor phase in that study. Inouye et al. (2001), reported that the EO of lemongrass produced a strong inhibitory effect against *H. influenzae* with MID 1.56 mg/L air in the gaseous phase. The author evaluated data at the minimum inhibitory dose which is required to inhibit the organism growth. The difference in the results compared to ours could be due to the difference in the measured values, as the vapor concentration was constantly changing during incubation in the study of Inouye et al. Only a few studies examined MIC in the liquid phase using the broth microdilution method which showed results of MIC 0.21 µg/mL (Majewska et al. 2019).

It is supposed, that the antibacterial effect of *T. ammi* could be due to the presence of thymol (Salimiraad et al. 2019). In a study by Paul et al. (2011), EO from the fruit of *T. ammi*, which was obtained by hydrodistillation has shown an antibacterial effect against *S. aureus* ATCC 6538 and KCTC 1916 in the liquid phase with the MICs 162.5 µg/mL and 175 µg/mL, respectively. In our experiments, we observed significantly higher MIC values. The distinction in the results achieved may be due to the different susceptibility of bacterial strains used. In correspondence to the study of Grădinaru et al (2018), who found that the strains of *S. pneumoniae* were sensitive to *T. ammi* EO (MIC = 0.25 µg/mL), our result also showed a substantial antibacterial effect related to this

respiratory pathogen. However, our results showed lower inhibitory activity than reported in the Grădinaru et al. Study of Kim et al. (2016) reported that there is less information on the antibacterial effect of *T. ammi* in the vapor phase, but they found that the EO of *T. ammi* has significant antifungal activity. According to our best knowledge, the growth-inhibitory activity of *T. ammi* in the vapor phase against pathogenic microorganisms of the respiratory tract was tested for the first time.

The main chemical components of *C. cyperus* EO, namely cyperene, longifolene, caryophyllene oxide and longiverbenone, could be responsible for its antibacterial activity (Kumar et al. 2016). Although it has been observed that *C. cyperus* has antibacterial and antifungal properties (Alam et al. 2011), there is no evidence on the antimicrobial activity of *C. cyperus* EO against pathogenic bacteria of the respiratory tract.

Table 2. Antibacterial activity of EOs from Indian medicinal plants against respiratory pathogens in liquid and vapor phase

Plant species	Plant part	Bacteria/growth medium/MIC (µg/mL)							
		Haemophilus influenzae 49247		***Staphylococcus aureus 29213***		***Streptococcus pyogenes 19615***		***Streptococcus pneumoniae 49619***	
		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	256	512	512	512	1024	512	1024
Positive Antibiotic control									
Amoxicillin		-	-	-	-	-	-	0.25	0.25
Ampicillin		1	1	-	-	-	-	-	-
Oxacillin		-	-	0.5	0.5	-	-	-	-
Tetracycline		-	-	-	-	0.25	0.25	-	-

MIC: minimum inhibitory concentration; -: not tested

6. Conclusion

In this study, *in vitro* growth-inhibitory activity of Indian medicinal plants has been tested against respiratory bacteria, namely *H. influenzae, S. aureus, S. pneumoniae*, and *S. pyogenes*. 9 EOs were hydrodistilled from various plant parts of 9 different species. Their antimicrobial effect was evaluated using the broth microdilution volatilization method. Among all EOs tested, only *C. citratus, C. scariosus*, and *T. ammi* possessed a considerable growth-inhibitory effect against at least one bacterium strain. The most effective results against *H. influenzae* produced *T. ammi* in the liquid phase and *C. citratus* in the vapor phase. *C. scariosus* showed low antibacterial effect against *S. aureus, S. pyogenes*, and *S. pneumoniae* in both phases. Only *T. ammi* produced a significant degree of antibacterial activity against *S. aureus* in the vapor phase. The other EOs showed no inhibitory activity in agar. Although the use of EO in practice is likely to be limited by its relatively low efficacy, further safety tests may refine these results. EOs could be further tested for other microorganisms that cause respiratory diseases, such as *Mycobacterium tuberculosis*.

