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Evaluation of *in vitro* antibacterial effect of essential oils from Philippine plants in liquid and vapour phase

BACHELOR'S THESIS

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Declaration

I hereby declare that I have done this thesis entitled Evaluation of *in vitro* antibacterial effect of essential oils from Philippine plants in liquid and vapour 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 11th May

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Abstract

Haemophilus influenzae, Staphylococcus aureus and *Streptococcus pyogenes* are pathogenic bacteria, which can cause life-threatening infectious diseases. 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 (EOs) possessing antimicrobial activity.

Within this thesis *in vitro* growth inhibitory effect of Philippines essential oil-bearing plants were tested against *Haemophilus influenzae, Staphylococcus aureus* and *Streptococcus pyogenes.* Firstly, EOs were distilled, subsequently, their antibacterial activity was assayed using broth microdilution volatilisation method and the minimum inhibitory concentrations were determined. It was found, that 9 EOs from 14 samples possessed certain degree of antibacterial activity in liquid or vapour phase. The most effective against *H. influenzae* was *Cinnamomum iners* with MIC 256 µg/mL in liquid phase and with MIC 1024 µg/mL in vapour phase. EO from *C. iners* was the only one from tested EOs that showed some antibacterial potential in vapour phase. All the others EOs showed some inhibitory effect in liquid phase only. The second most effective was *Alpinia cumingii* with MIC 512 µg/mL. *Alpinia elegans* and *Xanthostemon verdugonianus* possessed low antimicrobial activity against *H. influenzae* at MIC 1024 µg/mL. The most effective against *S. aureus* was *A. elegans* at MIC 128 µg/mL, followed by *A. cumingii*.with MIC 256 µg/mL and *Etlingera elatior* with MIC 512 µg/mL. Low antimicrobial effect with MIC 1024 µg/mL showed *A. cumingii, A. elegans, Vitex turczaninowii* and *X. verdugonianus.* Against *S. pyogenes* were effective only *A. elegans* and *E. elatior*, both at MIC 1024 µg/mL. None of the samples showed antibacterial effect against *S. aureus* and *S. pyogenes* in vapour phase.

Key words: Antimicrobial activity, broth microdilution volatilization method, essential oils, Philippines.

Abstrakt

Haemophilus influenzae, Staphylococcus aureus a *Streptococcus pyogenes* jsou patogenní bakterie, které mohou způsobovat život ohrožující infekční onemocnění. Vzhledem k tomu, že resistence k antibiotikům stále stoupá, je zde potřeba objevovat nové antiinfekční látky. Rostliny představují bohatý zdroj sekundárních metabolitů s antimikrobiální aktivitou, mezi které patří i silice.

V rámci této práce byl zkoumán *in vitro* inhibiční účinek esenciálních olejů z filipínských rostlin vůči růstu *Haemophilus influenzae, Staphylococcus aureus* a *Streptococcus pyogenes*. Nejprve byly vydestilovány esenciální oleje a následně proběhlo testování jejich antibakteriální aktivity pomocí bujónové mikrodiluční volatilizační metody, kdy byly stanoveny jejich minimální inhibiční koncentrace (MIC). Bylo zjištěno, že 9 ze 14 silic vykazují antibakteriální aktivitu v kapalné nebo plynné fázi. Nejúčinnější proti růstu *H. influenzae* byla *Cinnamomum iners* s MIC 256 µg/mL v kapalné fázi a s MIC 1024 µg/mL v plynné fázi. Všechny ostatní silice vykazovaly určitý inhibiční účinek pouze v kapalné fázi. Druhý nejúčinnější proti *H. influenzae* byla *Alpinia cumingi* s MIC 512 µg/mL a nízkou antibakteriální aktivitu prokázaly *Alpinia elegans* a *Xanthostemon verdugonianus* s MIC 1024 µg/mL. Proti *S. aureus* měla největší inhibiční účinek *A. elegans* s MIC 128 µg/mL, následována *A. cumingii* s MIC 256 µg/mL a *Etlingerou elatior* s MIC 512 µg/mL. Nízký antibakteriální účinek s MIC 1024 µg/mL prokázaly *A. cumingii, A. elegans, Vitex turczaninowii* and *X. verdugonianus.* Proti *S. pyogenes* byly účinné pouze *A. elegans* a *E. elatior* s MIC 1024 µg/mL. Žádný ze vzorků neprokázal antibakteriální účinnost proti *S. aureus* a *S. pyogenes* v plynné fázi.

Klíčová slova: Antimikrobiální aktivita, bujónová mikrodiluční volatilizační metoda, silice, Filipíny.

Contents

List of tables

List of figures

List of the abbreviations used in the thesis

ATCC American Type Culture Collection

BC Before Christ

CLSI Clinical and Laboratory Standards Institute

DMSO Dimethyl sulfoxide

EO Essential oil

MIC Minimum inhibitory concentration

1. Introduction

1.1. Bacterial respiratory diseases

1.1.1. Epidemiology

Respiratory diseases epidemiology encompasses pathological conditions affecting the organs and tissues that make gas exchange difficult in air-breathing animals. They include conditions of the respiratory tract including the trachea, bronchi, bronchioles, alveoli, pleurae, pleural cavity, and the nerves and muscles of respiration (Leitch 2000). Respiratory diseases are one of the top ten causes of death and they are main cause of death in low income countries. Range of these diseases is from mild, such as the common cold to life-threatening disease such as bacterial pneumonia, pulmonary embolism, acute asthma and lung cancer. Lower respiratory infections include bronchitis, pneumonia, tuberculosis, remained the deadliest communicable disease, causing 3.0 million deaths worldwide in 2016. Chronic obstructive pulmonary disease claimed 3.2 million lives in 2016 (World Health Organization 2018). These infections introduce risk factor for other health difficulties such as cardiovascular diseases too (Horvath & Acs 2015). The occurrence of these illnesses can influence series of environmental and behavioural factors such as an air pollution, bad hygienic practises, malnutrition and tobacco smoking, therefore pose the highest risk to those, who living in developing countries, where is poorly access to health care (Prüss-Üstün & Corvalan 2006). It is estimated that 235 million people suffer from asthma, more than 200 million people have chronic obstructive pulmonary disease (COPD), 65 million endure moderate-to-severe COPD, 1–6% of the adult population (more than 100 million people) experience sleep disordered breathing, millions live with pulmonary hypertension and more than 50 million people struggle with occupational lung diseases. Totalling more than 1 billion persons suffering from chronic respiratory conditions. Nine million children under 5 years of age die annually and lung diseases are the most common causes of these deaths ((Forum of International Respiratory Societies 2017).

Infectious diseases are caused by viruses, bacteria or other pathogenic microbes and it can cause many different diseases as a pneumonia, cystic fibrosis, varicella, rubella, tuberculosis, measles, influenza and more. It includes also coronavirus infections SARS-CoV, SARS-CoV-2 and MERS-CoV. SARS-CoV-2 is a new virus from Wuhan, (China) and causes COVID-19 disease. Since December 2019 to February, there are more than 160,000 deaths worldwide to 19th April 2020. Lower respiratory infections causing 3.0 million deaths worldwide, 8.7 million people develop tuberculosis annually and it kills 1.3 million people every year. 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 808 694 children under the age of 5 in 2017. The most common locations are attacking Sub-Saharan Africa and South Asia, but get infected, is possible everywhere. For example, in Philippines causes yearly death of approximately 8,900 children. In this research was examined 3 types of bacteria that are involved in pneumonia, which is one of the most widespread type of illnesses affecting respiratory system. Nowadays, it kills millions of people every year and it is most common reason of child death (World Health Organization 2019).

