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Vapours of essential oils and their constituents as *in vitro* inhibitors of pneumonia causing microorganisms

DOCTORAL THESIS

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Declaration

I, Markéta Houdková, hereby declare that I have done this thesis entitled "Vapours of essential oils and their constituents as *in vitro* inhibitors of pneumonia causing microorganisms" 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, August 28, 2018

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Ing. Markéta Houdková

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Abstract

With aim to develop effective proof-of-concept approach, which can be used in a development of new preparations for inhalation therapy, we designed a new microdilution volatilization method for *in vitro* fast screening and simple simultaneous determination of antibacterial potential of plant volatiles in the liquid and the vapour phase at different concentrations. In addition, EVA (ethylene vinyl acetate) capmat™ as vapour barrier cover was used as reliable modification of thiazolyl blue tetrazolium bromide (MTT) assay for cytotoxicity testing of volatiles on microtiter plates. First, antibacterial activity of six plant volatile compounds, namely carvacrol, cinnamaldehyde, eugenol, 8-hydroxyquinoline, thymol and thymoquinone, was determined against pneumonia causing bacteria (*Haemophilus influenzae*, *Staphylococcus aureus*, and *Streptococcus pneumoniae*) using optimized novel method and the cytotoxicity of these compounds was evaluated using MTT test in lung fibroblast cells MRC-5 (Medical Research Council cell strain 5). Thereafter, essential oils (EOs) hydrodistilled from seven Cambodian (*Alpinia oxymitra*, *Boesenbergia rotunda*, *Cinnamomum cambodianum*, *Citrus lucida*, *Limnophila aromatica*, *Rhodamnia dumetorum*, and *Sindora siamensis*) and six Philippine (*Alpinia brevilabris*, *Alpinia cumingii*, *Alpinia elegans*, *Callicarpa micrantha*, *Cinnamomum mercadoi*, and *Piper quinqueangulatum*) plant species were tested for their *in vitro* growth-inhibitory effect against *H. influenzae*, *S. aureus*, and *S. pneumoniae* with aim to verify practical usability of novel broth microdilution volatilization method. Additionally, MTT assay was performed for evaluation of their cytotoxic activity to human lung cells. The most effective antibacterial agents were 8-hydroxyquinoline and thymoquinone with the lowest minimum inhibitory concentrations (MICs) ranging from 2 to 128 µg/mL, but they also possessed the highest toxicity in lung cell lines with half maximal inhibitory concentration (IC_{50}) values 0.86–2.95 µg/mL. The lowest cytotoxicity effect was identified for eugenol with IC_{50} 295.71 µg/mL, however this compound produced only weak antibacterial potency with MICs 512–1024 µg/mL. In case of EOs, all samples exposed some antibacterial efficacy; however, only *A. oxymitra* rhizome EO was active against all bacteria tested. *A. oxymitra* pericarp EO was found as the most effective antibacterial agent against *H. influenzae* in liquid and solid medium with the respective lowest MIC of 64 and 32 µg/mL. Due to its high value for 80% inhibitory concentration of proliferation (> 512 µg/mL), this EO may be considered as safe to human lung cell lines. Other EOs showed moderate or weak

antibacterial effect with MIC values ranging from 128 to 1,024 µg/mL and from 64 to 1,024 µg/mL in liquid and vapour phase, respectively. Using dual-column/dual-detector system for gas chromatography-mass spectrometry (GC-MS) analysis of Cambodian EOs, β-pinene was identified as the main constituent of *A. oxymitra* leaves, pericarp and rhizome EOs, while volatile oil from *A. oxymitra* seeds consisted predominantly of shyobunol. The major constituents of *B. rotunda*, *C. lucida*, *L. aromatica*, *R. dumetorum*, and *S. siamensis* EOs were ocimene, decyl acetate, limonene, caryophyllene epoxide, and β-bourbonene, respectively. Chemical analysis of EOs from Philippine plant species determined hedycaryol, β-pinene, caryophyllene epoxide, aristolochene, borneol, and linalool were identified as the major components of *A. brevilabris*, *A. cumingii*, *A. elegans*, *C. micrantha*, *C. mercadoi*, and *P. quinqueangulatum* leaves EOs, respectively. The results demonstrate validity of our novel broth microdilution volatilization method, which allows cost and labour effective high-throughput antimicrobial screening of volatile agents (EOs and their constituents) without need of special apparatus. Based on results of growth-inhibitory and lung toxicity effects, *A. oxymitra* pericarp EO can be considered as an effective antibacterial agent with application potential for the development of inhalation therapy against respiratory infections. However, further research focused on *in vivo* evaluation of pharmacological effects and toxicological safety, will be necessary before its possible practical use.

