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Determination of *in vitro* antistaphylococcal effect of Cambodian essential oil-bearing plants using broth microdilution volatilization method

Master thesis

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Declaration

I, Ingrid Faltová, hereby declare that this diploma thesis, submitted in partial fulfilment of the requirements for the degree of Master of Science at the Faculty of Tropical AgriSciences of the Czech University of Life Sciences is my own independent work unless otherwise referenced or acknowledged.

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Abstract

Staphylococcus aureus is 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 possessing antimicrobial activity.

Within this thesis *in vitro* growth inhibitory effect of Cambodian essential oil-bearing plants from Lauraceae and Zingiberaceae families were tested against 5 representatives of *Staphylococcus aureus.* Firstly, essential oils were distilled, subsequently, their antistaphylococcal activity was assayed using novel broth microdilution volatilisation method and the minimum inhibitory concentrations were determined. It was found, that three essential oils possessed certain degree of antibacterial activity in liquid or vapour phase. The most effective were *Amomum pierreanum*, which inhibited growth of 4 strains of *S. aureus* in broth medium with MIC 1024 μg/mL and *Cinnamomum dimorphandrum* active against two strains in liquid phase and another two strains in vapour phase, both at MIC 1024 μg/mL. According to our results this method confirmed to be suitable for rapid simultaneous determination of antibacterial potential of EOs in the liquid and the vapour phase at different concentrations.

A. pierreanum and *C. dimorphandrum* are suggested as prospective plant materials for further study regarding the isolation and identification of active compounds which are responsible for antistaphyloccocal activity. These findings indicate that further studies on chemical and biological properties of their active components should be performed.

Keywords: Antimicrobial activity, broth microdilution volatilization method, essential oils, Cambodian aromatic plants.

Abstrakt

Staphylococcus aureus je patogenní bakterie, která může způsobovat život ohrožující infekční choroby. 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 rovněž silice.

V rámci této práce byl zkoumán *in vitro* inhibiční účinek esenciálních olejů z kambodžských rostlin z čeledí Lauraceae a Zingiberaceae vůči růstu 5 kmenů *S. aureus.* Nejprve byly vydestilovány esenciální oleje, následně proběhlo testování jejich antibakteriální aktivity pomocí bujónové mikrodiluční volatilizační metody, přičemž byly stanoveny jejch minimální inhibiční koncentrace. Výzkum ukázal, že tři esenciální oleje vykazují určitý stupeň antimikrobiální aktivity v kapalné či plynné fázi. Nejúčinnější byl esenciální olej z *Amomum pierreanum,* který potlačil růst čtyř kmenů *S. aureus* v bujónovém médiu s koncentrací 1024 μg/mL a *Cinnamomum dimorphandrum* účinný vůči dvěma kmenům v bujónu a zároveň dvěma dalším v plynné fázi. U všech byla stanovena minimální inhibiční koncentrace 1024 μg/mL. Na základě výsledků můžeme tuto metodu zhodnotit jako vhodnou pro stanovení antibakteriálního potenciálu esenciálních olejů v kapalné a zároveň plynné fázi v různých koncentracích.

Druhy *A. pierreanum* a *C. dimorphandrum* lze doporučit k dalšímu výzkumu týkajícího se identifikace a izolace aktivních látek, které jsou zodpovědné za antibakteriální aktivitu olejů. Výsledky práce poukazují na příhodnost dalšího studia chemických a biologických vlastností látek, které tyto rostliny obsahují.

Klíčová slova: Antimikrobiální aktivita, bujónová mikrodiluční volatilizační metoda, silice, kambodžské aromatické rostliny.

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List of abbreviations

ATCC	American Type Culture Collection
BC	Before Christ
CLSI	Clinical and Laboratory Standards Institute
DMSO	Dimethyl sulfoxide
EO	Essential oil
MIC	Minimum inhibitory concentration
MRSA	Methicillin-resistant *Staphylococcus aureus*

1. Introduction and literature review

1.1. *Staphylococcus aureus*

Staphylococcus aureus is a gram-positive bacterium discovered in 1880 by Scottish surgeon Alexander Ogston, as a cause of acute suppuration in humans (Ogston & Witte 1984) and four years later the German bacteriologist F. Rosenbach gave it a Latin name (Rosenbach 1884). *S. aureus* very often causes various infections ranging from mild skin and soft tissue inflammation to life-threatening sepsis such as blood poisoning, leading to septic shock associated with organ failure, toxic shock syndrome and necrotizing pneumonia. The pathogenicity of this bacterium results from the production of toxins namely exfoliative toxins, enterotoxins and other, that cause destroying tissues of infected organism (Murray et al. 1999). *S. aureus* forms a yellow pigment from which generic name aureus was derived, also it causes fermentation of saccharide mannitol (Anonymous 2018). Staphylococci are widespread in nature and present in approximately one third of the human population, especially on the skin and mucous membranes. 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).

1.1.1. Classification

Staphylococcus aureus belongs to Staphylococcaceae family which is divided into five genera of gram positive bacteria including genus *Staphylococcus*. The complete taxonomy is according to Bergey's Manual of Systematic Bacteriology following: Phylum: Firmicultes, Class: Bacilli, Order: Bacillales, Family: Staphylococcaceae, Genus: *Staphylococcus* (Garitty et al. 2005).

Staphylococci are traditionally divided according to their ability to produce plasma coagulase enzyme into coagulase-positive and coagulase-negative species. The group of the coagulase-positive staphylococci includes most of the clinically important species in human as well as veterinary microbiology. The most important opportunistic pathogen in humans is *S. aureus*, which has got many factors of virulence, the ability to produce large quantities of biologically active substances. Nowadays, *S. aureus* subs. *aureus*

belongs among the most studied species, also because of the presence of methicillin resistant strains (Van Belkum et al. 1993; Smeltzer et al. 1996; Gourd 2005).

The coagulase-negative group includes species that are generally clinically less significant, nonpathogenic or conditionally pathogenic. However, many studies of recent years emphasized that coagulase-negative species are mostly involved in nosocomial infections caused by *Staphylococcus* (Weinstein et al. 1998; Von Eiff et al. 2001). Coagulase-negative staphylococci are now known to comprise over 30 species namely *Stahpylococcus epidermidis, S. haemolyticus, S. hominis* with two subspecies – *S. hominis* subsp. *hominid* and *S. hominis* subsp, *novobiosepticus* (Petráš 2004).

Staphylococci are mostly susceptible to furazolidone (100 μg) and resistant to bacitracin (0.04 IU) (Falk & Guering 1983). Based on susceptibility to novobiocin, species can be divided into a group of novobiocin-sensitive and novobiocin-resistant (Slaughter 2001). In addition, increasing trend in resistance to antibiotics, mainly to methicillin and oxacillin, has been observed in the coagulase-negative staphylococci (Van Belkum et al. 1993; Kloss & Bannerman 1994; Slaughter et al. 2001).

Kloss divided species according to production of plasma coagulase and resistance to novobiocin into 6 following subgroups (Kloss et al. 1992):

1) Coagulase-negative, novobiocin susceptible: *Staphylococcus epidermidis, Staphylococcus hominis, Staphylococcus warneri, Staphylococcus capitis, Staphylococcus caprae, Staphylococcus lugdunensis*

2) Coagulase-negative, novobiocin resistant: *Staphylococcus saprophyticus, Staphylococcus xylosus, Staphylococcus kloosii, Staphylococcus arlettae, Staphylococcus cohnii, Staphylococcus equorum, Staphylococcus galinarum*

3) Coagulase-negative, novobiocin sensitive, producing beta-galactosidase: *Staphylococcus simulans, Staphylococcus carnosus, Staphylococcus felis*

4) Coagulase-positive, novobiocin sensitive: *Staphylococcus intermedius, Staphylococcus aureus, Staphylococcus schleiferi, Staphylococcus delphini*

5) Coagulase-variable, novobiocin-resistant: *Staphylococcus hyicus, Staphylococcus chromogenes*

6) Coagulase-negative, novobiocin-resistant, oxidase-producing: *Staphylococcus sciuri, Staphylococcus lentus*

According to Cowan (1951) coagulase-negative species can also be sub-divided based on ability or inability to ferment sugars. Most accurately, staphylococci can be divided on the basis of their genome relatedness using genetic kinship by DNA-DNA hybridization and monitoring thermostability of DNA heteroduplexes (Kloos et al. 1992). The DNA kinship of the individual genes is plotted by dendrograms. Despite the indisputable benefits of molecular biological analyses, phenotypic analysis is also very important. Due to the development of molecular-biological methods, the number of validated staphylococci has increased significantly in last decade. The genus *Staphylococcus* today comprises 38 species, of which 9 were found to have 2 subspecies and one with three sub-species (Place et al. 2003; Petráš 2004).

A more detailed division was performed using the 16S-rRNA gene sequencing method. Using this method, staphylococci were divided into 11 groups, namely: *Staphylococcus pidermidis, Staphylococcus haemolyticus, Staphylococcus warneri, Staphylococcus simulans, Staphylococcus carnosus, Staphylococcus sciuri, Staphylococcus lugdunensis, Staphylococcus auricularis, Staphylococcus aureus, Staphylococcus hyicus-intermedius a Staphylococcus saprophyticus* (Takahashi et al. 1999).

1.1.2. Morphology

S. aureus and other bacteria have a prokaryotic type of cell. The nucleus is absent and genetic information is contained in a single circular structure of the chromosome. Resistance genes are either present on the chromosomes or on extra-chromosomal elements (Schaberg & Zervos 1986).

The cell wall provides a supportive and protective function and its structure is adapted to transport of substances inside and outside of the cell. The majority of the *S. aureus* strains have outermost layer of the cell covered with a polysaccharide capsule (Votava 2003). Capsule is protecting the bacteria by inhibiting phagocytosis of the organisms

using polymorphonuclear leukocytes. A loose-bound, water-soluble film containing monosaccharides, proteins and peptides is produced and is able to bind the bacteria to tissues or foreign bodies. In such a way the staphylococci can create so called biofilm-thick layer of cells which provides protective barrier to bacteria. This biofilm protects bacteria against desiccation, but also against destruction by immune system. Bacteria strains with thickened layer are usually resistant to antibiotics (Madigan et al. 2010; Murray 2016).

Around 50% of the cell wall is created by peptidoglycan which consists of layers of chains composed of alternating subunits of N-acetylglucosamine and N-acetylmuramic acid with 1.4-β linkages. The peptidoglycan chains are cross-linked by tetrapeptide chains bound to N-acetylmuramic acid and by a pentaglycine bridge, which is specific for *S. aureus*. Peptidoglycan may have endotoxin-like activity leading to the release of cytokines by macrophages (Dmitriev et al. 2014).

Teichoic acids are other major components of the cell wall. They are species-specific water soluble polymers composed of glycerol, phosphate or ribitol phosphate. These polymers are bound covalently to N-acetylmuramic acid residues of the peptidoglycan layer or to the lipids in the cytoplasmic membrane (lipoteichoic acids). Teichoic acids are partially responsible for negative charge of the cell. Also, they are able to significantly affect adsorption of the cell surface (Murray 2016).

Other important proteins present in the cell wall are proteins A, collagen, fibronectin, fibrinogen and laminin, which also significantly contribute to cell adhesion. Detection of these proteins is essential for the identification of *S. aureus* (Gyles et al. 2011).