1.1.2. Types and forms

There are infectious and chronic forms illnesses. Pulmonary infections are mostly bacterial or viral and less commonly caused by another organism. In the viral type, a pathogen replicates inside a cell and causes a disease, such as the flu, which is typical for upper respiratory tract (Baron 1996). Bacteria cause disease by secreting or excreting toxins by producing toxins internally, which are released when the bacteria disintegrate, or by inducing sensitivity to their antigenic properties. Chronic diseases, such as asthma, are persistent and long-lasting (Basnayake et. al. 2017)

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. For example, in Philippines causes yearly death of approximately 8,900 children (World Health Organization 2015).

1.1.3. Bacterial species

As was said, bacterial pathogens such as *Haemophilus influenzae*, *Moraxella catarrhalis*, *Mycoplasma pneumoniae*, *Mycobacterium tuberculosis*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Staphylococcus aureus*, and *P. aeruginosa* could be the primary cause of the disease, but more often, it follows a viral infection. Except for invasive pulmonary aspergillosis caused by *Aspergillus* species that have emerged as an important cause of life-threatening infections in immunocompromised patients, fungal respiratory infections are quite rare (Kokoska et al. 2019). *H. influenzae, S. aureus, and Streptococcus pyogenes* are bacterial species commonly involved in pneumonia infections (Eddy, 2005).

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 commonly be 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. Encapsulated strains were classified on the basis of their distinct capsular antigens. The six generally recognized types are: a, b, c, d, e, f. Before the use of effective vaccines, serotype b strains caused the vast majority of invasive *Haemophilus* infections. After widespread vaccination, serotype b was reduced to a minimum and we can see increase of serotype f (Murray et al.1999). There are also not encapsulated forms, which are less invasive, but they can, however, produce an inflammatory response in humans, which can lead to many symptoms (Slack et. al. 1998). *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. In severe cases, cefotaxime and ceftriaxone delivered directly into the bloodstream are the elected antibiotics, and, for the less severe cases, an association of ampicillin and sulbactam, cephalosporins of the second and third generation, or fluoroquinolones are preferred. Macrolide antibiotics may be used in patients with a history of allergy to beta-lactam antibiotics, but macrolide resistance has also been observed (James & Jansen). For hospitalized patients particularly if there is associated respiratory failure the parenteral route of administration may be preferred (King 2012).

Staphylococcus aureus

This gram-positive, facultatively anaerobic, round-shaped bacterium, belongs to Staphylococcaceae family (Garitty et al. 2005). *S. aureus* was discovered in a pus from open injuries by Scottish surgeon Alexander Ogston in 1880.It is a common member of the human body and usually acts as a commensal. It can be found on a skin, in a microbiome and in the upper respiratory tract. Despite of this, *S. aureus* very often causes skin and respiratory infections, including life-threatening sepsis leading to toxic shock syndrome associated with organ failure. The pathogenicity comes from producing enterotoxins, exfoliative toxins and others. *S. aureus* forms a yellow pigment from which generic name aureus was derived. Staphylococci are widespread in nature and present in approximately one third of the human population. Transmission of genes of antibiotic resistance among strains of *S. aureus* is most often realized by transduction via temperate bacteriophages from the Siphoviridae family (Murray et al. 1999). Since majority of infections is caused by MRSA (Methicillin-resistant *S.aureus*), therapy should include antibiotics active against methicillin resistant strains. Until the first years of the 1990s, when MRSA was uncommon, attempts to treat and eradicate S. aureus were mainly based on a combination of two antibiotics from a choice of a semi-synthetic β-lactamase-resistant drug (flucloxacillin or dicloxacillin), rifampicin and fusidic acid (Döring &

Hoiby 2004). Nowadays, per-oral treatment can include long-acting tetracycline such as doxycycline, minocycline or linezolid, which is however limited by its toxicity and high cost (Liu et al. 2011).

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 in 1874, when he described the organism in cases of erysipelas and wound infections (Billroth 1874, Billroth 1877). Additional refinement of the name streptococcus came from Friedrich Julius Rosenbach in 1884, who examined bacteria isolated from suppurative lesions, and the species was named *Streptococcus pyogenes* (Evans 1936). *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 (Efstratiou & Lamagni 2016). 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 penicillin and clindamycin should be given (Stevens et al. 2014). *S. pyogenes* has been and is still extremely sensitive to penicillin-based antibiotics. In fact, despite being the treatment of choice for decades because of its efficacy and low toxicity, streptococci resistant to penicillin have not evolved. The reason for this is still unknown, but may be related to the inability of naked DNA to enter the streptococcal cell because of the presence of DNases in the cytoplasmic membrane, or that beta-lactamase may not be expressed or may be toxic to the organism (Horn et al. 1998). Resistance to clindamycin has only rarely been reported as well as erythromycin. Clindamycin is more efficacious in experimental models of necrotizing fasciitis and myonecrosis (Stevens et al. 2014).

1.2. Essential oils (EOs)

These natural substances are plant 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. They play very important role in plant defence and signalling processes and mediate communication with other plants (Harborne 1993; Bowsher et. al. 2008). Another important function is also their phytoncide effect, which is based on protection of plant against microbial, fungal and animal pathogens. Today we know about 3000 types of EOs, of which approximately 10% are commercially important (Silva et al. 2013). 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). They can be extracted by steam distillation, solvent extractions or hydro distillation from aromatic plants. These plants can grow all over the world, but most of them occur in tropical rainforests because of the biggest biodiversity (Buchbauer 2000).

1.2.1. 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 (Hunter 2009; Baser 2015).

1.2.2. Physico-chemical properties

It is known, that EOs are physical in nature and composed of complex mixtures of chemicals. They must be volatile since they are removed by distillation, usually fairly hydrophobic and generally from colourless to deep yellow colour. Each plant produces its own specific mixture of 20-60 chemical constituents at fluctuate concentrations with two or three major compounds representing 20–70 % of all content that usually define the biological properties of EOs (Bakkali 2008).

1.2.3. Antimicrobial activity

EOs possess a range of biological properties including antimicrobial effect contributing to human health (Raut et. al. 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 (Memar et. al. 2017). One of the first systematic *in vitro* examinations of the antimicrobial activity dates back to the late nineteenth 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), which was in use as surgical antiseptic at that time (Ashhurst 1927). 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).

Besides the well-documented and very often used antimicrobial and antifungal properties of nearly all EOs, this group of natural compounds also possesses distinct antiviral properties. Viruses are sub-microscopic particles (ranging from 20 to 300 nm) that can infect cells of a biological organism. They replicate themselves only by infecting a host cell and cannot reproduce on their own. Unlike living organisms, viruses do not respond to changes in their environment (Hunnius 2007). EOs can suppress the viruses in different ways. They can inhibit their replication, or they can prevent their spread from cell to cell. In 1999, a study was published about results on the antiviral activity of *Santalum album* against Herpes simplex virus type 1 and type 2. The authors found that the EO inhibited the replication of the viruses. HSV-1 was more influenced than HSV-2 dose dependently (Benencia et. al. 1999).