Key words: antibacterial activity; cytotoxicity; GC-MS analysis; plant volatile compounds; respiratory infections

Abstrakt

S cílem vyvinout efektivní koncept, který lze použít při vývoji nových přípravků pro inhalační terapii, byla navržena nová mikrodiluční volatilizační metoda umožňující rychlý *in vitro* screening a jednoduché stanovení antibakteriálního potenciálu rostlinných těkavých látek současně v kapalné a plynné fázi v několika různých koncentracích. Jako součást konceptu byl také modifikován thiazolyl blue tetrazolium bromide (MTT) test pro hodnocení cytotoxicity těkavých látek na mikrotitračních destičkách použitím EVA (ethylenvinylacetát) capmat^{TM} jako krycí vrstvy zabraňující úniku par. Za účelem optimalizovat podmínky nově vyvinuté metody, byla v rámci této disertační práce nejprve stanovena antibakteriální aktivita šesti rostlinných těkavých látek (karvakrol, cinnamaldehyd, eugenol, 8-hydroxychinolin, tymol a tymochinon) proti bakteriím způsobujícím pneumonii (*Haemophilus influenzae*, *Staphylococcus aureus* a *Streptococcus pneumoniae*). Dále byla hodnocena cytotoxicita zmíněných sloučenin pomocí MTT testu s plicními buňkami MRC-5. V další části výzkumu byla ověřena praktická využitelnost nové mikrodiluční volatilizační metody pro testování rostlinných silic, jejichž vzorky byly získány vodní destilací ze sedmi kambodžských (*Alpinia oxymitra*, *Boesenbergia rotunda*, *Cinnamomum cambodianum*, *Citrus lucida, Limnophila aromatica*, *Rhodamnia dumetorum*, *Sindora siamensis*) a šesti filipínských (*Alpinia brevilabris*, *Alpinia cumingii*, *Alpinia elegans*, *Callicarpa micrantha*, *Cinnamomum mercadoi*, *Piper quinqueangulatum*) rostlinných druhů, které byly následně testovány proti *H. influenzae*, *S. aureus* a *S. pneumoniae*. K tomu byl proveden MTT test pro hodnocení jejich cytotoxického účinku vůči lidským plicním buňkám. Nejúčinnějšími antibakteriálními látkami byly 8-hydroxychinolin a tymochinon s nejnižšími minimálními inhibičními koncentracemi (MIC) v rozmezí od 2 do 128 µg/mL, avšak zároveň u nich byla zaznamenána nejvyšší toxicita s hodnotou inhibiční koncentrace (IC_{50}) 0,86–2,95 µg/mL. Nejnižší cytotoxický účinek byl zjištěn pro eugenol (IC_{50} 295,71 µg/mL), ale tato látka vykazovala pouze slabou antibakteriální aktivitu (MIC 512–1024 µg/mL). V případě silic byl u všech vzorků zaznamenán určitý antibakteriální efekt; avšak pouze silice z oddenků *A. oxymitra* byla účinná proti všem testovaným bakteriím. Jako nejúčinnější antibakteriální prostředek se projevil esenciální olej z oplodí *A. oxymitra* proti *H. influenzae* v kapalném a pevném médiu s hodnotami MIC 64 a 32 µg/mL. Vzhledem k vysoké hodnotě 80% inhibiční koncentrace proliferace (IC_{80} > 512 µg/mL)