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Appendices

List of the Appendices:

Appendix 1: Photographic illustrations of plant samples

Figure 3. *Trachyspermum ammi* Sprague (Kumar 2022)

Figure 4. dried seeds of *Trachyspermum ammi* (Hvězdová 2022)

Figure 5. *Cyperus scariosus* R.Br. (Kasana et al. 2013)

Figure 6. dried roots of *Cyperus scariosus* (Hvězdová 2022)

Figure 7. *Cymbopogon citratus* Stapf. (Mans 2016)

Figure 8. dried leaves of *Cymbopogon citratus* (Hvězdová 2022)

Appendix 2: Photographic illustrations of plant distillation

Figure 9. Clevenger apparatus (Hvězdová 2021)

Figure 10. Plant material before crushing (Hvězdová 2021)

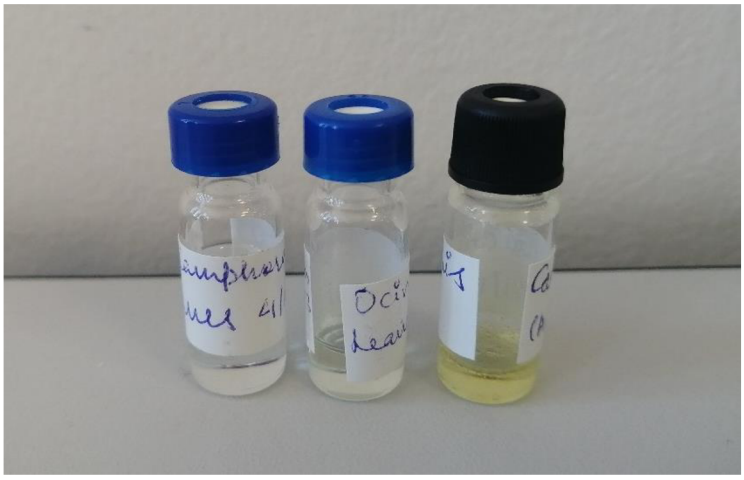

Figure 11. Examples of extracted plant material (Hvězdová 2021)

Appendix 3: Photographic illustrations of antimicrobial assay

Figure 12. Agar dilution (Houdková et al. 2017)

Figure 13. Fastened plate and lid together (Hvězdová 2021)

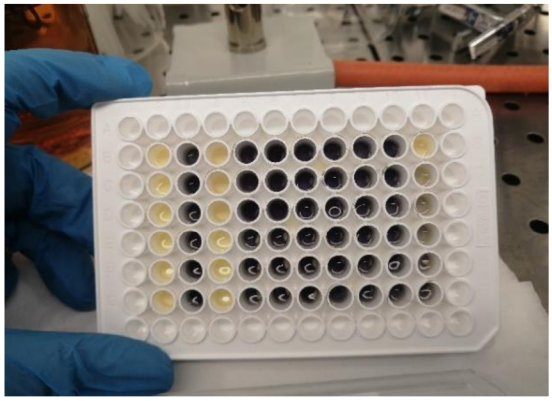

Figure 14. Determination of MIC on the plate (Hvězdová 2021)

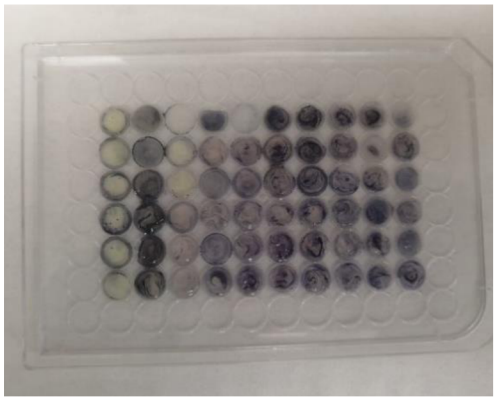

Figure 15. Determination of MIC on the lid (Hvězdová 2021)