Among other, some EOs have demonstrated a wide range of natural fungicidal effect. For example, great antifungal activity against very common mould *Botritis cinera* provide species of *Allium* and *Capsicum*. A 10% dilution of these extracts registered under 40.00 OD in fungal growth and completely inhibited spore germination of after 24 and 48 hours (Wilson et al. 1997). In general, fungi are more susceptible than bacteria to some EOs for example from plants like *Achillea millefolium* (Fierascu et. al. 2015), *Cinnamomum sp.*(Hilli 1997), *Daucus carota sativa* (Dwiwedi et. al. 1991), *Piper nigrum* (Khallouki et. al. 2000), while they are more resistant to others, e.g. *Juniperus sp.* (Stassi et. al. 1996) and *Tanacetum sp.* (Kalodera et. al. 1998). Dermatophytes are the most resistant, although numerous essential oils demonstrate high effectiveness against them. Among 22 samples of essential oils from 11 species of *Cinnamomum* genus, the oil of *Cinnamomum suvabenium* are the most active against *Microsporum canis*, *Trichophyton mentagrophytes* and *Trichophyton rubrum* as well as some candidiasis (*Candida albicans* and *Candida glabrata*), producing MIC values of 470-2 520 µg/ml (Mastura et. al. 1999). The antifungal activities of EOs could be also applied in the vapour phase for food storage (Tripathi et al. 2008). Carvacrol and thymol were reported to be effective against food-borne fungi, including *Aspergillus niger, Aspergillus flavus,* and *Aspergillus parasiticus* (Razzaghi-Abyaneh et al. 2009). Grow and aflatoxin production of *A. parasiticus* have been inhibited by the EOs of *Thymus vulgaris* and *Citrus aurantifolia*. *Mentha spicata, Foeniculum miller, and Artemisia dracunculus* inhibit the growth of *A.parasiticus* only, while *Carum carvi* conversely control aflatoxin production without effect on fungal growth (Razzaghi-Abyaneh et al. 2009).

A number of reports have been published in recent years on the antibacterial activity of some EOs. It is commonly known that Gram-positive bacteria are more susceptible to EOs and

Gram-negative ones are less sensitive. Many researches confirmed this phenomenon, but there are some exceptions. For example, *E. coli* is more susceptible to *Malaleuca alternifolia* oil and many other oils than *S. aureus* (Hayes 1997; Hilli 1997). In traditional medicine, the inhalation of *Mentha piperita*, *Salvia officinalis* and *Thymus vulgaris* volatile oils have been used traditionally to treat respiratory tract infections (Dorman 2000; Fabio 2007). They may inhibit the growth of bacteria or destroy bacterial cells. Their active compounds were shown to have strong antibacterial activity against *Salmonella typhimurium*, *S. aureus*, and *P. aeruginosa.* The *Syzigium aromaticum* oil was found to be the most effective (Conner et al. 1993). There are known many other EOs and their constituents have been effective against pathogenic bacteria. For example, *Carissa carandas*, *Curcuma zedoaria*, *Grewia asiatica* and *Punica granatum* effectively inhibit *in vitro* growth of *Streptococcus pneumoniae* causing pneumonial infection (Israr et. al. 2012). Carvacrol, eugenol and thymol are known to inhibit *Escherichia coli*, *Salmonella typhimurium* and *Listeria monocytogenes* (Kim et al. 1995). *Salvia officinalis* containing α-thujone, camphor, and 1.8-cineole as the major chemical constituents was reported to inhibit *S. aureus* and *Providencia stuartii* (Fraternale et al. 2005). Antimicrobial activity of thymol, carvacrol, cinnamaldehyde and their combination was tested on bacteria involved in upper respiratory tract infection, namely *S. aureus, P. aeruginosa, E. coli, K. pneumoniae, H. influenzae, S. pneumoniae* and *Branhamella catarrhalis*. A broad spectrum of activities of individual compounds and a synergistic effect of their mixtures was observed (Benes-Marshall 2001). Carvacrol, eugenol and thymol were also reported to inhibit *Escherichia coli*, *S. typhimurium* and *L. monocytogenes* (Kim et al. 1995). There are also many reports regarding antibacterial activity against *S. aureus*. Most of them are based on determination of antistaphylococcal activity in liquid phase. However, several studies recently confirmed that vapour phases of EOs are more effective (Inouye et al. 2003).

Recently, there have been studies confirming that vapour phases of EOs are more effective antimicrobials than their liquid phases including *Eucalyptus globulus* (Inouye et al. 2003), *Melaleuca alternifolia* (Mondello et al. 2009) *Cymbopogon citratus* (Tyagi & Malik 2010) and a range of others including *Thymus vulgaris*, *Foeniculum vulgare* and *Lavandula sp.* EOs (Soylu et al. 2006). One of the reasons that has been suggested for the vapour phase being more effective is that the lipophilic molecules in the aqueous phase associate to form micelles and thus suppress that attachment of the EOs to the organism, whereas the vapour phase allows free attachment (Inouye et al. 2003). However, compared with the wealth of evidence for the effectiveness of EOs in liquid phase, the potential of EO vapours is relatively less researched, although gaining interest. Inhalation of EO vapours in the form of smoke produced by bay leaves was apparently responsible for visions that came to the oracle at Delphi (Thompson 2003). The first record of EOs having medicinal uses was by Theopharustus in 4th century BC when they were used as antidotes to poisons and inhaled in vapour form to ease the throat. The use of EOs and EO vapours as a cure to be either consumed or inhaled before eating for nausea and stomach cramps caused by food consumption was suggested by Pliny in 23–72 AD (Arias & Ramon-Laca 2005). They were also used by the ancient Egyptians for medicine, perfumery and spiritual life (Edris 2007).

1.3. Philippines

Geography

This Philippines is an island nation located in south-eastern Asia. It is composing of 7 107 islands called the Philippine Archipelago, with an area of approximately 300 000 km^2. The largest of these islands is Luzon at about 105,000 km^2 and the second largest island is Mindanao at about 95,000 km^2 (WorldAtlas 2017). The archipelago is bounded by the Bashi Channel in the north, the Philippine Sea (Pacific Ocean) in the east, the Sulu and Celebes Seas in the south and the South China Sea in the west. Its northernmost islands are approximately 240 km south of the island of Taiwan, and the southernmost islands lie 24 km of the coast of Borneo (Malaysia). The islands are commonly divided into three island groups, which are further divided into regions, provinces, cities and municipalities and barangays. The islands and their respective administrative regions are Luzon, Visayas and Mindanao. The Philippines has a varied topography with highlands and numerous valleys. Its four major lowland plains are the central plain and the Cagayan valley in Luzon, and the Agusan and Cotabato valleys in Mindanao. These lowlands contrast sharply with the adjacent high mountain areas of the central and east Cordilleras and the Zambales mountains (FAO 2016). The Philippines consists of volcanic islands, including active volcanoes, with mostly mountainous interiors surrounded by flat lowlands and alluvial plains of varying widths along the coasts. The elevation ranges from sea level to the highest point of Mount Apo on Mindanao Island, at 2,954 meters above sea level. Principal Rivers: The longest river is the Cagayan on Luzon, about 350 kilometres in length (U.S. Library of Congress 2006).

Climate

The climate is tropical and monsoonal with uniform temperature, on average 27°C throughout the year. Humidity is relatively high, above 70% everywhere all year except in southern Tagalog, where it falls to 65% in March/April. There is low solar radiation, diversity of rainfall and high frequency of tropical cyclones. The average annual rainfall is about 2 348 mm/year, but it varies from around 960 mm in General Santos City in southeast Mindanao to more than 4 050 mm in Infanta in central Luzon. The annual amount is influenced mainly by altitude and wind. The archipelago lies in the typhoon belt, and many islands are liable to extensive flooding and damage during the typhoon season from June to December. The frequency of typhoons is greater in the northern portion of the archipelago than in the south. Usually, two or three typhoons reach the country each year (FAO 2016).

Population

There is a continuous population growth in Philippines. In 2018, lived there approximately 206 million people. Employment rate in October 2019 was estimated at 95,5 % and most of the people work in services (57,7 %), then in agriculture (23,5%) and industry (18,8 %) (Philippine Statistics Authority 2019). The Philippines is one of the most dynamic economies in the East Asia Pacific region. With increasing urbanization, a growing middle class, and a large and young population, the Philippines' economic dynamism is rooted in strong consumer demand supported by a vibrant labour market and robust remittances (The World Bank 2019). Because of the colonization by Spaniards for more than 300 years, the most widespread religion is Catholics and due to USA domination in 20th century is the official language English (Tucker 2009). Most of the people in the Philippines are of Austronesian descent who migrated from Taiwan during the Iron Age. They are called ethnic Filipinos. The largest Filipino ethnic groups are Tagalog 24.4%, Bisaya 11.4%, Cebuano 9.9%, Ilocano 8.8%, Hiligaynon 8.4%, Bicol 6.8% and Waray 4%. Another local ethnicity is 26.1% and other foreign ethnicity make up 0.1% (CIA 2010). About 8% of all Filipinos of Austronesian descent are tribal peoples.