1.1.3. Diseases

S. aureus causes disease through production of toxins or through direct invasion and destruction of tissue. The symptoms are mostly the result of toxin activity leading to acute diseases such as staphylococcal scalded skin syndrome, staphylococcal food poisoning or toxic shock syndrome. Other diseases result from proliferation of the organisms, cause abscess formation and tissue destruction (e.g., cutaneous infections, endocarditis, empyema, pneumonia, septic arthritis). People suffering from immunodeficiency are more susceptible to staphylococcal diseases. Also presence of foreign body can

easily cause health complications since significantly less staphylococci are necessary to establish disease (Murray 1999).

Four types of exfoliate toxins (ETs) namely ET_A, ET_B, ET_C and ET_D have been identified and characterized for *S. aureus*. These epidermolytic toxins cause skin infections in humans and some animals. The most common human exotoxins are ET_A and ET_B. Staphylococcal scalded skin syndrome (bullous exfoliative dermatitis) is fully developed generalized form of blistering and peeling of the skin surface layers (Melish 1970). The disease was firstly described in 1878 by Gottfried Ritter von Rittershain in 297 infants younger than 1 month old (Murray 2016). The scalded skin syndrome is characterized by the abrupt onset of redness erythema and inflammation around the mouth that spreads over the body within two days following by large bullae and blisters, leading to desquamation of the top layer of epidermis. Ritter's disease mostly occurs in young children. Mortality rate is less than 5%, but eventually is caused by secondary bacterial infection of denuded skin (Ryan 2003).

S. aureus can also cause food poisoning. Intoxication is induced by toxin present in food, mostly meat or milk products. Storing these food products at room temperature will enable bacteria to grow and produce toxin, which is heat-stable. After consuming of contaminated food onset occurs after 4 hours with symptoms like nausea, vomiting, abdominal pain and diarrhea. Certain strains of *S. aureus* can also cause enterocolitis manifesting by water diarrhea, abdominal cramps and fever. Enterocolitis occurs mainly in patients with decreased colonic flora where *Staphylococcus* strains can multiply and produce enterotoxin A and leuktoxin LukE/LukD (Murray 2016).

The cause of toxic shock syndrome (TSS) is production of toxic shock syndrome toxin (TSST-1), superantigen, which is increasing permeability of endothelium and intensifies the lethal effect of endotoxin. Typical symptoms include fever, hypotension and polyorganic insufficciency; moreover, in severe cases can end up with death (Ryan 2013). TSS can be classified according to the type of *S. aureus* inducing disease: non-menstrual, which is induced by *S. aureus* type causing skin or mucous membrane infections and menstrual associated with *S. aureus occurring* in the cervico-vaginal or orally-mucous surface (Peterson et al. 2005).

Also several respiratory diseases are caused by *S. aureus.* Staphylococcal pneumonia is rather rare. However, it has high morbidity and mortality rate, and occurs mainly during influenza epidemics. Aspiration pneumonia, mostly seen in children can develop after the aspiration of oral secretions colonized by pathogenic bacteria and may cause lung abscess. Hematogenous pneumonia is common for patients suffering from bacteremia, presence of bacteria in the blood or endocarditis, an inflammation of the inner layer of the heart (Fisher et al. 1958).

Methicillin resistant *S. aureus* (MRSA) is an important pathogen in the clinical environment responsible for severe necrotizing pneumonia with hemoptysis or septic shock (Murray 2016). Even though it causes mostly mild skin infections it is difficult to treat because of its resistance to majority of commonly used antibiotics. *S. aureus* causes also several pyogenic cutaneous infections such as impetigo, folliculitis, furuncles and carbuncles (Ryan 2003).

1.1.4. Treatment

The mortality of patients suffering from diseases or infections caused by *S. aureus* before antibiotics were introduced was more than 70% (Skinner & Keefer 1941). In 1928, Alexander Fleming discovered antibiotic penicillin produced by ascomycetous fungi *Penicillium rubens* but already few years later, in 1942, the first penicillin resistant strains of staphylococci appeared (Rammelkamp & Mason 1942). In 1945, Fleming found out, that mutation and resistance of staphylococci are caused by misuse of antibiotics. However, penicillin was still available without prescription until 1950s. One decade later, less than 20% of staphylococcal isolates were still susceptible to penicillin. In 1960s methicillin resistant *S. aureus* emerged as a nosocomial pathogen (Brumfitt & Hamilton-Miller 1989). MRSA firstly appeared in hospitals; subsequently it was spread to the community. This pattern recurs with each new antimicrobial resistance wave (Chambers 2001). Two new drugs against MRSA were introduced- vancomycin and teicoplanin. Nevertheless *S. aureus* developed resistance against both of them as well (Hiramatsu 1997). Generally, MRSA is very resistant bacteria especially due to its protecting biofilm and the treatment, which is usually internal with chemotherapeutics, is becoming more difficult.

Infection control and prevention

Staphylococcal abscesses are most often able to resolve spontaneously with no need for antimicrobial therapy. Some more serious infections of soft tissue or localized skin require incision, abscess drainage and disinfection of infected tissue. In case of more severe syndrome, therapy by antibiotics is appropriate. Since majority of infections is caused by MRSA, therapy should include antibiotics active against methicillin resistant strains. Per-oral treatment can include long-acting tetracycline such as doxycycline, minocycline or linezolid, which is however limited by its toxicity and hight cost (Liu et al. 2011). Vancomycin is a drug used intravenously. Its use is gradually decreasing and being substituted since staphylococci have developed resistance in 1997 (Hiramatsu 1997). There are two forms of vancomycin resistance. Low-level resistance is observed in *S. aureus* with a thicker cell wall. Vancomycin is trapped in the cell wall matrix and is unable to reach cytoplasmic membrane and subsequently disrupt the wall synthesis. High-level resistance is mediated by *van*A gene operon that was acquired from vancomycin-resistant enterococci. These bacteria have a modified peptidoglycan layer that does not bind vancomycin. This form of resistance is uncommon. However, if these resistant staphylococci become widespread, then antibiotic treatment of these highly virulent bacteria could be difficult (Hiramatsu 2001).

Staphylococci are omnipresent organisms occurring on the skin, mucous membranes and their introduction through wounds occurs often. The number of organism required to establish an infection is large. Usually proper cleansing and disinfection of the wound using iodine solution or soap is sufficient to prevent infection. Spreading of infection from nasal carriers can be decreased by the combination of nasal creams containing topical antimicrobials such as mupirocin or neomycin and per-oral treatment with antimicrobials that are concentrated in phagocytes and nasal secretions (e.g., rifampin) (Hill et al. 1988; Drancount et al. 1993). The outbreak of infection from person to person; especially surgical wound infections is difficult to prevent. Cephalosporin or vancomycin can be given during or after surgical procedure to reduce the chance of infection. However, the risk can be minimized mainly by proper hygiene. Also, spread of MRSA can be difficult to control because asymptomatic nasopharyngeal carriage is the most common source of these organisms (Schweizer 2014).

The resistance to β-lactam antibiotics is caused by β-lactamase (penicillinase) enzyme produced by the bacteria (Abraham 1940). Penicillinase hydrolyses β-lactam ring and subsequently deactivates the antibacterial properties of molecules. Because of the penicillin resistant strains of staphylococci, penicillinase-resistant beta-lactams such as methicillin, dicloxacillin, nafcillin or oxacillin were developed, however during time staphylococci developed resistance to these types antibiotics as well. All strains of methicillin resistant *S. aureus* produce penicillin-binding proteins (PBPs) which stimulate the construction of the cell wall (Tomasz et al. 1989).

1.2. Plants-derived products for treatment of microbial diseases

The antibiotic era during the 20th century has significantly reduced the threat of infectious diseases. However, over time susceptibility of microbial pathogens decreased and resistance firstly to penicillin, subsequently to other drugs occurred. The scientists realised that the effective span of any antibiotic is limited and also the public started to became more aware of problems connected with the misuse of antibiotics. Nowadays, half of all deaths in tropical countries are still caused by infectious diseases. All these reasons are leading to renewed interest in discovery of new anti-infective compounds (Eisenberg 1993).

Drugs derived from plant sources have been empirically used in the treatment of various human disorders for thousands of years. Plants are rich in a wide variety of secondary metabolites, which provide a good source of anti-infective agents, highly effective instruments against microbial infections. About 80% of the people in developing countries still use traditional medicines for their health care such as primary resource available (Kim 2005). Western medicine is trying to duplicate their approach and recover the knowledge in order to discover new medicines but also to conserve biodiversity and improve life in poor rural communities (Cowan 1999). In fact, wide range of plant medicinal products is nowadays available on the market and popularity still increases. 25 to 50% of current pharmaceuticals are derived from plants, however very few are intended for use as antimicrobials. Also, possible treatment of some diseased caused by fungi and protozoa are limited and chemotherapeutics are inadequate due to their side effects or toxicity (Eisenberg 1993). There are 250,000- 5,000,000 plant species on the Earth. Only 1% of them have been phytochemically investigated. There is a great poten-

tial for discovering new bioactive compounds such a basis for the development on new lead chemicals for pharmaceuticals (Cowan 1999; Kalemba & Kunicka 2003). The medicinal use of plants as a raw material for future drugs and studying of bioactive chemical entities from natural sources are the subjects of scientific discipline called ethnopharmacology (Patwardhan 2005).

According to the development of phytomedicines, plant medicaments can be divided into three groups. The first generation of plant drugs were simple botanicals used in more-less crude form. Already in the fifth century B.C. Hippocrates mentioned 300-400 medicinal plants. Later, in the first century A.D., Dioscorides wrote a medicinal plant catalogue of medicinal plants that became a prototype of modern pharmacopoeias. Also the Bible provides description of around 30 healing plants such as frankincense and myrrh, known for their antiseptic properties (Stockwell 1988). Other effective medicines used in crude form were opium or aloe. Second generation of plant based drugs emerged during industrial revolution and was based on processing of plant extracts in order to isolate their active constituents. Some of the compounds were even more active than their synthetic substitutes. Remarkable example could be for instance quinine obtained from *Cinchona* or reserpine from *Rauvolfia* (Mukeshwar et al. 2011; Savoia 2012). Generally, development of pharmaceutics was in this case based on identification of active molecules, biological assay and determination of dosage, followed by studies dealing with safety and efficacy of drug. The third generation of phytotherapeutics is based on clinical evaluation of herbal medicine used in folk medicine. Evaluation should include toxicity, cytotoxicity and eventually detailed analysis of drug. Form and dosage is designed to mimic traditional use of the herb (Savoia 2012). Example of plant product used in traditional medicine and subsequent proof of its antimicrobial activity can be chewing stick used in remote areas of Africa and Asia instead of toothbrush to decrease oral bacterial infections. Chewing sticks from *Fagara zanthoxyloides* and *Azadirachta indica* was shown to contain alkaloids with antimicrobial properties. *A. indica* was already tested for its toxicity, dosage of active substance was determined and there are already several products on the market containing *A. indica* extracts (Odebiyi 1979).

Plant-derived compounds of therapeutic value are mostly secondary plant metabolites, traditionally used for medicinal purposes. They have a wide range of activity according to the species, topography and climate (Ahmad et al. 2006). Diversity in the chemical

composition modifies their antimicrobial activity. The most important types of active compounds are mentioned below.

Phenolic compounds are the simplest bioactive phytochemicals widely distributed in plants, which they protect against microbial infections. They are a group of aromatic compounds consisting of single substituted phenolic ring. Among typical representatives belong cinnamic and caffeic acid. Caffeic acid can be found for instance in tarragon and thyme and is effective against viruses, fungi and bacteria (Wild 1994). Generally, phenols have potential anti-oxidative and anti-infective properties (Saleem 2010). Phenolic compounds with a C_3 side-chain and no oxygen are classified as essential oils (EOs). Well characterised representative is eugenol from clove oil providing antimicrobial properties. Group of phenolics and polyphenols further consists of flavones, flavonoids and flavanols, quinones, tannins, polymeric phenolic substances, and coumarins (Thomson 1978).