může být tento esenciální olej považován za bezpečný pro lidské plicní buňky. Ostatní silice vykazovaly mírný nebo slabý antibakteriální účinek s hodnotami MIC v rozmezí 128–1024 µg/mL v kapalné a 64–1024 µg/ml v plynné fázi. Chemickou analýzou kambodžských esenciálních olejů za použití plynového chromatografu vybaveného dvěma kolonami a dvěma detektory byla identifikována jako hlavní složka olejů z listů, oplodí a oddenků *A. oxymitra* látka β-pinen, zatímco silice ze semen *A. oxymitra* byla zastoupena převážně látkou shyobunol. Jako hlavní složky ostatních kambodžských esenciálních olejů byly zjišteny následující látky *B. rotunda* (ocimen), *C. lucida* (decyl acetát), *L. aromatica* (limonen), *R. dumetorum* (karyofyllen epoxid) *a S. siamensis* (β-bourbonen). Chemickou analýzou silic z listů filipínských rostlin byly stanoveny tyto hlavní složky hedykaryol (*A. brevilabris*), β-pinen (*A. cumingii*), karyofylén epoxid v (*A. elegans*), aristolochen *(C. micrantha*), borneol (*C. mercadoi*) a linalool (*P. quinqueangulatum*). Získané výsledky ověřily účinnost nové mikrodiluční volatilizační metody, která umožňuje cenově dostupný a rychlý screening antimikrobiální aktivity těkavých látek (silic a jejich složek) bez speciálního vybavení. Na základě výsledků inhibice bakteriálního růstu a toxicity vůči plicním buňkám může být silice z oplodí *A. oxymitra* považována za účinnou antibakteriální látku s dobrým potenciálem pro vývoj inhalačních prostředků při léčbě respiračních infekcí. Pro další možné praktické využití této silice, však bude nutné provést další výzkum zaměřený na hodnocení farmakologických účinků a toxikologické bezpečnosti *in vivo*.

Klíčová slova: antibakteriální aktivita; cytotoxicita; GC-MS analýza; respirační infekce; rostlinné těkavé látky

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List of the abbreviations used in the thesis

AIDS	Acquired immune deficiency syndrome
ATCC	American type culture collection
CAS	Chemical abstracts service
CFU	Colony forming unit
CLSI	Clinical and Laboratory Standards Institute
DMSO	Dimethylsulfoxide
EMEM	Eagle's minimum essential medium
EO	Essential oil
EUCAST	European Committee on Antimicrobial Susceptibility Testing
EVA	Ethylene vinyl acetate
FBS	Foetal bovine serum
FDA	Food and Drug Administration
FID	Flame ionization detector
GC-MS	Gas chromatography-mass spectrometry
HIV	Human immunodeficiency virus
IC_{50}	Half maximal inhibitory concentration
IC_{80}	80% inhibitory concentration of proliferation
IP	Insect Pathology
KCCM	Korean Culture Center of Microorganism
MH	Mueller-Hinton
MIC	Minimum inhibitory concentration
MRC-5	Medical research council cell strain 5
MTT	Thiazolyl blue tetrazolium bromide
ND	Not determined
NIST	National Institute of Standards and Technology Library
PRC	Plasmid Reference Center
RI	Retention index
RT	Retention time
SD	Standard deviation
SPME	Solid-phase microextraction
Std	Standard
TI	Therapeutic index
tr	Trace
UPLB	University of the Philippines Los Baños
VSU	Visayas State University
WHO	World Health Organisation

1 Introduction

Pneumonia belongs to leading causes of morbidity and mortality, which pose the highest risk to the children under five years, elderly, and immuno-compromised individuals especially in low-income countries including Cambodia and Philippines [1]. This acute respiratory infection of lung parenchyma is caused by bacterial pathogens such as *Haemophilus influenzae*, *Staphylococcus aureus*, and *Streptococcus pneumoniae* [2]. Besides a systematic antibiotic treatment, an inhalation therapy is possible way of the cure for such ailments. This approach based on direct delivery of the antibacterial agents to the site of infection in the respiratory system maximizes their efficacy and simultaneously restricts systemic exposure and associated toxicity [3], moreover inhalation administration of therapeutics prevents the degradation of active components in the gastrointestinal tract as well [4].