The aborigines of the Philippines, called as Aeta, who are descended from Negritos of the Andaman Islands, now constitute only 0.003% of the entire population. Significant foreign minorities include the ethnic Chinese, Americans, and the South Asians, mostly Sindh and Pakistani. Other foreign ethnicities in the country include Spaniards, other Europeans, especially British and Dutch, then Koreans, Japanese, Indonesians, and Arabs (Johnson et. al. 2007). Various degrees of intermarriage between ethnic groups have resulted in the formation of a new vibrant class of peoples, collectively known as Filipino Mestizos. According to a Stanford University small-n study, only about 3.6% of all Filipinos have European genes, most probably Spanish Filipinos come from various Austronesian ethnolinguistic groups. Currently, there are more than 185 ethnolinguistic groups, each with its own language, identity, culture and history. The number of individual languages listed for Philippines is 185. Of these, 183 are living and 2 are extinct. Of the living languages, 175 are indigenous and 8 are non-indigenous. Furthermore, 39 are institutional, 67 are developing, 38 are vigorous, 28 are in trouble, and 11 are dying (Lewis & Melvyn 2009).

Agriculture

In 2016, there was 12440 ha of agriculture area and 8040 forest area (FAO 2016). Forestry, agriculture and fishing together contributes about 9% of GDP (World Bank 2018). The main agricultural crops are *Oryza sativa*, *Saccharum officinarum*, *Cocos nucifera*, *Manihot esculenta*, *Musa sp.* and *Ananas comosus*. The most common livestock raised are broiler chicken, carabao, cattle, duck and fishery products (Bureau of Agricultural Statistics 2004). In 1991, the agroforestry was widely applied to the Philippines as a response to increase of population and subsequent pressure to agriculture. Deforestation led to leaching of nutrients and annual yields decreased (Katayama & Luna 1998).

1.3.1. Diversity of essential oils bearing plants

Due to its geographical isolation, diverse habitats and high rates of endemism, Philippines are one of 18 mega-biodiverse countries of the world, containing two-thirds of the earth's biodiversity and between 70% and 80% of the world's plant and animal species. They rank fifth in the number of plant species and maintains 5% of the world's flora (U.S. Agency for International Development 2016). The endemic plant population of the

Philippines is considered to be the highest in Asia, accounting to approximately 65 % of the total plant species 16 in which, 1,500 are recognized to have medicinal properties (Madulid 2000). However, only 120 species are scientifically validated for safety and efficacy, and only 84 species are included in the Philippine National Drug Formulary. In past years, the Department of Health included ten medicinal plants in their program, among these are *Vitex negundo* and *Blumea balsamifera*, which are commercialized to treat cough and kidney stone, respectively (Philippine National Drug Formulary 2008). Other plant species are heavily commercialized as food supplements, although scientific validation of their pharmacological properties is lacking. Even though there are many plants producing EOs, the applications of them as potent antimicrobial agents are not extensively explored in the Philippines. The composition and bioactivities of EOs vary with species and are affected by cultivation techniques and climate (El-hawary et. al. 2013).

Here are some examples of plants producing EOs, that are typical for Philippines and grow there naturally or they are cultivated.

Abelmoschus moschatus is a medicinal plant, representative Malvaceae family occurs from India to southern China and through South-East Asia to northern Australia and the Pacific. On a small scale it is cultivated and occasionally occurs as a weed throughout the tropics and in warm temperate areas (Charrier 1984). *A. moschatus* is the source of ambrette seed oil used in luxury perfumery, cosmetic products and as an additive in the preparation of some kinds of chewing tobacco, baked products, sweets, alcoholic and non-alcoholic drinks. Tender leaves, shoots and pods are occasionally eaten as vegetable. It has many applications in traditional medicine too. In the Philippines a decoction of the roots and leaves is taken as an emollient remedy for gonorrhoea and rheumatism. The seed is said to have stomachic, tonic, diuretic, stimulating and antispasmodic properties (Perry 1980).

Acacia farnesiana a shrub or small tree, which comes from the northern part of tropical America, where its closest relatives can also be found. It is the most widely distributed *Acacia sp.* introduced to all tropical and subtropical regions of the world for its fragrant flowers and has become widely naturalized. It was first introduced to the Philippines from Mexico by the Spaniards. Roots are chewed for sore throat and in decoction they are employed as a remedy for tuberculosis. The tender leaves are bruised with a little water and swallowed against gonorrhoea and affections of the bladder (Clarke et. al. 1979).

Alpinia is a genus in the Zingiberaceae family (Simonetti & Schuler 1990). Most *Alpinia* spp. are plants of forest understory habitat. Most are pollinated by large bees, but some are pollinated by birds and bats. It is the largest genus in the Zingiberaceae family, with about 230 species. A number of those are commonly grown for their flowers, and others are used as spices (Kress et. al. 2005). *Alpinia malaccensis* is a robust herb. The origin is unknown ad it is widespread from the moister parts of the Himalayas, the western Ghats and the hills of Bengal and Chittagong in India to Malaysia, Indonesia and the Philippines. It is cultivated in north-eastern India, Indonesia and China. All parts of the plant are fragrant, and its leaves and rhizomes contain different EOs. Those from the leaves have been to perfuming women´s cloths and hair and now it is used in perfumery. The rhizome is occasionally used as a spice, while it is eaten as a vegetable in north-eastern India. The rhizomes were chewed together with betel nut (*Areca catechu*) to make the voice strong and clear. The pounded rhizome is applied to cure wounds, but it was also an ingredient in a kind of poison. The ripe and unripe fruits are well known in traditional medicine to control vomiting. Many *Alpinia* spp. are cultivated as garden plants and as pot plants for their attractive, often variegated leaves and striking inflorescences (Burtt 1972).

Aquilaria sp. are trees from Thymelaeaceae family. Decoction of the leaves is used as a bath against fever. All parts of these plants are fragrant, its leaves and rhizomes contain different EOs (Benival 1989). It is the major source of agarwood, which contain a resinous heartwood, used for perfume and incense. The fragrance produced by the burning agar wood has been highly valued for thousands of years, and its use as incense for ceremonial purposes in Buddhism, Confucianism and Hinduism (Ishihara et. al. 1993). Agarwood is said to be native to the Philippines. There are three species reported to have been found in the Philippines and these are *A. apiculina, A. acuminate, and A. cumingiana* (Akter et. al. 2013).

Blumea balsamifera from Asteraceae family occurs from India to southern China, Taiwan, Malaysia, Indonesia and the Philippines. In this region it is one of the most common and widely used medicinal plant. It can be used to treat bronchitis, arthritis, insomnia and dysmenorrhoea. In the Philippines a diuretic and kidney-stone medicine is prepared commercially from *B. balsamifera*. In Vietnam a decoction of the leaves is prescribed against influenza, cough and dyspepsia. Inhalation of the vapour from a boiling decoction of fresh leaves is used against the same afflictions. (Manalo & Coronel 1983).

Cananga odorata from Annonaceae family is a tree commonly known as ylang-ylang. There are two forms which are grown commercially. One group is Ylang-ylang, producing ylang-ylang oil and second group Cananga yielding cananga oil. Commercial cultivation for the production of oil started in the Philippines, later followed by the production of cananga oil in Indonesia. Both oils are distilled from the flowers and both of them are used to supply the dominant odour note of many perfumes (Acda et. al. 1995). Fragrant flowers are used for personal adornment and decoration at festivities and other celebrations. It also has medical properties, for example dried flowers are used in against malaria and the fresh flowers are made into a paste for treating asthma (Dassanayake & Fosberg 1987).