Flavonoids are significant phenolic compounds occurring as a C_6-C_3 unit linked to an aromatic ring (Stermitz et al. 2000). They can be divided into several groups including flavones and flavonols. Flavones are phenolic structures containing one carbonyl group. By adding of a 3-hydroxyl group flavonol is formed. Both of them have antimicrobial properties since they are able to bind extracellular and soluble proteins and to complex with bacterial cell walls and disrupt bacterial membranes. Flavonoids occurs in photosynthesizing cells and are mostly found in fruit, vegetables, nuts, seeds, stems, flowers, tea, wine, propolis and honey (Cazarolli et al. 2008).

The catechines belonging to flavonoids are present especially in tea plant *Camellia sinensis* where they form complexes with the bacterial cell wall and provide high antimicrobial activity (Friedman et al. 2006). Flavonoids in general are not only effective against viruses, they are also low in toxic so active concentration is not harmful to human body. Moreover, synergy has been demonstrated between active flavonoids as well as between flavonoids and existing chemotherapeutics (Kuhnau 1976).

Quinones are another group of ubiquitous secondary metabolites with potential antimicrobial properties. They consist of aromatic rings with two ketone substitutions. Quinones are coloured, highly reactive and responsible for the browning reaction in cut or

injured fruits and vegetable (Schmidt 1988). They are providing dyeing properties to henna *Lawsonia inermis* (Fessenden 1982). They also produce stable free radicals and irreversibly complex with nucleophilic amino acids in proteins, which leads to inactivation of the protein and loss of function. Due to this ability, quinone has significant antimicrobial effect. Kazmi described an antibacterial effect of anthraquinone produced by *Cassia angustifolia* against several bacterial strains including *S. aureus* (Kazmi et al. 1994). Anti-staphylococcal activity was also found in quinones produced by henna (*Lawsonia inermis*) and chamomile (*Matricaria chamomilla*), (Thomson 1978).

Tannins are polymeric phenolic substances mainly used for tanning leather and known for its astringency capability. They can be found in bark, wood, leaves, fruits and roots. Notable content of tannins is present in tea and red wine, where they are responsible for antimicrobial activity (Scalbert 1991). Tannins have the ability to complex with proteins as well as they can directly inactivate some organisms, modify the morphology or inhibit insect growth. They can be toxic to fungi, yeasts and bacteria (Schultz 1988).

Coumarins are phenolic substances composed of fused benzene and alfa-pyrone rings (O'Kennedy 1997). They are responsible for odours, for instance specific odour of hay. Coumarins have been determined to be anti-inflammatory, anti-thrombotic and antiviral and antibacterial, however they can have also toxic properties (Weinmann 1997). As a group, coumarins have been found to stimulate macrophages which could have an indirect negative effect on infections. Hydroxycinnamic acids, related to coumarins, seem to be inhibitory to gram-positive bacteria (Casley-Smith 1997).

Terpenoids and steroids; the fragrance of plants is carried in the EOs fraction. These oils are secondary metabolites that are highly enriched in compounds based on an isoprene structure. They are called terpenes, and they occur as diterpenes-hemiterpenes (C_5), sometimes also sesquiterpenes (C_{15}). If the compounds contain additional elements, usually oxygen, they are termed terpenoids. Such examples are menthol and camphor (monoterpenes), farnesol and artemisin (sesquiterpenoids) or capsaicin obtained from chilli peppers, possessing wide range of biological properties, including antibacterial activity (Cordell 1993). Terpenoids showed an activity against bacteria, fungi, viruses and protozoa. For instance, the ethanol-soluble fraction of purple prairie

flower produces petalostemumol, compound which is active against *S. aureus* (Hufford et al. 1993)

Alkaloids are heterocyclic nitrogen compounds characterised by different antimicrobial activities. Firstly, isolated alkaloid was morphine in 1805, obtained from *Papaver somniferum* (Fessenden 1982). From morphine, codeine and heroine are derived. Diterpenoid alkaloids especially form plants of Ranunculaceae family shown antimicrobial properties, while berberine alkaloid obtained from roots and stem bark of *Berberis* species was found to have mainly antiplasmodic activity (Omulokoli 1997). The isoquinoline alkaloid emetine obtained from the underground part of *Cephaelis ipecacuanha*, and related species, has been used for many years as an amoebicidal drug and treatment of abscesses due to the spread of infections (Iwu 1999).

Generally, various active compounds can be found in different parts of plants. For instance, ginseng roots contain saponins and EOs, leaves of eucalyptus provide EOs and tannins. Other active substances can be harvested from the bark of poplar or cinchona (Thomson 1978). The primary benefits of using plant derived medicines are that they are relatively safer than synthetic alternatives, providing therapeutic benefits, mitigate many of side effects often connected with synthetic antimicrobials and offer more affordable treatment (Murray 1995).

1.3. Essential oils

EOs, also known as etheric oils, volatile oils or essences are natural products formed by several volatile compounds. According to the International Standard Organisation on EOs an EO is defined as the product obtained from plant raw material by hydro distillation, steam distillation or dry distillation or by a suitable mechanical process (Baser & Demirci 2007; Zuzarte 2015). The definition of an EO excludes other aromatic products obtained by different extractive techniques (Berger 2007).

Aromatic plants produce fragrant essences in the secretory cells. These essences accumulate in intercellular spaces, channels, silt reservoirs, special trichomes, papillae and glands. EOs are liquid volatile substances, often considered to be waste products of metabolism. However, they play very important role in plant defence and signalling pro-

cesses (Harborne 1993; Bowsher et al. 2008). They are able to attract pollinators and repel insects and other pests at the same time. The production and the release time of fragrances may vary for different plants. An important function of EOs is also their phytoncid function, which is based on protection of plant against microbial, fungal and animal pathogens. Many aromatic plants have excretory cells near the ground, in flowers or leaves and essentials are released by contact. Nowadays about 3000 types of EOs are known, of which approximately 10% are commercially important (Silva et al. 1999). EOs are used in many fields for various purposes. Pharmaceutical and agronomical industries use them because of antimicrobial effects, whereas aromatherapy, cosmetics and perfumery profits from their aromatic properties (Buchbauer 2000).

EOs can be found in various plant organs such as flowers (*Rosa, Lavandula*), fruits (*Citruses),* seeds (*Elettaria, Amomum*), leaves (*Eucalyptus*, *Laurus*), resin (*Boswellia*), bark (*Cinnamomum)*, roots (*Zingiber*), grass roots (*Vetiveria*), stalk or stem (*Cymbopogon*). They are stored in secretory structures that differ in morphology, structure, function, and distribution. Internal secretory structures include secretory cells, cavities and ducts while external structures comprise glandular trichomes, epidermal cells and osmophores (Svoboda 2000; Baser 2015). The amount of produced EO depends on several extrinsic factors such as climatic conditions, altitude, air humidity and soil composition and intrinsic factors especially season and genetic variations (Procházka 1957).

The most economically important EOs are orange oil (*Citrus sinensis*), cornmit oil (*Mentha arvensis* L. var. *piperascens*), eucalyptus oil (*Eucalyptus globulus*, *E. polybractea*), citronella oil (*Cymbopogon winterianus*, *C. nardus*) and coriander oil (*Coriandrum sativum*).

1.3.1. Chemistry

EOs are substances typically containing 20-60 different components. Each EO (EO) is mostly composed of two to three major constituents present in high concentrations and trace amounts of minor ones. For example, EO obtained from peppermint (*Mentha x piperita)* may contain up to 60% of menthol and almost 20% of menthon. Further components in trace amounts are menthyl acetate, 1.8-cineole, limonene, beta-pinene and

beta-caryophyllene (Bakkali 2008; Schmidt 2009). Those major constituents determine the biological properties and activity of EO.

The EO components can be distinguished into two groups according to their biosynthetic origin. The first and major group is consisted of terpenes and terpenoids with skeleton composed of isoprene unit, the second group includes nonterpenoid volatile hydrocarbons (aromatic and aliphatic constituents), (Tisserand & Young 2013). Terpenes form structurally and functionally different classes. They are made from combinations of several 5-carbon-base units called isoprene. Terpenes can be divided based on the structure of the carbon chain to acyclic or cyclic; also according to the number of isoprene units per monoterpene. The monoterpenes contain two isoprene units, sesquiterpenes has three and diterpenes four. Other types of terpenes such as hemiterpenes, triterpenes and tetraterpenes occur rather rarely. If terpene contains oxygen it is called a terpenoid (Bakkali 2008; Tisserand & Young 2013). Monoterpenes are major representative molecules constituting 90% of the EOs offering great diversity of structures. They consist of following functions:

Acyclic terpenes: Carbures myracen (*Laurus nobilis)* and ocimen (*Ocimum basilicum*) belongs among the most frequently occurring acyclic monoterpenes in EOs. Acyclic monoterpenes include alcohols linalool (*Lavandula angustifolia*), geraniol (*Cymbopogon Martinii* var. *Motia)* citronellol, lavandulol and nerol. The aldehyde group comprise geranial, which creates 80% of EO from *Andropogon stratus* and is used in medicine for its antiseptic properties. Representatives of acyclic monoterpene esters are lineally acetate (*Lavandula angustifolia*), propionate or citronellal acetate (*Cymbopogon citratus*), (Bakkali 2008; Tisserand 2013; Bajalan 2014).

Monocyclic terpenes: Monocyclic terpenes include for instance carbures limonene (*Citrus* sp.), terpinenes, and p- cimene. Menthol is an alcohol contained in mint oils. Monocyclic alcohols are generally considered to have antiseptic property. Another monocyclic terpen is α-terpineneol (*Myristica fragrans*), carveol, terpinen-4-ol (*Melaleuca alternifolia*). Terpenic aldehyd perillaldehyde is cold-pressed from seeds of *Perilla frutescence.* From ketones it is important to mention menthone (*Mentha piperita*), carvone (*Carum carvi)*, piperitone (*Eucalyptus globulus)*, (Bakkali 2008; Tisserand 2013; Bajalan 2014).

Bicyclic terpenes: Bicyclic carbure sabinene occurs in *Myristica fragrans* and *Juniperus communis.* Others carbures are pinenes and camphene. *Artemisia* EO comprises alcohol thuyol and ketone thuyone, in which undesirable side effects such as mental disorders were identified. Another bicyclic terpene alcohol is borneol (*Rosmarinus officinalis*) or fenchol, example of byciclic ketone is camphor (*Cinnamomum camphora),* fenchone or thuyone (Velíšek 1999; Bakkali 2008; Tisserand 2013).

Sesquiterpenes: Sesquiterpenes are the main constituent of EOs containing 15 carbon atoms. The extension of the chain increases the number of cyclisation which allows a great variety of structures. Mostly, sesquiterpenes occur in monocyclic and bicyclic form. Usually, they are characterised by bitter and intense flavour. There are only few less significant representatives of acyclic sesquiterpenes.

Monocyclic sesquiterpenes: Among the most well-known monocyclic sesquiterpenes belongs y-bisabolen, which is found in *Origanum vulgare* and *Piper nigrum*, zingiberene and arcurcumene, are occurring in *Zingiber officinale* and α-humulen, which is part of the hops (Buckle 2003; Bakkali 2008; Baser 2010).