Although the timely antibiotic therapy can considerably reduce fatal cases of respiratory infections [5], many low-income countries have limited access to health services and synthetic drugs as well [6]. The natural substances including plant essential oils (EOs) and their volatile compounds are of great potential for development of novel antimicrobial drugs because of the broad spectrum of chemical diversity [7,8]. For bacteria, it is more difficult to develop resistance to the multi-component mixtures than to single-ingredient conventional antibiotics [9]. Nevertheless, due to their specific physico-chemical properties such as high volatility and hydrophobicity, conventional antimicrobial susceptibility testing methods like broth dilution and agar diffusion face specific problems in the drug research and development process.

In the past decades, several methods for testing of the antimicrobial effects of EOs have been developed with aim to study the potential of their volatile constituent for development of novel inhalation therapeutics [10]. Among *in vitro* methods, the vapour phase tests demonstrate the antimicrobial activity of EOs and their constituents in the most appropriate way because it was confirmed that their vapours are more effective antimicrobials than liquid phases [11]. Therefore, the results of these tests can be useful to understand the antibacterial potential of EOs in the respiratory tract [12].

With aim to establish effective proof-of-concept approach for development of new preparations for the inhalation therapy of the respiratory diseases, a new screening assay, named broth microdilution volatilization method was designed by our team for simple and rapid simultaneous determination of antibacterial potential of plant volatile compounds in the liquid and the vapour phase at different concentrations. This test is accompanied by MTT cytotoxicity assay modified for testing of volatiles on microtiter plates with an ethylene vinyl acetate (EVA) capmat™ as vapour barrier cover. With goal to verify appropriateness of both methods for assessment of plant-derived volatile constituents, carvacrol, cinnamaldehyde, eugenol, 8-hydroxyquinoline, thymol, and thymoquinone were tested as representatives of various classes of antimicrobially effective phytochemicals (Chapter 5). Thereafter, detailed examination of *in vitro* growth-inhibitory potential of EOs from seven Cambodian and six Philippine less explored plant species against pneumonia causing bacteria was performed to verify suitability of newly developed broth microdilution volatilization method for EOs testing (Chapters 6 and 7). Additionally, the cytotoxicity and chemical composition of tested EOs were analysed with aim to assess the relationship between their antimicrobial potential, chemistry, and safety for treatment of pneumonia (Chapters 5-7). For chemical analysis, dual-column/dual-detector system GC-MS was used to achieve a higher quality identification of detected constituents, when the volatile oils analyses were conducted on two columns with different polarities, non-polar HP-5MS and mid-polar DB-17MS (Chapters 6 and 7).

2 Literature Review

2.1 Pneumonia

Diseases of respiratory system are more frequent and more severe since they figure in the top positions of global causes of deaths. According to World Health Organisation (WHO) [13], chronic obstructive pulmonary disease claimed 3.2 million lives in 2015. In addition, lower respiratory infections (e.g. pneumonia, bronchitis) caused 3 million deaths worldwide in 2016 as they are the most deadly communicable disease. Moreover, these infections can also be an independent risk factor for other health difficulties such as cardiovascular diseases [14].

Respiratory infections pose the highest risk to children under five years, the elderly and immuno-compromised individuals, especially those living in developing countries, where it is associated with poverty, lack of access to health care, as well as with environmental, and behavioural influences [15]. Risk factors increasing a development of respiratory infections are malnutrition, tobacco smoking, use of solid fuels in a household, air pollution, crowding, bad hygienic practices, lack of exclusive breastfeeding, and low degree of maternal education [16].

2.1.1 Epidemiology

Pneumonia is an acute respiratory illness secondary to infection and inflammation of the lung parenchyma when lung alveoli are filled with pus and fluid, which makes breathing painful and limits oxygen intake. Community acquired pneumonia is pneumonia acquired outside of hospital or long-term care facility. By contrast hospital acquired pneumonia is pneumonia developing 48 hours after admission to the hospital [17,18]. Patients typically present with fever, cough, chills, fatigue, sputum production, fast and difficult breathing and pleuritic chest pain. Other presentations may include headache and myalgia [19,20].