Clausena anisum-olens is evergreen shrub or small tree endemic to the Philippines, belonging to Rutaceae family. It is distributed all over the archipelago and is also cultivated occasionally. Leaves of *C. anisumolens* are used as a condiment in preparing local dishes and beverages and to flavour cigarettes (Brown 1941). The EOs from the leaves are a substitute of anise oil, which is used for the preparation of the Philippine drink 'Anisado'. It is also well known in traditional medicine. The leaves are stuffed into pillows for a soporific effect, they are used in baths against rheumatism or in decoction for nausea during pregnancy (Molino 1993).

Citrus spp. are usually shrubs or small trees from Rutaceae family and they are one of the ancient, most traded and most popular crops. The earliest records of its cultivation date back to 2100 BC (Moore 2001). The origin of genus *Citrus* is believed to have originated from Southeast Asia, but nowadays it is grown widely all over the world for its numerous health benefits. Furthermore, their EOs are used as natural preservatives due to their broad spectrum of biological activities including antimicrobial and antioxidant effects (Mitropoulou et. al. 2017). This genus includes many important crops both because of their fruit and their EOs. They are *Citrus aurantifolia*, *Citrus aurantium*, *Citrus bergamia*, *Citrus japonica*, *Citrus junos*, *Citrus limon*, *Citrus reticulata*, *Citrus sinensis*, and many more. For example, EO from *C. sinensis* was reported to inhibit the growth of several bacteria including *S. aureus, L. monocytogenes, Vibrio parahaemolyticus, S. typhimurium, E. coli,* and *P. aeruginosa* as well as several fungal species (Viuda- Martos et. al., 2008). There are also some species native to Philippines, like *C. microcarpa* and it is predominantly cultivated there as an economically important citrus. It is also known as calamondin or Philippine lime. Calamondin is ubiquitous in traditional Filipino cuisine. It is used in various condiments, beverages, dishes, marinades, and preserves (Mabberley 2004).

Cymbopogon citratus is a herb from *Poaceae* family, widely used in tropical countries (Weiss 1997). It is commonly used in Filipino cuisine, in teas, soups and curries. Like all other plants listed here, also *C. citratus* possesses various pharmacological activities such as antibacterial against *B. subtilis, E. coli, S. aureus* (Onawunmia et. al. 1984) and antifungal properties (Wannissorn 1996). Various other effects like antimalarial, antimutagenicity, antimycobacterial, antioxidants, have also been studied.

Gaultheria genus contains about 135 species and it is member of Ericaceae family (Mabberiey 1997). The leaves and flowers of several *Gaultheria* sp. yield wintergreen oil, which has an intensely sweet-aromatic fragrance and flavour. The oil was formerly used extensively to flavour drinks, in perfumes and in medicine. Its best-known use is in cola drinks, while herbal tea is made from fermented leaves. It is a mild antiseptic in mouth care products, e.g. against toothache and sore throat. The leaves are used in traditional Filipino medicine to treat coughing, tuberculosis, fever and pain. Edible portion is the enlarged calyx (Arcander 1960).

***Pogostemon* spp.** comprises about 80 species. One important member of the genus is *Pogestemon cablin*. Origin of this plant is uncertain, the border region between South-East Asia and China has been suggested as the most likely and the Philippines as a secondary centre of diversity. Cultivation of *P. cablin* has a long history of use in Philippines as incense, body and garment perfume and as a repellent of insects and leeches. Patchouli oil steam distilled from leaves is almost universally used as a fixing agent in perfumery, blending beautifully with an exceptionally wide range of fragrance and body-care materials (Weiss 1997).

Obviously, there is much more species in this region and outside of the region too. EOs bearing plants grow worldwide, but they occur more especially in the tropics and subtropics regions because of their need to protect themselves is bigger than in temperate zones (Hawksworth 1996).

1.3.2. Traditional use of spices, aromatic and medicinal plants

Ever since, plants have played an important role in the Philippines in many fields. On the first place, it is a food source. It can serve as a main part and as seasonings and Filipinos are very generous with spices during cooking traditional meals. Virtually every Filipino dish can be spiced up with some of *Capsicum* spp., from rich meat viands to everyday soups and noodles. Bicol, a region in south-eastern Luzon, is known for using chili peppers in most of its dishes. *Zingiber officinale* is used in most of Filipino food recipes, both for flavour and aroma, although the flesh of the root is not always eaten. As in rest of the world, also here people use huge in their cuisine huge amount of *Allium sativum* and *Allium cepa.* Native variety of *A. sativum* comes in smaller bulbs, with cloves less than half the size of other types and *A. cepa* is strong and pungent, making them a great source of flavour. One of very significant spices is *Cymbopogon citratus* which has strong-smelling leaves and stalks commonly used in soups, teas and sauces. The leaf is slightly sweet with a hint of citrus. There are several ways to use it, but the most common method is cooking the fresh leaves or sometimes the entire stalk or bulb with the food to release the flavour. *Pandanus amaryllifolius* is mostly an aromatic ingredient, commonly used with plain white rice. Couple of leaves are add to rice as it boils, and it comes out with a strong, inviting aroma. Some regions even weave it onto rice pots for an even stronger scent. It is also common condiment in rice cakes, puddings, and other Filipino desserts recipes. The strong, pungent taste of *Laurus nobilis* makes it a perfect fit for Filipino cooking recipes. It has a wide range of uses, from meat sauces and dips to main traditional dishes like adobo, menudo and mechado. Dried bay leaves are traditionally used, fresh is seldom available in local markets (Villamayor 2020).

Another important area of plant use is in traditional medicine. The development of traditional healing was influenced by the history and the location of the country. The geopolitical position is like a gateway to either enter or exit Southeast Asia and that has allowed the exchanging of medical knowledge between immigrants, whether they are colonial predecessors or neighbouring countries. The trade between China and the

Philippines was recorded as early as the eighth century and enhanced in the sixteenth century and it is believed that Chinese traders introduced some medicinal plant there (Apostol 2012). Also, the methods used by some healers is similar to the Chinese acupuncture study, the idea that the body through fluids of energy known as a yin and yang. Native Filipino medicine uses the four elements: earth, water, fire, and air to diagnose conditions, while Traditional Chinese medicine views the conditions of the body through the five-element theory: fire, earth, metal, water, and wood (Furth 2009). Other similar approaches to diagnosing include herbs, taking patient history, facial diagnosis, and tongue diagnosis (Apostol 2012).

During Spanish regime was written many books about traditional medicine of Philippines. Later, during years of occupation by Americans, further research on medicinal plants was conducted. During the Second World War, indigenous people of Philippines were depended entirely on plants as sources of medicine due to lack of medicaments (World Health Organization 1997).