Bicyclic sesquiterpenes: Bicyclic sesquiterpene azulenes are found in *Matricaria chamomilla* and *Artemisia absintium* such as matricin or matricarin. After steam distillation and acidification, these substances convert to chamazulene, which causes a blue discoloration of the resulting EO. *Artemisia* is containing absintin, artabsin and in food industry it is used to produce absinth alcoholic beverages. Another example is β-kadinen (*Juniperus*), β-selinen (*Apium graveolens*) and β-caryophyllene (*Syzygium aromaticum*), (Berger 2003; Bakkali 2008; Baser 2010).

Aromatic compounds: They contain a basic frame of carbon and hydrogen with functional group, which is group of atoms creating shape determining the chemical properties of the molecule. The aromatic compounds occur less frequently than the terpenes. They include aldehydes mostly from EOs of *Cassia fistula, Citrus hystrix, Cymbopogon citratus* and also cinnamaldehyde from *Cinnamomum zeylanicum.* Aromatic alcohols are for instance cinnamic alcohol; phenols comprise chavicol and eugenol (*Syzygium aromatic*). Aromatic ethers occurring in EOs are estragol (*Artemisia dracunculus, Foeniculum vulgare),* safrol (*Sassafras albidum),* isosafrol (*Murraya koenigi)* and

myristicin (*Myristica fragrans*) in higher dosage hallucinogenic (Velíšek 1999; Berger 2007; Bakkali 2008).

1.3.2. Taxonomical distribution

The major EO bearing plant families with representative genera include Apiaceae (*Coriandrum, Foeniculum, Pimpinella*) annual, biennial, and perennial plants, with EOs stored in tubular ducts; Asteraceae (*Matricaria, Echinacea*) comprising of evergreen shrubs, rhizomatous herbs, tuberous perennials, and tree herbs; Cupressaceae (*Juniperus, Cupressus*) a group of conifers producing resin and EOs within woods; Lamiaceae (*Lavandula, Thymus, Rosmarinus*) aromatic herbs and shrubs with volatile compounds stored in glandular trichomes; Lauraceae (*Cinnamumum, Laurus*) mostly tree or shrubs containing large number of aromatic trees with EOs in cells within the bark, wood and leaves; Myrtaceae (*Syzygium, Melaleuca, Eucalyptus, Myrtus*) group of highly aromatic plants, including also fruits species; Pinaceae (*Picea, Cedrus*), conifers producing resin with acids, turpentine, and terpenoids; Piperaceae (*Piper, peperomia*) vascular plants and vines; Santalaceae (*Santalum*) with EOs in wood; and Zingiberaceae (*Zingiber, Amomum, Elettaria*) with aromatic rhizomes (Hunter 2009; Baser 2015).

1.3.3. Biological activity

The biological activities of EOs have been known since ancient times. They were used for prevention and treatment of diseases, food flavouring and preservation, and aromatherapy. During 20th century, the use of EO diminished as a result of development of organic chemistry; however, the demand for safe and natural alternative medicine had risen again with the concern about toxicity of synthetic compounds (Zink 1997; Gaysinsky & Weiss 2007). EOs provide a wide range of biological activity, especially antimicrobial, antioxidant, anti-carcinogenic, anti-inflammatory and many others described below. The antimicrobial effect of EO is due to its significance devoted to the following chapter.

Antioxidant activity:

Antioxidants are substances whose molecules are able to reduce the activity of free oxygen radicals and thus reduce the probability of their formation or convert them into

less reactive states. By this action, antioxidants reduce the oxidation process in the body of the organism or the environment in which they occur. The reactive forms of oxygen act on living organisms; the deleterious effects include cell membrane disintegration, mitochondrial disruption or disruption of membrane enzymatic activity. These reactive forms may also be a factor in the development of some degenerative diseases. A common form of reactive oxygen is hydrogen peroxide which causes, for example, lipid peroxidation as well as oxidative changes of deoxyribonucleic acid (DNA) in cells. Significant antioxidants that are found in nature are vitamins or EOs, especially flavonoids and terpenoids (Berger 2007; Tomaino et al. 2005).

Two main ingredients of *Thymus* spp. and *Origanum* spp. EOs, thymol and carvacrol, are shown to act as strong antioxidants (Tepe et al. 2004; Miguel 2010). In addition, *Curcuma zedoaria* EO, citronellal obtained from *Cymbopogon citratus* and eugenol were found to be excellent radical scavengers. Scavenging effect has been also described for neral and geranial from *Melissa officinalis* (Mau et al. 2003). According to Tomaino, the order of efficacy among the most common EOs with good radical-scavenging and antioxidant properties is in the order, clove>cinnamon>nutmeg>basil>oregano>thyme (Tomaino et al. 2005). In many cases, the antioxidant activity of the EOs could not be attributed to their major constituents, and minor compounds might play a significant role in the antioxidant activity, and synergistic effects were reported. For instance, in *Melaleuca* species, EO containing 1.8-cineole (34%) and ter-pinen-4ol (19%) exhibited stronger antioxidant activity than those with high methyleugenol (97%) or 1.8-cineole (64.30%) contents (Farag et al. 2004).

Antimutagenic activity:

Some of the EO has a clear antimutagenic ability, which is often associated with an anti-carcinogenic activity. It was found out that pro-oxidative activity of some EO components is very effective in reducing growth of localised tumours and in proliferation and apoptosis (cell death) of tumour cells, nevertheless they do not show any unfavourable changes nor toxic effects on healthy tissues (Schwartz 1996; Bakalli 2006). Generally, mutations can be prevented in various ways, including inhibiting the penetration of mutagens into cells, adding antioxidants, which inactivate the free radicals produced by mutagens, activating cell antioxidant enzymes, and detoxificating mutagens by activat-

ing enzymes with plant extracts (Ramel et al. 1986; Odin 1997). For instance, *Matricaria chamomilla* oil proved to inhibit compounds inducing mutagenic errors in mouse bone marrow cells (Hernandez-Ceruelos et al. 2002), *Melaleuca alternifolia* and *Lavandula angustifolia* EOs showed inhibitory effect against mutations induced in *E. coli* (Evandri et al. 2005). In addition, chromosomal damage in human lymphocytes can be protected by *Curcuma longa*, *Piper betel* or *Acacia catechu* (Ghaisas & Bhide 1994). Significant antimutagenic properties have been observed for many other plants such as *Helichrysum italicum, Ledum groenlandicum, Cinnamomum camphora* and *Origanum compactum,* which reported to be active against the urethane-induced mutations in *Drosophila melanogaster* (Idaomar et al. 2002; Mezzoug et al. 2007).

Anticarcinogenic activity

One of the most difficult challenges in chemotherapy is treatment of malignant cell growth leading to cancer. Plant molecules like taxol or eugenol are effective against cancerous cell proliferation. Various types of malignancies are reported to be lowered after treatment with plant EOs. For instance, geraniol from *Cymbopogon martini* was found to inhibit DNA synthesis and reduce the size of colon tumours, (Carnesecchi et al. 2004) whereas limonene and periodic acid remarkably reduce the lung metastatic tumour by around 65% (Bardon et al. 1998). Citral from *Cymbopogon citratus* and myristicin (*Myristica fragrans)* has been reported to be active against early stages of hepatocarcinogenesis. In addition, components of *Allium sativum* EO proved to have anticancer properties (Morit et al. 2000; Puatanachokchai et al. 2002). It was found out that poor-nutrient diet can lead to carcinogenesis. Isoprenoids contained in diet can play an important role in the prevention of civilisation diseases. Limonene obtained from citruses especially *Citrus limon* exhibited chemo-preventive effects against gastric cancer. EOs have also the ability to act as antioxidants and reduces overproduction of oxidative stress or increased cellular metabolism which can otherwise lead to tumour development (Czarnecka et al. 2006).

Anti- inflammatory activity:

EOs of *Ocimum sanctum* and *Aloe vera* are known to possess activity against inflammatory reactions for a long time (Singh & Majumdar 1997). EOs from *Eucalyptus globu-*

lus, *Rosmarinus officinalis*, *Commifora myrrha*, *Lavandula angustifolia* and S*yzygium aromaticum* provide inflammation preventive abilities (Darshan & Doreswamy 2004; Barbieri et al. 2013). Their antimicrobial ability is used during inflammatory reaction, when unfavourable reactive oxygen species are formed. According to Miguel, also *Citrus aurantium, Cinnamomum zeylanicum, Juniperus communis* and *Matricaria chamomilla* are considered to have anti-inflammatory properties (Miguel 2010).

Analgesic activity:

An analgesic is a substance used to relieve pain or achieve analgesia, painless condition without loss of consciousness. These substances have ability to reduce perception of pain but do not eliminate its cause. One of the analgesics contained in EOs is menthol, characteristic by its peppermint smell and taste. Usually, its use is external and topical. Menthol promotes blood circulation and cooling effect (Berger 2007). Other EOs that provide analgesic effects are obtained from the *Eucalyptus* species or *Syzygium aromaticum* (Silva et al. 2003; Cortés-Rojas 2014). Higher analgesic efficacy was also exhibited by *Lavandula hybrida,* administrated through the inhalators route. The responses to chemical and thermal stimuli was significantly reduced (Barocelli 2004).

Digestive activity:

One of the major uses of aromatic plants in medicine is for digestive disorders. Aromatic plants are usually used as an infusion or tea, and thus are delivered directly to the gastrointestinal system (Berger 2007). Aromatic plants and their EOs exert their digestive action by inhibiting gastric motility, releasing of bile from the gall bladder inducing the expulsion of gases from the stomach and intestine and more indirectly protecting liver function. For example, lavender EO is reported to affect the gastrointestinal function through activation of the vagus nerve (Barocelli 2004). The olfactory stimulation generated by lavender oil scent and its main component linalool activates gastric nerves that enhance food intake by rodents, while grapefruit oil fragrance and its main component d-limonene show the opposite effect (Shen 2005). *Satureja obovata*, *Acalypha phleoides*, *Melissa officinalis* and peppermint were reported to inhibit gastric motility (Hills & Aaronson 1991; Sadraei 2003).

Repellent activity:

The EOs have been extensively tested for their repellent activities, which are mostly linked to the presence of monoterpenes and sesquiterpenes (Sukumar 1991). In some cases, these chemicals can work synergistically (e.g. *Cymbopogon winterianus* with 5% vanillin), improving their effectiveness (Tawatsin et al. 2011). Among the plant families producing EO with repealing properties belong *Cymbopogon spp., Ocimum spp., and Eucalyptus spp.* The active compounds present in mixtures are α-pinene, limonene, cineole, citronellol, citronellal, camphor and thymol. These natural products have the potential to provide efficient protection against insects and are more human- and environmentally safer than synthetical ones (Gbolade 2000; Oyedele 2002; Yang 2004; Carroll 2006).

Toxicity:

Generally, alcohols, aldehydes and phenolic constituents are responsible for the cytotoxicity which is active against variety of virus, bacteria and fungi (Burt 2004; Rota et al. 2004; Hammer & Carson 2006). However, undesirable side effects such as irritation and sensitization of cells, acute toxicity to organ system, phototoxicity or carcinogenicity can occur and limit the medicinal use. Compounds such as thujone from *Artemisia absintihium* are known to exert toxic effect in humans leading to hallucinations, hepatic necrosis or ataxia (Vigan 2010). Some EOs are known to contain photoactive compounds. For example, *Citrus bergamia* contains psoralens which cause mutagenicity and cytotoxicity under the UV light (Averbeck 1990). Generally, EOs may be safe at low concentrations; however, they can display toxicity to humans at high concentrations (Sinha et al. 2014).