Pneumonia is very common disease with WHO estimating annually 450 million episodes and 4 million deaths worldwide. Childhood pneumonia is leading cause of children mortality and morbidity; it kills more children than HIV/AIDS, malaria or measles. Every year there are nearly 155 million cases of this illness and over 2 million

children die under five years of age [20,21]. It is expanded mainly in developing countries of South Asia and sub-Saharan Africa. The estimated incidence of children pneumonia is 0.28 and 0.05 episodes per child/year in developing and developed countries, respectively [20]. For instance in Cambodia, about 9,100 children die from pneumonia every year [22] and according to the WHO [18] only 64.2% of children with pneumonia symptoms are taken to an appropriate healthcare provider. Similarly, in Philippines, where pneumonia causes yearly death of approximately 8,900 children [1], an access to the health services is the most important health problem.

2.1.2 Microbial pathogens

Pneumonia occurs due to invasion of the lower respiratory tract by bacterial, viral, fungal or parasitic microbes as well as by non-infectious agents. The most common bacterial pathogens responsible for severe cases of pneumonia are *Streptococcus pneumoniae*, *Chlamydia pneumoniae*, *Mycoplasma pneumoniae*, *Haemophilus influenzae*, *Legionella* sp., *Staphylococcus aureus*, and *Moraxella catarrhalis* [23,24]. However, the mixed viral-bacterial infections are highly prevalent with an increase in atypical pathogen co-infections [19].

2.1.3 Treatment

2.1.3.1 Systemic antibiotics

In general, lower respiratory tract infections are difficult to treat due to the sequestration of microorganisms deep within the airways, where only limited portions of drug gain access after traditional systemic treatment [25]. Nevertheless timely antibiotic therapy can considerably reduce fatal cases of pneumonia case [5]. Combination of beta-lactams and macrolides have become the standard care for patients with pneumonia [26]. Co-trimoxazole and amoxicillin are recommended as the first line drugs for treatment of non-severe pneumonia, then benzylpenicillin, ampicillin, gentamicin, ceftriaxone, and oxacillin are used as agent for the cure of severe pneumonia [20,27]. Nevertheless, it is well understood that effective antimicrobial therapy requires drug concentrations at the target site of infection. There is a risk of degradation of active components in the gastrointestinal tract as well. To reach the deep airways in sufficient concentrations, often toxic doses of drugs would need to be given systemically [28,29].

2.1.3.2 Inhalation therapy

Since systemically administered antibiotics used to treat respiratory infections often have poor penetration into the lung parenchyma and narrow therapeutic windows between efficacy and toxicity, direct access of antibiotics to the site of infection in the lung parenchyma via inhalation could overcome these obstacles [25]. Inhaled antimicrobial agents have the capability of directly targeting the airways, creating increased and more sustained local concentrations and thereby increasing the therapeutic index, improving efficacy, minimizing toxicity, and decreasing the time of onset for the administered drug without the systemic exposures [30,31]. Optimal characteristic of inhaled antimicrobial are lipophilicity, positive charge and high molecular mass, these properties should be balanced against and appropriate particle size [25]. There are several types of devices for effective delivery of inhaled medications to the lungs: soft-mist nebulizers (jet, vibrating mesh, or ultrasonic), pressurized metered-dose inhalers, and dry-powder inhalers [32]. However, there are several problems with the use of inhaler devices, such as the deposition of aerosolized particles in the oropharyngeal region and upper airways while the deposition of medication in the lungs is reduced due to patient-specific respiratory tract physiology, especially in children and elderly [33]. As well as, the distribution of antibiotic agent can be limited because of lung morphology, clearance mechanisms (mucociliary and alveolar macrophages) [34].

Due to the inappropriate use of antibiotics increase antibiotic resistance, occurrence of side effects and falling eradication rates, there is a need to develop novel agents. Moreover, many low-income countries have limited access to health services and synthetic drugs as well, for example less than 40% and 50% of children are treated with antibiotics in Cambodia and Philippines, respectively [13,18,35]. Some studies have emphasized that cost of treatment, the lack of medical equipment and specialized medical doctors, and geographical constraints belong to the major barrier of health security for patients in many developing countries [36].