Nowadays, in rural and remote areas of Philippines the traditional medicine is still very popular and, in many cases, indispensable. There are either a handful practicing herbalists or licenses modern medicine doctors. Herbal remedies are conducted in a variety of ways including decoction, like a tea, expression, which means pounding of the plant and then applying the extract and infusing plants in water for a certain period of time and applying the result to affected areas. These botanical remedies involve extracting the essential parts out of the plant material, and can be transformed into oil, ointment, and other forms of medicine. Plant materials consist of leaves, tree bark, and roots. Herbal extracts can either be consumed or applied to affected area (Fierro & Nolasco 2013). Traditionally used plants include *Allium sativum*, which is able to lower blood cholesterol, *Blumea balsamifera* has diuretic properties, *Carmona retusa* helps in the treatment of cough, colic, diarrhoea and dysentery, *Cassia alata* is used for skin because of anti-fungal properties and against itching, *Mentha cordifolia* has analgesic effects, *Momordica charantia* is serve as hypoglycemic agent, *Ouisqualis indica* is anti-parasitic agent, *Peperomia pellucida* can lower blood uric acid, *Psidium guajava* is used as disinfection for wound healing and *Vitex negundo* as anticough medicament and against asthma. In Philippines, there is also approximately 250,000 unregulated traditional herbalists. Their prescription may occasionally involve prayers or incantations in addition to using natural substances. Although such practices an often considered superstitious, they can carry potential for anthropological and pharmacological research in the future. These local herb doctors are respected members in their community and medicinal plants are part of the cultural heritage. Knowledge is of traditional practice is pass from one generation to another and based on nature principles. When modern Western drugs became available, many locals, especially those in urban centres lost contact with their herbal heritage. This leads to loss of attraction about knowledge and difficult work of herbalists and researchers. Especially the widespread adoption of medicinal plants became to be not easily accessible. A number of western trained doctors is now prescribing herbal and plant-based medicines for their patients and to continue providing Philippine health care, especially in remote areas and islands where drug shortage is critical (World Health Organization 1997).

2. Aims of the Thesis

The main aim of this work is to determine *in vitro* growth-inhibitory effect of EOs distilled from various parts of Philippine plants species belonging to Lamiaceae, Lauraceae, Myrtaceae, Piperaceae and Zingiberaceae families, against 3 standard bacterial strains, namely *Haemophilus influenzae*, *Staphylococcus aureus*, and *Streptococcus pneumoniae* by the broth microdilution volatilization method.

The specific objectives are:

a) Isolation of EOs from plant species

b) Determination of MICs of isolated EOs in liquid and vapour phase.

3. Material and Methods

3.1. Plant material

Plant material was selected based on chemotaxonomic criteria, whereas eleven plant species were chosen as phytochemically less explored representatives of taxa containing EOs. Then, reference specimen sheets with botanical descriptions, natural habitat, and illustrations were elaborated. The plant materials were collected on the island Leyte. Most of the samples come from foothills of Pangasugan mountain, but *Alpinia haenkei* was collected in Lake Danao National Park and *Cinnamomum. iners* at Tree planting site by Visayas State University (VSU) in Baybay city. All species (see Figure) were collected from wild populations, except *C. iners*. Ethnobotanical expert Prof. Ladislav Kokoška (Czech University of Life Sciences - Department of Crop Sciences and Agroforestry) and Marlito Jose M. Bande (Visayas State University - Institute of Tropical Ecology and Environmental Management) identified plant species. Voucher specimens were deposited in the herbarium of Department of Botany and Plant Physiology of the Faculty of Agrobiology, Food and Natural Resources of the Czech University of Life Sciences Prague, Czech Republic. Full list of plants with place and date of collection see in Table 1.

Table 1. Characteristics of plant material

Plant species	Plant part	Place of collection	Date of collection
Alpinia cumingii	flowers	Mt Pangasugan, Leyte	08.05.2018
Alpinia cumingii	pericarp	Mt Pangasugan, Leyte	08.05.2018
Alpinia elegans	pericarp	Mt Pangasugan, Leyte	24.04.2018
Alpinia elegans	seeds	Mt Pangasugan, Leyte	24.04.2018
Alpinia haenkei	pericarp	Lake Danao National Park, Leyte	30.04.2018
Alpinia haenkei	rhizome	Lake Danao National Park, Leyte	30.04.2018
Cinnamomum iners	bark	Tree planting site, VSU, Baybay, Leyte	04.06.2019
Cinnamomum iners	leaves	Tree planting site, VSU, Baybay, Leyte	04.06.2019
Etlingera elatior	leaves	Mt Pangasugan, Leyte	23.04.2018
Litsea leytensis	leaves	Mt Pangasugan, Leyte	15.05.2018
Piper philippinum	leaves	Mt Pangasugan, Leyte	12.05.2018
Piper retrofractum	leaves	Mt Pangasugan, Leyte	12.05.2018
Xanthostemon verdugonianus	leaves	Mt Pangasugan, Leyte	12.05.2018
Vitex turczaninowii	leaves	Mt Pangasugan, Leyte	12.05.2018

VSU: Visayas State University

3.2. Distillation of essential oils

Plant material was dried and finely 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 European Pharmacopoeia (European Pharmacopoeia 2013). The EO was then collected and stored in sealed glass vials at 4°C.

3.3. Microorganism and media

For the cultivation of bacteria were used American Type Culture Collection (ATCC): *H. influenzae* ATCC 49247, *S. aureus* ATCC 29 213, and *S. pyogenes* ATCC 19615. Cultivation and assay media (broth/agar) were Mueller-Hinton (MH) complemented by yeast extract and Haemophilus Tested Medium (*H. influenzae*), MH (*S. aureus*), and Brain Heart Infusion (*S. pyogenes*). Broths pH were equilibrated to final value 7.6 using Trizma base (Sigma-Aldrich, Prague, CZ). All microbial strains and cultivation media were purchased from Oxoid (Basingstoke, UK). Stock cultures of bacteria were cultivated in appropriate medium at 37°C for 24 h prior the testing and then a turbidity of the bacterial suspension was adjusted to 0.5 McFarland standard using Densi-La-Meter II (Lachema) to get the final concentration of 10^7 CFU/mL. The susceptibilities of *H. influenzae, S. aureus, and S. pyogenes* to oxacillin (≥86.3%), respectively (purchased from Sigma-Aldrich, Prague, CZ), were checked as positive antibiotic controls (Clinical and Laboratory Standards Institute 2015).

3.4. Antimicrobial test

The antibacterial potential of plant EOs in liquid and vapour phase was determined using a broth microdilution volatilization method (Houdkova et. al., 2017). The experiments were performed in 96- well immune plates, covered by tight-fitting lids with flanges designed to reduce evaporation (SPL Life Sciences, Naechon-Myeon, Republic of Korea). Briefly, 30 μL of agar was pipetted into every flange on the lid, except the outermost flanges, and inoculated with bacterial suspension in amount of 5 µL (see Figure 2). In the second part of this method, each sample of EOs was dissolved in DMSO (Sigma-Aldrich, Prague, CZ) at maximum concentration of 1% and diluted in an appropriate broth medium. Seven two-fold serially diluted concentrations of samples starting from 1,024 µg/mL were prepared for all EOs (see Figure 1). The plates were then inoculated with bacterial suspensions using a 96-pin multi-blot replicator (National Institute of Public Health). The wells containing inoculated and non-inoculated broth were prepared as growth and purity controls simultaneously. Finally, clamps (Lux Tool, Prague, CZ) were used for fastening the plate and lid together, with the handmade wooden pads for better fixing (see Figure 12). The microtiter plates were incubated at 37°C for 24 h. The minimum inhibitory concentrations (MICs) were evaluated by visual assessment of bacterial growth after colouring of a metabolically active bacterial colony with thiazolyl blue tetrazolium bromide dye (MTT) (Sigma-Aldrich, Prague, CZ) at a concentration of 600 µg/mL when the interface of colour change from yellow and purple (relative to that of colours in control wells) was recorded in broth and agar (see Figures 13 and 14). The MIC values were determined as the lowest concentrations inhibiting bacterial growth compared with the compound-free control and expressed in µg/mL (in the case of vapour phase also in µg/cm3 , where 256; 128; 64; 32; 16; 8; 4; and 2 µg/cm3 are real values for 1,024; 512; 256; 128; 64; 32; 16; and 8 µg/mL, respectively). The DMSO assayed as the negative control at concentration 1% did not inhibit any of the strains tested either in broth or agar media. With exception of *C. iners*, EO, which was tested in only one repetition because of small amount of sample, all experiments were carried out in three independent experiments and results were expressed as median/modal MICs values.