1.3.4. Antimicrobial activity

Nowadays, number of studies are investigating biologically active substances containing EOs. The reason is still increasing resistance of microorganisms against many antimicrobial agents. Antimicrobial activity of EO 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). Thus, EOs containing phenolic substances such as thymol or carvacrol

are the most effective in terms of effectiveness. Scientific literature states that these substances are active against a wide range of micro-organisms (Kalemba & Kunicka 2003).

According to Zaika (1988), *Cinnamomum verum* and *Syzygium fragrans* reported inhibitory activity against growth of the microorganism. The antimicrobial activity of cinnamon and clove oil is ascribed to eugenol, and cinnamaldehyde, which are the main compounds of EO of these spices. Cinnamon contains 0.5-1% of an EO containing 65-75% cinnamaldehyde and 8% eugenol. The cloves contain an average of 17% of EO, and this is composed of 93-95% eugenol (Farrell 1990; Vigil 2005).

It can be inaccurate to compare the results of similar studies, because the authors use various methods and different cultivation conditions to evaluate the antibacterial activity, nevertheless most of the results coincides with the antimicrobial effect of higher phenolic compounds (Zaika 1988; Vigil 2005).

As already mentioned, some of phenolic compounds have a broad antimicrobial spectrum of action. These include thymol, cinnamaldehyde and eugenol. Their antimicrobial activities have been reported against many bacteria whereas gram-positive bacteria were found to be more susceptible to action of phenolic compounds. Phenol derivatives are compounds which, in their structure, contain a phenol molecule and one or more substituents. It has been shown that this chemical change may lead to an increase in the antimicrobial activity of phenolic compounds (Beuchat & Golden 1989; Vigil 2005).

Cinnamaldehyde, EO obtained from the bark of *Cinnamomum verum* have a strong inhibitory effect as well on the eukaryotic microorganisms namely *Aspergillus flavus, Aspergillus parasiticus, Aspergillus versicolor*, and *Aspergillus ochraceus*. The inhibitory effect was also observed by a Bullerman (1974) who stated, that the content of 1-2% ground cinnamon in the liquid medium did not completely inhibit the growth of *Aspergillus parasiticus,* however, it reduced the production of aflatoxins by 99%. The same author, in his next study, demonstrated that cinnamic acid had an inhibitory effect on *A. parasiticus* already at a concentration of 200 ppm and cinnamaldehyde with MIC 150 ppm (Bullerman 1974; Mahmoud 1994; Vigil 2005).

Usually, major components are mainly responsible for the antibacterial activity in most of the EOs; nevertheless, there are some studies where whole EOs have a higher antibacterial activity than the combination of the major isolated components, indicating that minor components are critical to the activity, probably by producing a synergistic effect (Burt 2004; Mastelic et al. 2005).

Synergistic effect was reported for thymol and carvacrol. The combination of citral with vanillin, eugenol, thymol or carvacrol on growth inhibition of *Zygosaccharomyces bailii* showed better results (Rivera-Carriles 2005). Also 1.8- cineole and camphor had higher antimicrobial effect on *Candida albicans* than if tested individually (Viljoen et al. 2003). On the other hand, antagonism was observed between *p*-cymene, thymol and carvacrol in the oil of *Lippia chevalieri* (Bassole 2003).

Antimicrobial activity can be divided according to the microorganisms they act primarily against.

Antiviral activity

Viral diseases are still a major problem for human health worldwide. With the increasing virus resistance there is a need for new active compounds against those infections. Natural products can offer a new source of antiviral agents. It is evident, that many EO possess antiviral properties against many DNA and RNA viruses, such as herpes simplex virus type 1 and 2 (HSV-1, HSV-2) or dengue virus type 2 (Wagstaff et al. 1994; Allahverdiyev et al. 2004; Reichling et al. 2009). Inhibitory activity against herpes virus was reported for Eucalyptus globulus and Thymus vulgaris (Reichling et al. 2005; Schnitzler et al. 2007). Melissa officinalis containing citral and citronellal inhibited the replication of HSV-2, whereas lemongrass reported activity against HSV-1 at concentration 0.1% (Armaka et al. 1999). Melaleuca alternifolia exhibits efficacy against recurrent herpes virus infections (Carson et al. 2001). Origanum vulgare showed antiviral activity against yellow fever virus at 3.7 g/mL (Meneses et al. 2009).

Antifungal activity

Some EOs have demonstrated a broad range of natural fungicidal effects against postharvest pathogens. Three members of apiaceae family showed variable anti-Candida

albicans activity with a trend of coriander > anise > fennel; with the MICs of 0.25%, 0.5% and 1%, respectively (Hammer et al. 1999). Other EO active against *C. albicans* with the concentration between 0.01- 0.15% are from *Cinnamomum verum, Cymbopogon citratus*, aerial parts from *Zingiber officinale*, clove oil, geraniol (Devkatte et al. 2005; Hammer & Carson 2011). The antifungal activities of EOs could be 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). *Aspergillus parasiticus* growth and aflatoxin production have been inhibited by the EOs of *Thymus vulgaris* and *Citrus aurantifolia*, whereas *Mentha spicata*, *Foeniculum miller*, and *Artemisia dracunculus* inhibited fungal growth only. *Carum carvi* controlled aflatoxin production without effect on fungal growth (Razzaghi-Abyaneh et al. 2009).

Antibacterial activity

Plant EO and their major chemical constituents are potential candidates as antibacterial agents (Nazarro et al. 2013). They may inhibit the growth of bacteria or destroy bacterial cells. Active compounds from *Cinnamomum verum*, *Syzygium aromaticum*, *Thymus vulgaris*, *Origanum vulgare*, and *Rosmarinus officinalis* oils were shown to have strong antibacterial activity against *Salmonella typhi*, *S. aureus*, and *Pseudomonas aeruginosa*. The clove oil was found to be the most effective (Conner et al. 1993). Active compounds of these plants were carvacrol, thymol, cinnamic aldehyde, eugenol, and *p*-cymene. Carvacrol, eugenol and thymol were also reported 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 and was reported to inhibit *S. aureus* and *Providencia stuartii* (Fraternale et al. 2005).

Activity against *S. aureus*

There are many reports regarding antibacterial activity of EO-bearing plants 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. The main reason is that the lipophilic molecules in the aqueous phase associate to form micelles and thus suppress the attachment of the EOs to the organisms,

whereas the vapour phase allows free attachment (Inouye et al. 2003). Several authors described antibacterial activity of EO in vapour phase. For exemple in study of Nedorostova et al. (2009) *Armoracia rusticana* and *Mentha piperita* showed some degree of antistaphylococcal effect. Other study presented that mixture of geranium with lemongrass or grapefruit EO significantly reduced MRSA. It was found that the citrus EO vapour reduces formation of biofilms by both- methicillin resistant and methicillin susceptible strains of *S. aureus* (Laird & Phillips 2012). In addition, thymoquinone vapors have been reported to inhibit growth of *S. aureus* (Novy et al. 2014; Rondevaldova et al. 2017).

1.4. Chemotaxonomical approach to drug discovery

The search for new, natural compounds is growing, mainly due to the acquired resistance of microorganisms to commonly used drugs and because nosocomial infections caused by these microorganisms have increasingly resulted in public health problems. It is estimated that microbial resistance develops within seven to eight years of regular antibiotic use (Wang 2008).

Medicinal plants constitute a valuable source of potential medicines for all of humanity. It is well known that many of these plants contain potent biologically active compounds and at least 25% of the drugs presently used in modern medicine are derived from plants. The use of medicinal plants for the benefit of mankind is timeless and is undoubtedly increasing and their taxonomic categorization is essential for the better understanding of plant community, their origin and association with each other (Silva et al. 2013).

Nature consists of variable living components of the environment having useful, harmful and inactive chemical constituents in form of secondary metabolites, (e.g. alkaloids, steroids, amino acids, etc.) that are derived from primary metabolites. The chemical structure of these compounds is often specific and restricted to taxonomically related organisms. Despite the fact that plants are usually classified according to the morphology and anatomy, there is a new chemotaxonomical approach that categorise plants based on chemical constituents. This concept was partly used in past centuries when variation in metabolic profile was used for classification purposes by folk taxonomist (Singh

2016). Chemotaxonomic knowledge can undoubtedly contribute to existing data about some genera and bring new information about secondary metabolites of plant species to the taxonomic classification. Also the methods used for this type of categorisation is much more sophisticated, and are able to estimate quantitative and qualitative composition of plant material up to trace amount of compounds (Jantan 2004).

Currently, one of major challenges is the selection of plants for a bioprospecting study because this process is the first research step. There are three main methods used to select plants for biological or phytochemical investigation. Namely they are ethnoparmacological, chemotaxonomical and random selection approaches (Lahlou 2003). Random selection, involves the arbitrary collection of the species without consideration, and ethnopharmacological applies information about the traditional use of plant drugs to treat specific diseases. Firstly, the validity of indigenous use is confirmed, subsequently, the search for modern drugs from plants on the basis of their indigenous uses follows. Chemotaxonomic approach relies upon the fact that taxonomically related plants often biosynthesize chemically similar secondary metabolites and studies plants of the same family or genus from which active compounds have been already isolated (Silva et al. 2013). Certain families known to be rich in the kind of specific secondary compounds that tend to be medicinally relative are for example, *Rubiaceae, Solanaceae, Leguminosae, Raunuculaceae, Berberidaceae, Papaveraceae,* containing biodynamically active alkaloids. Screening of the members of taxonomic alliances might yield more positive results than random selection approach (Wang 2008). The collection of different parts of plants with medicinal activity and of plants belonging to the same genus as those active plants is the primary focus of many reseachers. This strategy seems to be a most promising approach for the efficient use of renewable natural resources.

The rich source of secondary metabolites, especially effective terpenes are EO- bearing plants belonging mostly to families Apiaceae, Asteraceae, Cupressaceae, Lamiaceae, Lauracaee, Myrtaceae, Pinaceae, Piperaceae and Zingiberaceae (Baser 2005; Hunter 2009).

The methods of antimicrobial susceptibility testing in vapour phase

Until now, only a few methods have been used for the assessment of the antimicrobial

activity of EOs in vapour phase. The most frequently used is the disc volatilization test, where the EO impregnated filter disc is placed on the lid of the Petri dish and inhibition zones are measured (Maruzzella & Sicurella 1960). Its various modifications using sterile adhesive tape, agar sealing on the lid or four section Petri dishes have also been developed. Within other assay, the EO and microorganisms are placed separately into a sealed environment such as jar which means that several organisms can be tested for susceptibility to one EO vapour at the same time (Laird 2011). Seo (2015) introduced airtight experimental apparatus for simultaneous evaluation of antimicrobial activities of EO gases at various concentrations. Apparatus consists of upper chamber with seven wells containing a solid medium with the target microorganism and lower chamber containing gaseous EO generated from liquid EO. Although all of these assays are commonly used, they are not designed for high-throughput screenings.

Recently, novel method enabling the rapid and simultaneous evaluation of growth inhibitory properties of volatile compounds (such as carvacrol, cinnamaldehyde, eugenol, 8-hydroxyquinoline, thymol, thymoquinone) in liquid and vapour phase was introduced by Houdková (2017). This broth microdilution volatilization method uses 96 microplates with tight- fitting lids, alows testing more agents against different strains of organisms at the same time. Howewer, the application of this method for testing of EOs has not experimentaly been verified yet. Thus, we deciced to determine antistaphylococcal effect of EOs obtained from Cambodian aromatic plants using the broth microdilution volatilization method.