2.1.3.3 Traditional folk medicine

Recently, the attention has been paid to the medicinal plants, which are considered as valuable sources of wide spectrum of secondary metabolites possessing biological activities that may contribute to effective therapeutic treatment and human health [37]. Medicinal plants have played important role for curing various diseases in folk medicine

for thousands years. In the developing countries, about 80% of the people still rely on plant therapy and the tendency of using ethnomedicine has been gradually increasing in the developed countries. Over 21,000 plant species were recorded by WHO for their medicinal uses throughout the world, especially the flora of the tropical areas by virtue of its diversity plays a significant role in being able to provide rich source of phyto-medications [38,39]. The natural products derived from medicinal plants have proven to be an abundant source of biologically active compounds and many of them have been the basis for the development of new pharmaceuticals [40]. Nowadays, it is estimated that plant materials are present in 50% pharmaceutical preparations [41]. As there are approximately 500,000 plant species occurring worldwide, only 1% has been phytochemically investigated, therefore there is a great potential for discovering novel bioactive substances [40]. The multi-component plant extracts helps to prevent the bacterial resistance as well as it can lead to pleiotropic, synergistic or additive effects in the organism [42]. The primary benefits of using plant derived medicines are relative safety and affordable cure, thus demands of use of herbal remedies is increasing nowadays [41,43].

In many regions of the world, folk medicine using healing effects of plants has a long tradition. Besides unique systems of traditional Chinese, Ayurvedic, and Unani medicines, several other indigenous traditional medicine practices have also been developed in history by various cultures in other continents, such as traditional Khmer herbal medicine and Filipino traditional medicine. The theory and application of these traditional ways of therapy differ significantly from those well-developed allopathic medicines [43]. *Blumea balsamifera* (leaves), *Centella asiatica* (stem, leaves), *Chromolaena odorata* (leaves), *Citrus* × *aurantiifolia* (fruit), *Coleus amboinicus* (leaves), *Cymbopogon nardus* (leaves), *Imperata cylindrical* (root), *Melicope pteleifolia* (leaves), *Mentha arvensis* (leaves), *Piper betle* (leaves), *Tagetes erecta* (leaves), *Zingiber montanum* (rhizome) are examples of species used by Khmer and Filipino traditional healings for treatment of cough, which can refer to other respiratory diseases such as pneumonia, tuberculosis, bronchitis, and laryngitis as well [44,45]. Since these species are representatives of families characterized by a high content of EOs (Asteraceae, Lamiaceae, Poaceae, Rutaceae, and Zingiberaceae), they could have a potential for inhalation way of remedies as well.

2.2 Essential oils

2.2.1 Taxonomical distribution

Plants producing EOs belong to various genera distributed to around 60 families such as Alliaceae, Apiaceae, Asteraceae, Lamiaceae, Lauraceae, Myrtaceae, Poaceae, Piperaceae, Rutaceae, and Zingiberaceae that are well known for their ability to produce EO of medicinal and industrial value [46]. EOs are extracted by steam distillation, hydrodistillation or solvent extractions from various aromatic plants generally localized in temperate and warm countries like Mediterranean and tropical countries. They can be produced in all plant organs (buds, flowers, seeds, fruits, leaves, stems, bark, wood and roots) where are stored in secretory cells, canals, epidermic cells or glandular trichomes [8]. It is estimated that there are known 3,000 EOs, of which about 300 are commercially important especially for the flavours and fragrances market [47]. In nature, EOs play an important role in the protection of the plants against pathogens, herbivores, and insects by reducing their appetite for such plants. On the other hand, they can attract pollinators or they mediate communication with other plants [8].

2.2.2 Chemistry

Chemically, the EOs are volatile aromatic liquids usually colourless substances, generally of lower density than water. They are lipophilic and soluble in organic solvents [48]. These complex mixtures of low molecular weight compounds are produced as plant secondary metabolites. As each plant species has evolved to protect itself against predator and stress condition, each plant produces its own specific mixture of EO chemical constituents. This can contain 20–60 constituents at varying concentrations with two or three major compounds representing 20–70 % of all content that usually define the biological properties of EOs [49,50].