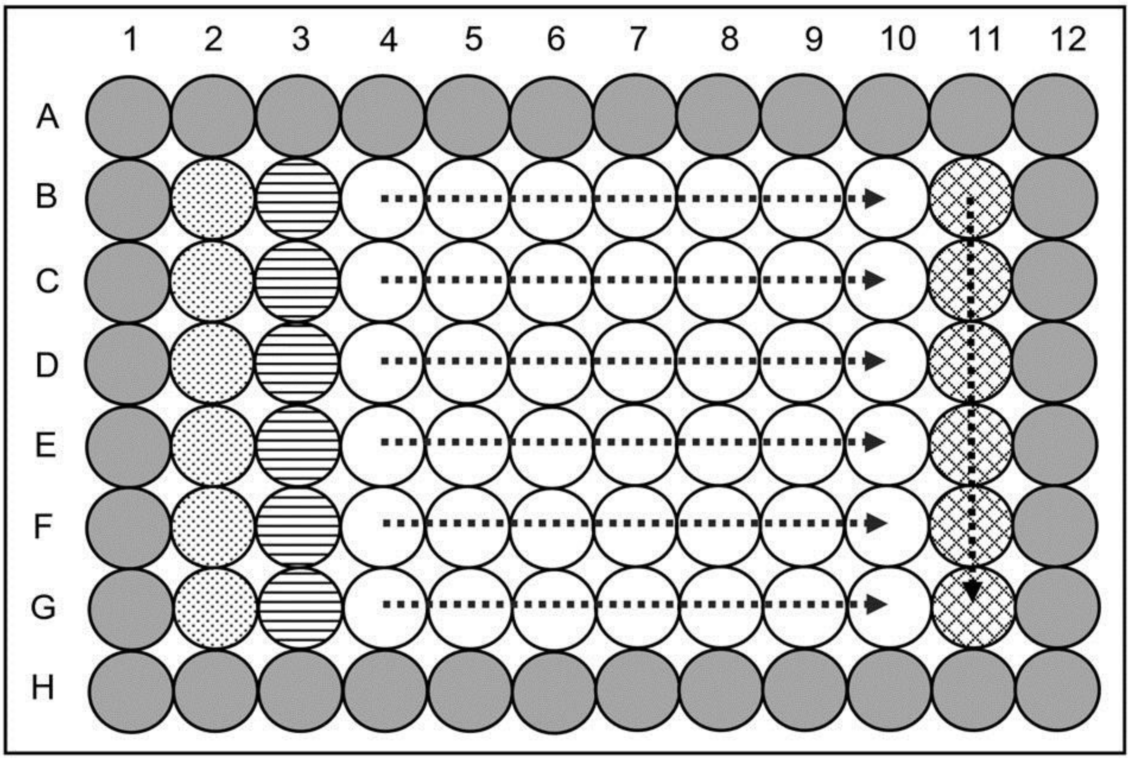

Figure 1. Schematic design of experiment: flat-bottom wells (Houdkova 2017) demonstrating: Grey-coloured wells: empty wells (not used); dotted wells: purity control; striped wells: growth control; white coloured wells: serial two-fold dilution of tested volatile compounds; gridded wells: serial two-fold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

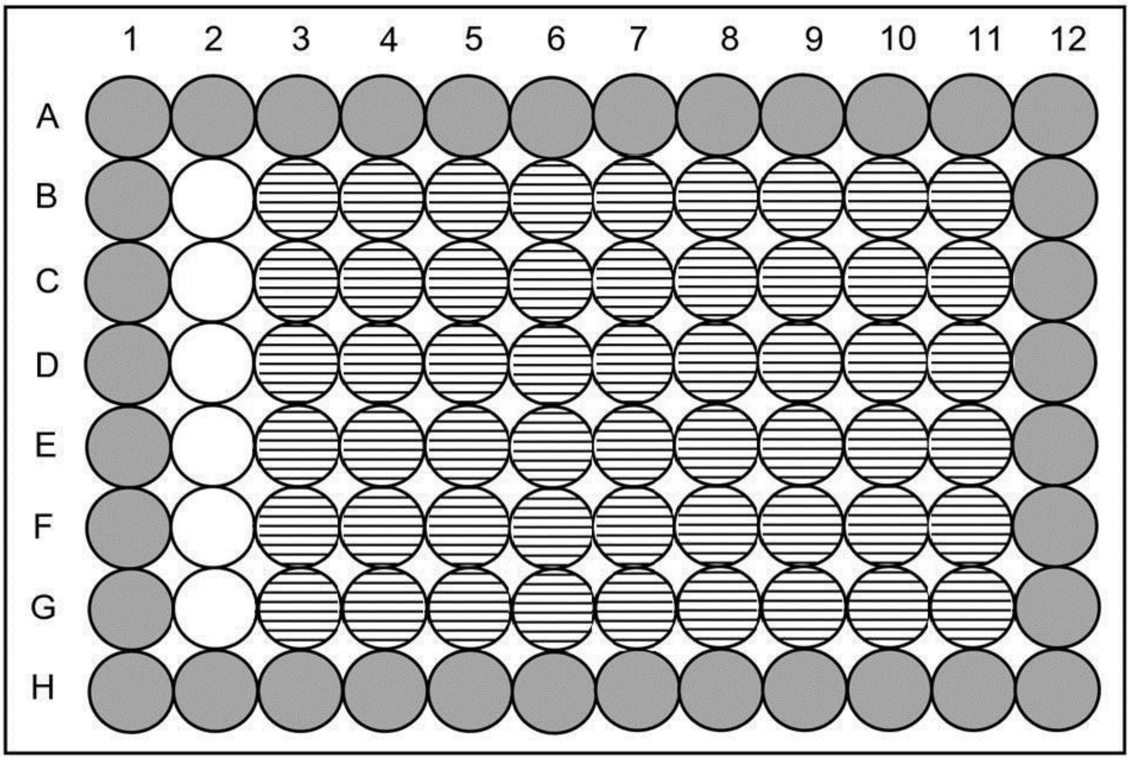

Figure 2. Schematic design of experiment: flanged lids demonstrating: Grey-coloured wells: empty wells (not used); white coloured wells: purity control (agar); striped wells: agar and bacteria (Houdkova 2017).

4. Results and discussion

Antibacterial effect of EOs in liquid and vapour phase

In total, 14 EOs from different plant parts (bark, flowers, leaves, pericarp, rhizomes and seeds) of 11 species were distilled. The results of antibacterial activity of EO-bearing plants are displayed in the Table 2. From all tested compounds, nine EOs possessed certain degree of antibacterial activity in broth, only one of them in agar as well.

In comparison with other microorganisms, *H. influenzae* was the most susceptible to all EOs tested. EO from *C. iners* (leaves) was the most effective against *H. influenzae* in liquid phase with MIC 256 µg/mL and in vapour phase with MIC 1024 µg/mL. EO from *C. iners*- leaves was the only one from tested EOs that showed some antibacterial potential in vapour phase. All the others EOs, showed some inhibitory effect against *H. influenzae* in liquid phase only. EO from *A. cumingi* (pericarp) possess second lowest MIC (512 µg/mL). *A. elegans* (pericarp), *A. elegans* (seeds), *C. iners* (bark) and *X. verdugonianus* (leaves) produced only low antibacterial effect with MICs 1024 µg/mL. Pericarp EO of *A. haenkei* did not show any antibacterial effect against *H. influenzae*.