2. Objectives

The main aim of this work is to determine *in vitro* growth-inhibitory effect of EOs distilled from various parts of Cambodian plants species belonging to Lauraceae and Zingiberaceae families, against 5 representatives of standard strains and clinical isolates of *Staphylococcus aureus* by the broth microdilution volatilization method.

The specific objectives are:

a) Isolation of EOs from Cambodian chemotaxonomically related plant species;

b) Evaluation of broth microdilution volatilization method for testing of EOs in vapour and liquid phase;

c) Determination of MICs of isolated EOs for *S. aureus* in liquid and vapour phase.

3. Material and methods

3.1. Plant material

The plant species were selected according to their chemotaxonomical relationship belonging to Lauraceae and Zingiberaceae families. Then, reference specimen sheets with botanical descriptions, natural habitat, and illustrations were elaborated. Plant samples were subsequently collected in several regions of Cambodia, namely in Kampot (Bokor hill), Pursat (Pramaoy) and Aoral (marked in Figure 1.) during the end of April- May 2017. Collected plant species (see Figure 10.-16.) were identified by Mr. Phourin Chhang from Institute of Forest and Wildlife Research and Development, The Forestry Administration, Mininstry of Agriculture Forestry and Fisheries, Phnom Penh, Cambodia (Lauraceae) and Dr. Jana Leong- Škorníčková (Research & Conservation, Singapore Botanic Gardens, Singapore) - Zingiberaceae. Voucher specimens were also collected for further deposition at Czech University of Life Sciences. All of the collected plant material (leaves, rhizomes) were air- dried at room temperature and subsequently send by post to CULS, where further laboratory processing and testing took place.

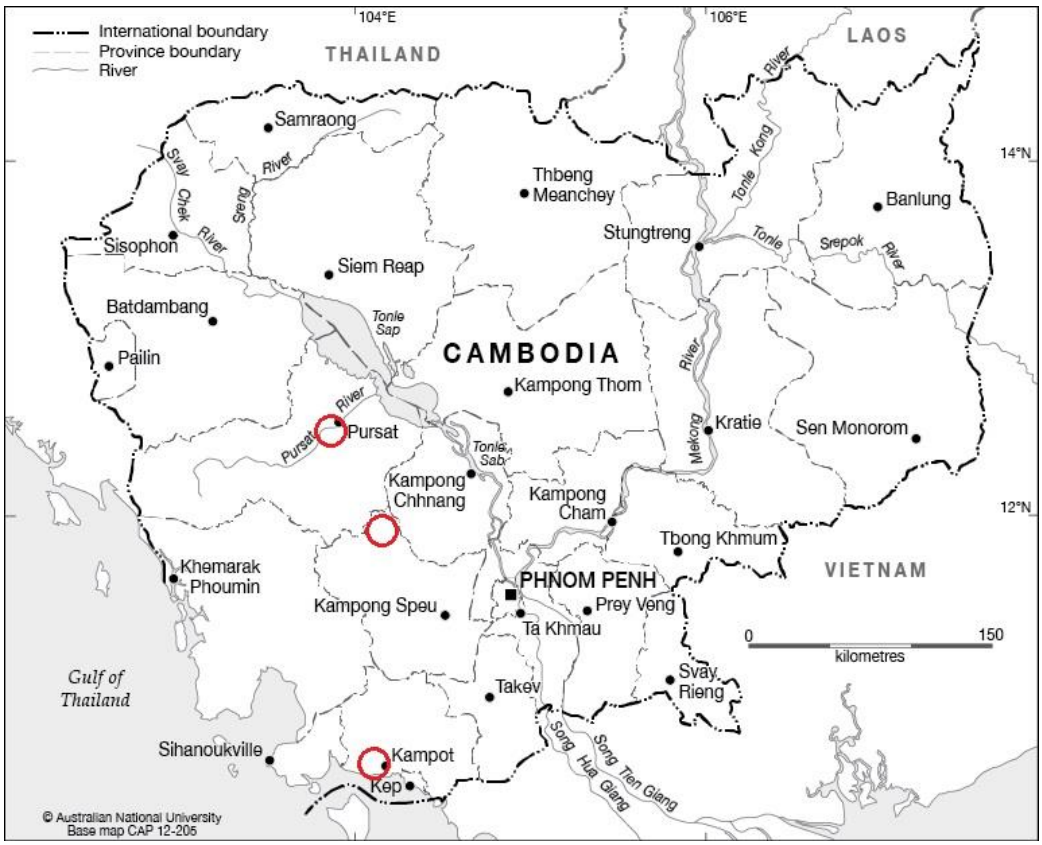

Figure 1. Areas of collection of the plant material

3.2. Distillation of EOs

Plant material was dried (see Figure 9.) 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 H_2O in ratio 1:20 using Clevenger apparatus (Merci, Brno, Czech Republic according to the procedures described in the European Pharmacopoeia (European pharmacopoeia 2013). The EO was then collected, dried using anhydrous sodium sulphate (Merck, Darmstadt, Germany) and stored at 4°C in airtight glass vials.

3.3. Chemicals

For preparation of media and subsequent antimicrobial assay following chemicals were used. Dimethyl sulfoxid- DMSO (Penta, Prague, CZ), ethanol 96% pharmacological grade (Penta, Prague, CZ), Mueller-Hinton agar (Oxoid LTD., Basingstoke, UK), Mueller-Hinton broth (Oxoid LTD., Basingstoke, UK), NaCl- Sodium chloride (Sigma-Aldrich, Prague, CZ), KCl- Potassium chloride (Sigma- Aldrich, Prague, CZ), Tris base (Sigma- Aldrich, Prague, CZ), Oxacilin monohydrate sodium salt (Sigma- Aldrich, Prague, CZ) and Thiazol blue tetrazolium bromide (Sigma- Aldrich, Prague, CZ).

3.4. Microorganisms and media

Antistaphylococcal activity was tested against 5 strains of gram-positive *S. aureus*, namely SA 29213, SA 25923, SA 43300, TRSA1 and TRSA2, which were selected according to their resistance pattern and obtained from the American Type Culture Collection – ATCC, (Oxoid, Basingstoke, United Kingdom).

For preparation of bacteria, 7 mL of pure Mueller-Hinton broth was inoculated by 1 mL of selected *S. aureus* strain and stored for 24 hours at constant temperature (37°C) in thermostat. Then, immediately before testing, prepared microorganism strain was suspended in 10 mL of pure broth and turbidity of suspension was increased to 0.5 McFarland standard, using Densi-La-Meter II, purchased from Lachema, Brno, CZ (McFarland 1907).

In order to test anti-staphylococcal effect in volatilisation stage, agar solution as a solidifying agent had to be prepared. 3.8 g of agar was suspended in 100 mL of distilled wa-

ter, shortly boiled to dissolve the medium properly and sterilised by autoclave at 121°C for 15 minutes. When the temperature cooled down to room temperature, agar was stored at 4°C in refrigerator.

Two types of cultivation media were prepared. The first- pure broth- was prepared by disolving of 2.1g Mueller-Hinton broth in 100 mL of distilled water. Subsequently, bufferd broth was prepared by mixing 0.8 g NaCl, 0.02 g KCl, and 0.61 g Tris Base with 100 mL of distilled water. Solution was equilibrated by 35% HCl to obtain pH 7.6. Both media were sterilized by autoclave at 121°C for 15 minutes and stored in refrigerator.

3.5. Antimicrobial assay

In vitro antistaphylococcal activity was measured by the broth microdilution method described by Clinical and Laboratory Standards Institute (CLSI 2009) with modifications for simultaneous determination of antibacterial potential of plant volatiles in the liquid and vapour phase according to Houdkova et al (2017). For the antimicrobial assay microtiter plates with 96 flat-bottomed wells and flanged lids were used. During the test outer wells and flanges were left empty to prevent edge effect that could influence the results of an assay.

Flanges of the lids were inoculated by 5 µL of bacteria except 2nd column which was skipped as an indicator of purity control. Subsequently, 30 µL of autoclaved liquid agar was pipetted (see Figure 3. and 4.). In the second part of this method, each sample of EO was dissolved in calculated amount of DMSO (100%) to create stock concentration 102400 µg/mL and further diluted in buffered broth medium. Seven two-fold serially diluted concentrations of samples starting from 1024 µg/mL were prepared for all compounds (see Figure 2.). The final volume in each well was 100 µg. The plates were then inoculated with bacterial suspensions. The wells containing inoculated and non-inoculated broth were prepared as growth and purity controls. To evaluate susceptibility of microorganism strain, oxacillin (86.3% efficiency) as an antibiotic control was used. Low amount of oxacillin was dissolved in required amount of distilled water to achieve stock concentration (200 µg/mL). Subsequently, six two-fold serially diluted concentrations starting from 2 µg/mL was prepared. Finally, microtiter plates were fastened by

wooden pads (see Figure 5.) and metal clamps, purchased from Lux Tool, Prague, CZ and incubated at 37°C for 24h.

Minimum inhibitory concentrations were evaluated by visual assessment of bacterial growth after colouring of metabolically active bacterial colony with thiazolyl blue tetrazolium bromide dye (MTT) in concentration 600 μg/mL (Sigma-Aldrich, Prague, CZ). Dye in volume of 20 μL and 25 μL was applied into flanges of the lid and wells, respectively. Wells with absent antimicrobial activity stained blue, however those with positive antimicrobial activity stayed yellow (see Figures 6. and 7.). The wells with the lowest concentration that had the same colour as the purity control were identified as the MIC.

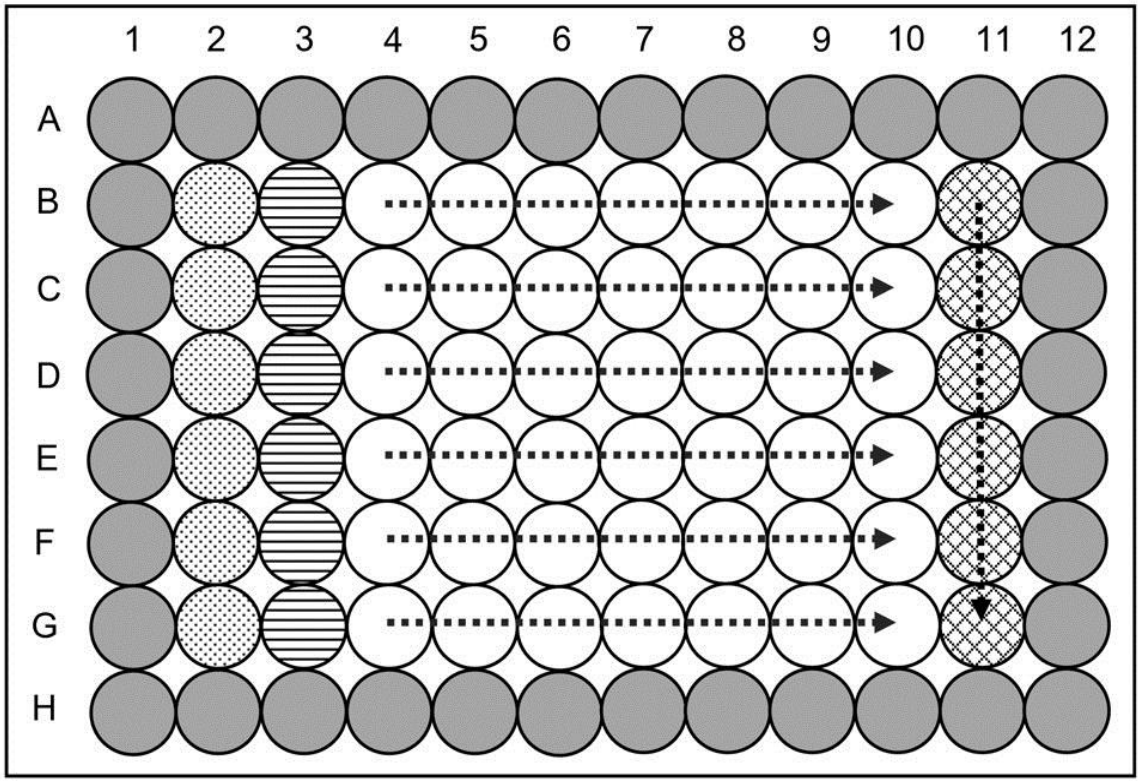

Figure 2. Schematic design of experiment: flat-bottom wells (Houdkova 2017) demonstrating: Grey-coloured wells: empty wells (not used); dotted wells: purity control; striped wells: growth control; white coloured wells: serial two-fold dilution of tested volatile compounds; gridded wells: serial two-fold dilution of positive antibiotic control.