Based on chemical composition of EOs, they are broadly classified into oxygenated compounds and hydrocarbons. Oxygenated compounds include esters, aldehydes, ketones, alcohols, phenols, and oxides, whereas hydrocarbons are composed of components with an isoprene structure, which are called terpenes [51]. Terpenes consist of several 5-C base units known as isoprene, their carbon limit is ranging from C_{10} to C_{40}. Major classes of terpenes include monoterpenes and sesquiterpenes (including

two or more isoprene units, respectively) [52], but monoterpenes contribute to 90 % of EOs overall. When these substances contain additional elements (e.g. oxygen), they are termed terpenoids. Terpenoids are synthetized from acetate units, and as such they share their origins with fatty acids but they are extensive branched and cyclized [53]. In fact, composition of EOs is largely affected by extraction method. It has been found that traditional techniques used for their extraction can cause losses of some volatiles and degradation of unsaturated or ester compounds through thermal or hydrolytic effects, while the use of solvent extraction can cause presence of toxic solvent residue. Moreover, obtained EO are susceptible to degradation by several factors, such as light, heat, oxidation, and hydration [54].

2.2.3 Analytical methods

Study of chemical composition and identification of individual constituents of EOs is important for understanding the origin of their biological activity, however chemical analysis of EOs can become difficult task. Besides the number of compounds present in each EO, most of them are present in minor quantities. Moreover, extensive group of monoterpenes include many compounds with similar molecular formulas and different type of structure, as well as great number of isomers. Thus, their analysis requires methods with low detection limits.

The chemical analysis of the components can be achieved by fractional distillation, gas chromatography, high-performance liquid chromatography, mass spectrometry, and nuclear magnetic resonance [50,52]. However, gas chromatography coupled with mass spectrometry detector (GC-MS) is the most used technique to identify and quantify the compounds of EOs. GC-MS technique achieves the highest resolution of EOs; therefore, it belongs to the most suitable methods for volatile compounds analysis. Although GC-MS is widely used, there are many studies using liquid chromatography as well. Another method, fractional distillation is frequently used to purify EOs or to concentrate the desirable parts of EOs for specific applications. The main goal is to separate substances through the volatility difference within them [55]. Application of solid-phase microextraction (SPME) is effective sampling technique for chemical analysis of volatile compounds in various matrices when it integrates sampling, extraction and concentration into a single solvent-free step. It is based on sorption of

analytes on or into a polymeric material that coats a silica fiber. During the analysis process, SPME can be matched with other analytical methods, e.g. GC-MS [56,57].

2.2.4 Pharmacological and medicinal uses

It has been long recognised that EOs possess a range of biological properties including antimicrobial, antiviral, antimutagenic, anticancer, antioxidant, antiinflamatory, immunomodulatory, and antiprotozoal effects contributing to human health [46]. It was reported that 60% of EOs inhibited fungi while 30% inhibited growth of bacteria. The mechanism of their action is not fully understood but it is speculated to involve membrane disruption by the lipophilic compounds [51]. Oxygenated terpenoids, particularly phenolic terpenes, phenylpropanoids and alcohols are the main classes of components responsible for the antimicrobial activity of plant EOs [58].

A number of reports have been published in recent years on the antibacterial activity of some EOs derived from plants. In traditional popular medicine inhalation of black pepper (*Piper nigrum*), cinnamon (*Cinnamomum zeylanicum*), cloves (*Syzygium aromaticum*), mint (*Mentha piperita*), sage (*Salvia officinalis*) or thyme (*Thymus vulgaris*) volatile oils have been used traditionally to treat respiratory tract infections [59,60]. Several EOs and their constituents have been effective against pathogenic microorganisms causing pneumonial infection. For example, *Carissa carandas*, *Curcuma zedoaria*, *Grewia asiatica* and *Punica granatum* effectively inhibit *in vitro* growth of *Streptococcus pneumoniae* [61]. Considering their antimicrobial activity and high volatility as they have ability to vaporize spontaneously, EOs are of a great potential to be used for inhalation therapy.