S. aureus has been susceptible to EO tested in liquid phase only. The most effective was EO of *A. elagans* (seeds) with MIC 128 µg/mL, followed by *A. cumingii* (pericarp) with MIC 256 µg/mL. Moderate activity was shown by *E. elatior* (leaves) with MIC 512 µg/mL. The low inhibitory effect with MIC 1024 µg/mL was produced by *A. cumingii* (flowers), *A. elegans* (pericarp), *V. turczaninowii* (leaves) and *X. verdugonianus* (leaves). None of the EOs showed antistaphylococcal activity in vapour phase.

The only two EOs effective against *S. pyogenes* were *A. elegans* (seeds) and *E. elatior* (leaves) with MIC 1024 µg/mL in liquid medium. In vapour phase, none of the EOs showed antistreptococcal potential.

Table 2 - Antibacterial activity of essential oils and antibiotics in liquid and vapour phase in broth and agar media

Plant species	plant part	Bacteria/growth medium/MIC (µg/mL)					
		Haemophilus influenzae		***Staphylococcus aureus***		***Streptococcus pyogenes***	
		broth	**agar**	**broth**	**agar**	**broth**	**agar**
Alpinia cumingii	flowers	-	-	1024	>1024	>1024	>1024
Alpinia cumingii	pericarp	512	>1024	256	>1024	>1024	>1024
Alpinia elegans	pericarp	1024	>1024	1024	>1024	>1024	>1024
Alpinia elegans	seeds	1024	>1024	128	>1024	1024	>1024
Alpinia haenkei	pericarp	>1024	>1024	>1024	>1024	>1024	>1024
Alpinia haenkei	rhizome	-	-	>1024	>1024	-	>1024
Cinnamomum iners	bark	1024	>1024	>1024	>1024	>1024	>1024
Cinnamomum iners	leaves	256	1024	>1024	>1024	>1024	>1024
Etlingera elatior	leaves	-	-	512	>1024	1024	>1024
Litsea leytensis	leaves	-	-	>1024	>1024	>1024	>1024
Piper philippinum	leaves	-	-	>1024	>1024	>1024	>1024
Piper retrofractum	leaves	-	-	>1024	>1024	>1024	>1024
Vitex turczaninowii	leaves	-	-	1024	>1024	-	>1024
Xanthostemon verdugonianus	leaves	1024	>1024	1024	>1024	>1024	>1024
Positive Antibiotic control							
Oxacillin		0.5	>4	0.25	>4	2	>4

MIC: minimum inhibitory concentration; - : not tested

In this study, the microdilution volatilization method was used for evaluation of *in vitro* antibacterial effect of essential oils from Philippine plants in liquid and vapour phase. The fact that the oils comprise several volatile components makes them ideal for research of their antibacterial activity in vapour phase. For bacteria, it is more difficult to develop resistance to the multicomponent mixtures like EOs than to single-ingredient conventional antibiotics (Santos & Novales 2012). There have been studies confirming that vapour phases of some EOs can be more effective antimicrobials than their liquid phase such as *Eucalyptus globulus*, *Melaleuca alternifolia*, and *Cymbopogon citratus* (Laird & Phillips 2012). Our results showed that EO from leaves of *C. iners* possess some potential in vapour phase, but lower than in liquid phase.

Some of the tested plant species or their constituents have been tested for antibacterial effect before. *A. cumingii* and *A. elegans* were reported as antimicrobial active against *H. influenzae* and *S. aureus* (Houdkova et. al. 2018). Several studies have been performed on *P. retrofractum*. It has been proven efficacious as antimicrobial agent against *S. aureus* (Jamal et. al. 2013). Several studies also proved antibacterial effect of *E. eleatior* against *S. aureus* (Susanti et. al. 2013; Wijekoon et. al. 2013). Growth- inhibitory effect against *S. aureus* was also detected in *C. iners* (Buru et.al. 2014). If we compare our results with previous studies, we can generally conclude that they are in agreement, except of *P. retrofractum.* Our results did not show any antibacterial activity against *S. aureus*, nor against other bacteria. Explanation of different results can be caused by testing different part of the plant with different solvents and methods, as well as use of different antimicrobial assays and the strains of used microorganisms. Furthermore, in some studies, other parts of the plants were used for testing and the region and date of the collection also differed. In contrast with several studies on antibacterial activity of volatile compounds in liquid media, there are only few reports on their effect in vapour phase. According to our best knowledge, the antimicrobial of *A. haenkei*, *L. leytensis*, *Piper philippinum*, *Xanthostemon verdugonianus* and *Vitex turczaninowii* as been determined for first time in this study.

5. Conclusion

In this study, antibacterial effects of EO-bearing Philippine plants have been tested against *H. influenze*, *S. aureus* and *S. pyogenes*. 14 EOs from various plant parts of 10 species were obtained. The broth microdilution volatilization method was used for assessment of their growth inhibitory effect. The most promising results of all the tested plants were achieved by *A. elegans*. It was most effective EO against *S. aureus* and it also showed certain degree of antimicrobial activity against *H. influenzae* and *S. pyogenes*, in all cases in liquid phase. *E. elatior and A. elegans* showed low antibacterial effect against *S. pyogenes. C. iners* was the most effective against *H. influenzae* in liquid and vapour phase. *C. iners* was the only EO, that possess low antibacterial effect in vapour phase. All the other species were inactive in agar medium. *A. cumingii*, *A. elegans*, *C. iners*, *E. elatior*, *V. turczaninowii* and *X. verdugonianus* are suggested as prospective EO-bearing plants with antibacterial effect. However further research focused on their compositions and determination of active compounds will be needed prior to its possible pharmacological application. Our findings could contribute to the development of new medicinal, that are based on volatile antimicrobials designed to overcome the resistance of *H. influenzae*, *S. aureus* and *S. pyogenes*.

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Appendix 1.Photographic illustrations of plant samples

Figure 3. *Alpinia elegans*

Figure 4. *Alpinia cumingii*

Figure 5. *Alpinia haenkei*

Figure 6. *Cinnamomum iners*

Figure 7. *Etlingera eleatior*

Figure 8. *Vitex turczaninowii*

Figure 9. *Litsea leytensis*

Figure 10. *Xanthostemon verdugonianus*

Figure 11. *Piper retrofractum*

Original photos by Marie Netopilova, (2018) and Marketa Houdkova (2017, 2019).

Appendix 2.Photographic illustrations of antimicrobial assay

Figure 12. Fixing plate and lid together

Figure 13. Determination of MIC-wells

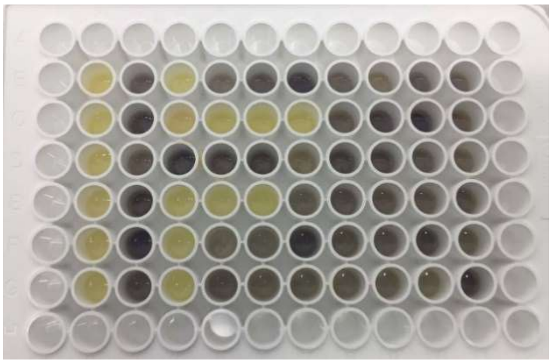
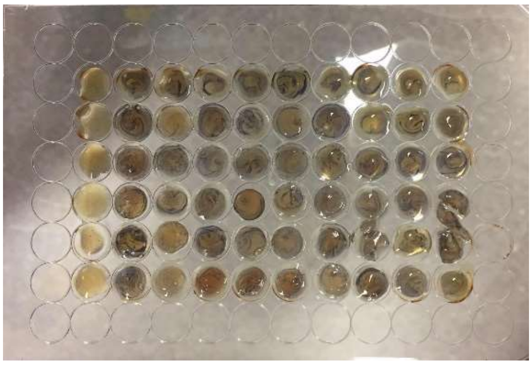


Figure 14. Determination of MIC-flanges



Purple flanges/wells: infected medium; yellow flanges/wells: non-infected medium; white flanges/wells: not used

Original Photos by Jana Zimova, 2019.