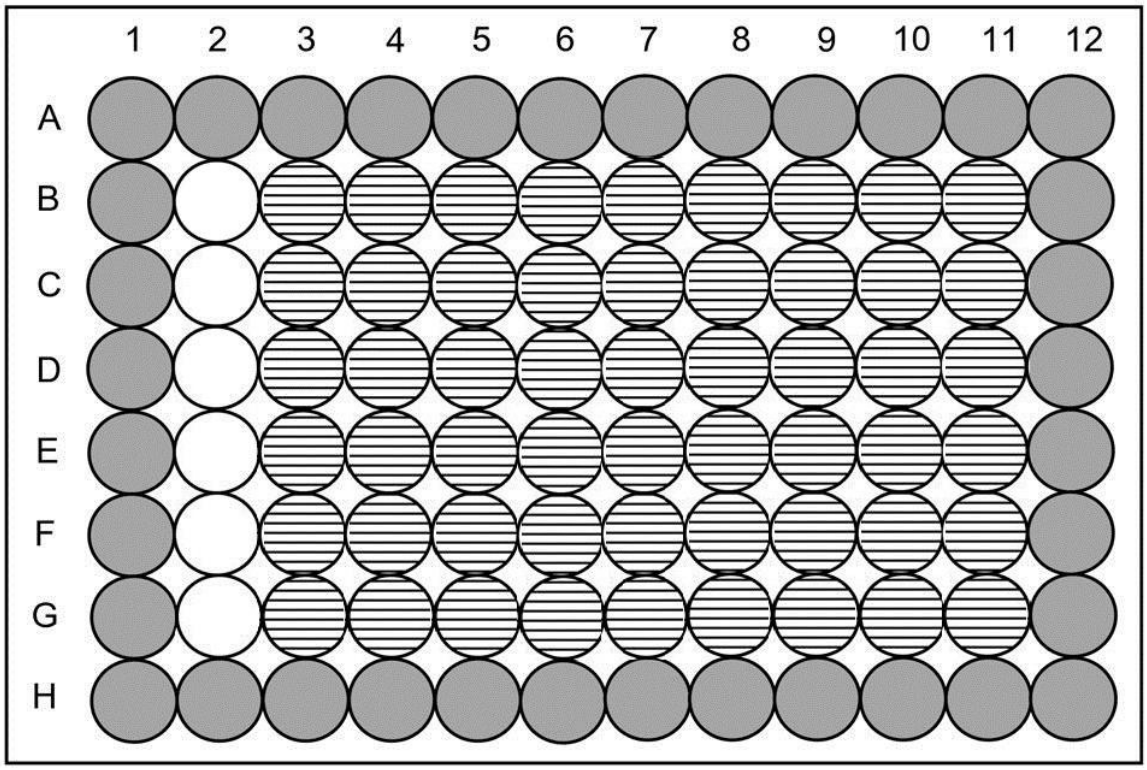

Figure 3. Schematic design of experiment: flanged lids demonstrating: Grey-coloured wells: empty wells (not used); white coloured wells: purity control (agar); striped wells: agar and bacteria (Houdkova 2017).

4. Result and discussion

Physical characteristics of EOs

In total, 12 EOs from different plant parts (leaves or rhizomes) of 10 species were distilled. The highest yields of EO were obtained from dried leaves of *C. bonni* (4.14%), *C. dimorphandrum* (3.20%) and *N. zeylanica* (1.08%). The yield of remaining oils was less than 1% in the order *A. pierreanum rhizomes* > *C. plicata* rhizomes > *G. pierreana* rhizomes > *C. plicata* leaves > *G. adhaerens* leaves > *C. singularis* leaves > *A. pierreanum* leaves > *Machilus bokorensis* leaves > *G. pendula*. Not only yield, but also the density and colour of EOs were different. In correspondence with our results, various authors have previously isolated EOs from *C. singulars* (Cuong et al. 2017), *C. plicata* (Rachkeeree et al. 2018), *G. pendula* (Shaari 2009) in amounts lower than 1%. According to our best knowledge, EOs from *A. pierreanum, C. bonni, C. dimorphandrum, G. adhaerens, G. pierreana,* and *M. bokorensis* were distilled for the first time. However, physical characteristics of previously distilled EOs are not described in the literature.

Most oils have less density compared to water, nevertheless two exceptions were found- EOs from *C. dimorphandrum* and *C. plicata* rhihomes. Interestingly, EO obtained from another part (leaves) of *C. plicata* did not show this characteristic. The oils also possess a different colour and smell. In general, all the EOs were limpid and colourless after 20 minutes of distillation, however with a prolonged time the colour changed. Interestingly, the oil obtained from rhizomes of *C. plicata* had blue-violet colour (see Figure 8.). *N. zeylanica* and *C. bonii* had pleasant smell and were almost colourless. Other oils had more or less intense shade of yellow. *C. dimorphandrum* oil had a pale yellow colour and eugenol-like smell. Oils of *A. pierreanum*, leaves of *C. plicata* and *G. pendula* were pale yellow and clear, *G. pierreana* slightly turbid. *M. bokorensis* had a pale greenish yellow colour, *G. adhaerens* intense yellow and *C. singularis* yellow-orange.

Table 1. Characteristics of plant materials and EOs

Plant species	VSN[a]	Area of collection	Date of collection	Plant part	Yield (%)	Colour
Amomum pierreanum Gagnep.	2521KBFR1	Aoral	13.5.2017	L	0.25 %	pale yellow
				R	0.96 %	pale yellow
Cinnamomum bonii Lecomte	2522KBFR2	Bokor	1.6.2017	L	4.14 %	colourless
Cinnamomum dimorphandrum Yahara & Tagane sp.nov.	2523KBFR3	Bokor	29.4.2017	L	3.20 %	pale yellow
Curcuma plicata Wal. Ex Baker	2524KBFR4	Pramaoy	25.5.2017	L	0.40 %	pale yellow
				R	0.73 %	blue- violet
Curcuma singularis Gagnep.	2525KBFR5	Aoral	12.5.2017	R	0.27 %	yellow- orange
Geostachys pierreana Gagnep.	2526KBFR6	Bokor	1.6.2017	R	0.44 %	pale yellow
Globba adhaerens Gagnep.	2527KBFR7	Bokor	1.6.2017	L	0.30 %	intense yellow
Globba pendula Roxb.	2528KBFR8	Pramaoy	23.5.2017	L	0.12 %	pale yellow
Machilus bokorensis Yahara & Tagane	2529KBFR9	Bokor	29.4.2017	L	0.24 %	greenish yellow
Neolitsea zeylanica (Nees & T. Nees) Merr.	2530KBFR1	Bokor	29.4.2017	L	1.08 %	colourless

*yield calculated by formula EO (distilled) mL/g (weight of dry sample) x 100%; [a]VSN: voucher specimen number.

Table 2. Antistaphylococcal activity of EOs in liquid and vapour phase against five strains of *Staphylococcus aureus*

Plant species	PP[a]	Bacterium/growth medium/MIC[b] (μg/mL)									
		SA 29213		SA 25923		SA 43300		TRSA 1		TRSA 2	
		Broth	Agar	Broth	Agar	Broth	Agar	Broth	Agar	Broth	Agar
A. pierreanum	L	1024	-	-	-	1024	-	1024	-	1024	-
	R	-	-	-	-	-	-	-	-	-	-
C. bonii	L	1024	-	-	-	-	1024	-	1024	-	-
C. dimorphandrum	L	1024	-	-	-	-	1024	-	1024	1024	-
C. plicata	L	-	-	-	-	-	-	-	-	-	-
	R	-	-	-	-	-	-	-	-	-	-
C. singularis	R	-	-	-	-	-	-	-	-	-	-
G. pierreana	R	-	-	-	-	-	-	-	-	-	-
G. adhaeres	L	-	-	-	-	-	-	-	-	-	-
G. pendula	L	-	-	-	-	-	-	-	-	-	-
M. bokorensis	L	-	-	-	-	-	-	-	-	-	-
N. zeylanica	L	-	-	-	-	-	-	-	-	-	-

[a]PP, plant part (L, leaf, R, rhizome); [b]MIC: minimum inhibitory concentration.

Evaluation of broth microdilution volatilisation method for EOs testing

Detailed results of *in vitro* growth-inhibitory effect of plant essential oils against *S. aureus* in liquid and vapour phase using the broth microdilution volatilization method showed that differences in sample testing were always within the range of three concentrations which corresponds to the CLSI standards of the MICs determination (see Table 3.)(CLSI 2015). Therefore the method is generally suitable for testing of the essential oils.

The novel broth microdilution volatilization method which was used for our research was introduced by Houdkova et al. (2017), as a result of lack of reports regarding antibacterial activity of volatile compounds in vapour phase and absence of efficient methods for its evaluation. In the past decades, several methods for testing of the antimicrobial activity of EOs have been used, for example disc volatilization test with various modifications using Petri dishes, sterile adhesive tape and agar sealing on the lid, how-

ever they enable only screening of limited number of samples, some needs special equipment wich is not commonly available and increase material consumption (Kloucek et al. 2012). This is the first study using broth microdilution volatilization method for testing of EOs antimicrobial effect. According to our results this method was confirmed to be suitable for rapid simultaneous determination of antibacterial potential of EOs in the liquid and the vapour phase at different concentrations.

Anti-staphylococcal effect of isolated EOs in liquid and vapour phase

The final results of antistaphylococcal activity of essential oil-bearing plants are displayed in the Table 2. From all tested compounds three EOs possessed certain degree of antibacterial activity in broth, two of them in agar as well. The most effective and abundant antistaphylococcal activity possessed EO from *A. pierreanum*, which inhibited growth in 4 strains of *S. aureus* including MRSA (SA 43300) in liquid medium with MIC 1024 μg/mL. Further prospective EO from C. *dimorphandrum* was active against SA 29213 and TRSA 2 in liquid phase as well as against SA 43300 and TRSA1 in vapour phase at the concentration 1024 μg/mL. In addition, *C. bonii* proved activity against three strains, specifically against SA29213 in broth phase and in SA 43300 and TRSA1 in agar all with MIC 1024 μg/mL. *C. plicata, C. singulars, G. pierreana, G. adhaerens, G. pendula, M. bokorensis, N. zeylanica* did not show any antibacterial effect to *S. aureus*. There is no evidence of any previous antimicrobial testing of above mentioned EO-bearing plants, however, there are several reports confirming *Cinnamomum* and *Amomum* species to have antistaphylococcal property. For instance, cinnamaldehyde from *C. osmophloeum* leaves possessed strong activity with MIC 250 μg/mL using broth microdilution method. It has also been demonstrated that *C. zeylanicum* bark oil has an inhibitory activity against *S. aureus* at concentration 250 μg/mL MIC, and is able to prevent food spoilage and the growth of pathogenic bacteria (Ouattara et al. 1997; Chang 2001). Thomas (2012) reported that *C. verum* contains 75-85% of the microbial-active phenolic compound eugenol. Since *C. dimorphandrum* demonstrated the biological activity and is taxonomically related to *C. verum* there is a probability, that major compound of *C. dimorphandrum* oil is eugenol as well. According to Buru et al. (2014) *C. impressicostatum* stem-bark water extract demonstrated strong anti-microbial effect against MRSA as well as *A. subulatum* fruit extract (Yang et al. 2008). To our best knowledge this is the first report regarding biological activity

of *A. pierreanum, C.Bonii, C. dimorphandrum, G. pierreana, G. adhaeres* and *M. bokorensis.*

Although *C. plicata, C. singularis, G. pendula and N. zeylanica* did not show any antistaphylococcal properties, there are some studies about their antimicrobial activity or use in traditional medicine. *C.plicata* and *G. pendula* rhizomes are traditionally used in Asia to treat stomach ailment, flatulency and constipation (Sirirugsa 1998; Ahmad 2017). Furthermore, phytochemical investigation on *G. pendula* resulted in isolation of a compound isoandrographolide which has demonstrated strong cytotoxic properties towards a panel of cancer cell lines (Shaari et al. 2009). According to available literature, only *C. singularis* and *N. zeylanica* have been already screened for their chemical compositions. Rhizomes of *C. singularis* are reported to possess antibacterial, antifungal, antiviral, and anti-inflammatory activities important for the treatment of rheumatism, gastrointestinal illness, and dermatological diseases. EO comprises of 68 compounds and inhibits growth of *Bacillus subtilis* and *Escherichia coli*. The active compounds from *N. zeylanica* are mainly sesquiterpenes (zeylanicine, zeylanidine, linderalactone) with described anti-inflammatory activity. The roots of the plant are used to treat rheumatic arthralgia in folk medicine (Joshi 1967).

The microdilution volatilization method used for an antimicrobial assay was designed recently thus the scientific literature is still limited. The fact that the oils comprise a number of volatile components makes them ideal for research investigation. For bacteria, it is more difficult to develop resistance to the multi-component mixtures (e.g. essential oils) than to single-ingredient conventional antibiotics (Santos and 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* EO, *Melaleuca alternifolia* EO, *Cymbopogon citratus* EO and others including thyme, fennel and lavender (Laird and Phillips, 2012). This can be a case of *C. bonii*, which performed higher antistaphylococcal activity in agar than broth medium. According to our best knowledge this is the first time *A. pierreanum, C. bonii* and *C. dimorphandrum* were tested and proved to have staphylococcal activity in vapour or liquid phase.

5. Conclusions

In this study, antistaphylococcal effects of EO-bearing Cambodian plants have been tested against 5 strains of *S. aureus.* 12 Essential oils from various plant parts of 10 species were obtained. To our best knowledge, EOs from *A. pierreanum, C. bonni, C. dimorphandrum, G. adhaerens, G. pierreana,* and *M. bokorensis* were distilled for the first time. The novel broth microdilution volatilization method was used for assessment of their growth inhibitory effect. In liquid phase, the lowest MIC were observed for *A. pierreanum* against four strains of *S. aureus* with value 1024 μg/mL and *C. dimorphandrum* at the same concentration against two strains. Volatile phase showed positive results in case of *C. dimorphandrum* and *C. bonii* that inhibited growth of two strains including methicillin resistant one at MIC 1024 μg/mL. In addition, broth microdilution volatilization method was found to be promising for rapid simultaneous determination of antibacterial potential of EOs in the liquid and the vapour phase at different concentrations. *A. pierreanum* and *C. dimorphandrum* are suggested as prospective EO-bearing plants with antistaphylococcal effect, effective also against methicillin resistant strain. 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, veterinary and food preparations that are based on volatile antimicrobials designed to overcome the resistance of *S. aureus*.

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ANNEX 1: Detailed results of anti-staphylococcal activity of Cambodian plants

Table 3. Antistaphylococcal activity of EOs in liquid and vapour phase

Plant species	PP[a]	Bacterium/grown medium/MIC (µg/mL)									
		SA 29213		SA 25923		SA 43300		TRSA 1		TRSA 2	
		Broth	Agar	Broth	Agar	Broth	Agar	Broth	Agar	Broth	Agar
AP	L	1024	-	-	-	1024	-	1024	-	1024	-
		1024	-	-	-	1024	-	512	-	1024	-
		1024	-	-	-	1024	-	1024	-	1024	-
	R	-	-	-	-	-	-	-	-	-	-
		-	-	-	-	-	-	-	-	-	-
		-	-	-	-	-	-	-	-	-	-
CB	L	1024	-	-	-	-	1024	-	1024	-	-
		1024	-	-	-	-	1024	-	1024	1024	-
		1024	-	-	-	-	-	-	-	-	-
CD	L	1024	-	-	-	-	1024	-	1024	1024	-
		1024	-	-	-	-	1024	-	1024	1024	-
		1024		-	-	-	1024	-	512	1024	-
CP	L	-	-	-	-	-	-	-	-	-	-
		-	-	-	-	-	-	-	-	-	-
		-	-	-	-	-	-	-	-	-	-
	R	-	-	-	-	-	-	-	-	-	-
		-	-	-	-	-	-	-	-	-	-
		-	-	-	-	-	-	-	-	-	-
CS	R	-	-	-	-	-	-	-	-	-	-
		-	-	-	-	-	-	-	-	-	-
		-	-	-	-	-	-	-	-	-	-
GeP	R	-	-	-	-	-	-	-	-	1024	-
		-	-	-	-	-	-	-	-	-	-
		-	-	-	-	-	-	-	-	-	-
GA	L	-	-	-	-	-	-	-	-	-	-
		-	-	-	-	-	-	-	-	-	-
		-	-	-	-	-	-	-	-	-	-
GP	L	-	-	-	-	-	-	-	-	-	-
		-	-	-	-	-	-	-	-	-	-
		-	-	-	-	-	-	-	-	-	-
MB	L	-	-	-	-	-	-	-	-	-	-
		-	-	-	-	-	-	-	-	-	-
		-	-	-	-	-	-	-	-	-	-
NZ	L	-	-	-	-	-	-	-	-	-	-
		-	-	-	-	-	-	-	-	-	-

PP[a]: Plant part; L: Leaves; R: Rhizomes; *AP Amomum pierreanum*; *CB: Cinnamomum bonii; CD: Cinnamomum dimorphandrum*; *CP: Curcuma plicata; CS: Curcuma singularis; GeP: Geostachys pierreana*; *GA: Globba adhaerens; GP: Globba pendula; MB: Machilus bokorensis; NZ: Neolitsea zeylanica;* - ≥1024

ANNEX 2: Photographic illustrations of plant samples

Figure 4. Lid and plate preparation

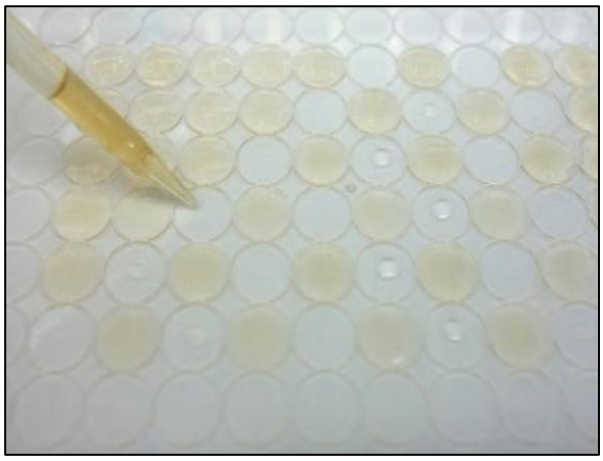


Figure 5. Fixing plate and lid together

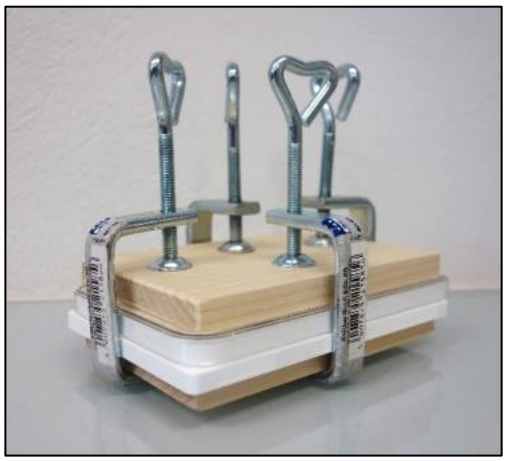


Figure 6. Determination of MIC- wells

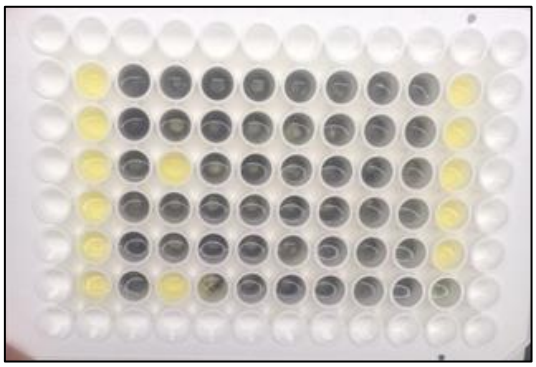
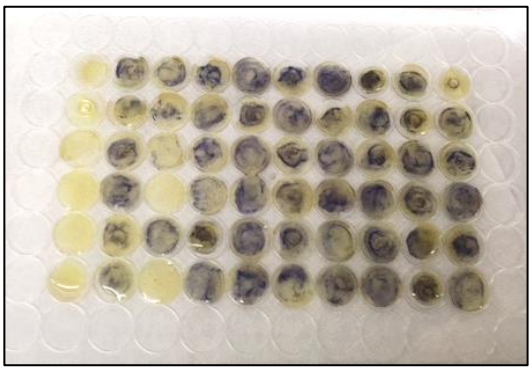


Figure 7. Determination of MIC- flanges



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

Figure 8. *C.plicata* EO

Figure 9. *G. pendula dried leaves*

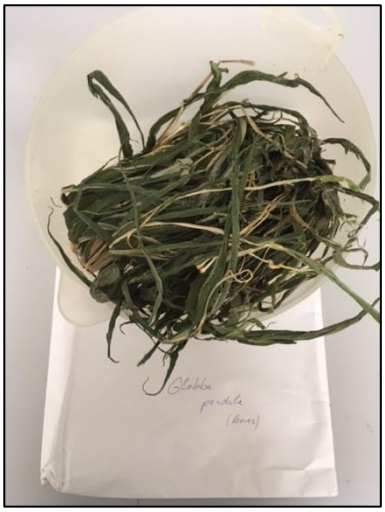


Figure 10. *A.pierreanum*

Figure 11. *C.singularis*

Figure 12. *G.adhaerens*

Figure 13. *C. plicata*

Figure 14. *M. bokorensis*

Figure 15. *G. pendula*

Figure 16. *G. pierreana*

Original photos by Ingrid Faltova and Marie Netopilova, 2017.