Several studies reporting the growth-inhibitory potential of EO's major constituents have recently been published. For example, a significant antibacterial effect of *α*- and *β*-pinenes has previously been reported against methicillin-resistant *S. aureus* with respective MIC values 4.15 and 6.25 µg/mL [62]. A certain degree of anti-*S. aureus* effect has previously been attributed to 1,8-cineole and terpinen-4-ol [63,64]. According to the previous studies [65,66], decanal and *β*-caryophyllene, produced antibacterial effect against clinical strains of *S. aureus* and other bacterial pathogens with respective MIC values 62.50–125.00 µg/mL and 0.61–2.86 µg/mL. Antibacterial properties were

also described for caryophyllene epoxide, limonene, ocimene, geraniol, and camphor [63,64,67,68].

Number of medicinal plants have been classified as capable of treating respiratory disorders. The scientific validation of herbal medicine has been well described in the literature [69]. The herbal remedies are usually available as non-prescription drugs, dietary supplements and confectionery in the form of capsules, tablets, lozenges, tinctures, syrups, nasal drops, inhalers and sprays. Especially the last three medications are beneficious for the treatment of respiratory diseases because their active components are delivered directly to the site of infection in the airways. Due to consumer demands for natural therapies over the last twenty years, the global herbal supplements and remedies market is forecast to reach US$115 billion by the year 2020 [70]. Examples of phytomedicinal products based on EOs of medicinal plant that are currently available on the market are mentioned below:

Biotussil is traditional herbal medicine used as a therapy for respiratory diseases, including rhinitis and inflammation of the nasal sinuses. This medicinal product in form of oral drops contains the following active substances: extracts of *Gentiana lutea* root, *Primula veris* flowers, *Plantago lanceolate* leaves, *Thymus vulgaris* herb, *Glycyrrhiza glabra* root, *Sambucus nigra* flowers, and EOs of *Foeniculum vulgare* fruit and *Pimpinella anisum* fruit [71].

GeloMyrtol is an herbal medicine sold in the form of enteric-coated soft gelatin capsules. Its medicinal ingredients are EOs of *Eucalyptus globulus*, *Citrus sinensis*, *Myrtus communis*, and *Citrus limon* (in the ratio 66:32:1:1), the major components are limonene, 1,8-cineole, and α-pinene. For its additional antioxidative, anti-inflammatory and antibacterial potential, this medication is recommended for cure of acute and chronic infections of the upper and lower airway system, such as rhinosinusitis, bronchitis and chronic obstructive pulmonary disease [72].

Pinio-Nasal is a mixture of EOs from *Pinus sylvestris*, *Mentha piperita*, Eucalyptus *globulus*, plant volatile compounds thymol and guaiazulene, and vitamin E (in the ratio 54:14:7:0.7:0.3:24). These nasal drops are indicated to treat rhinitis and other inflammatory diseases of the nose and nasopharyngeal mucosa. Inhalation of its active substances can favourably affect infectious inflammatory airway diseases (inflammation of the larynx, trachea and bronchi) [73].

2.2.5 Toxicology

Although the plant derived substances are considered as safe agents with beneficial properties on human health [74], there is potential risk of possible drug-induced pulmonary toxicity when they are administered in form of inhalation therapy as the EOs can exert cytotoxic effect on eukaryotic cells. This toxic activity is beneficial for the anticancer and chemotherapeutic application of EOs against virus, bacteria and fungi, however it can cause the undesirable side effects toward human tissues and cells [75,76]. However, EOs were found to be safe for human consumption at low concentrations [77]. Nevertheless, the development process of respiratory system therapeutic preparations should involve determination of their safety to lung tissue [10]. Thiazolyl blue tetrazolium bromide (MTT) colorimetric assay is one of the most commonly used methods for evaluation of *in vitro* cytotoxicity using microtiter plate design, which is applicable to cytotoxicity assessment of natural products [78] including volatiles [79].

2.3 Methods for evaluation of growth-inhibitory effect of EOs

Due to the specific physico-chemical properties of EOs such as high volatility and hydrophobicity, conventional antimicrobial susceptibility testing methods like broth dilution and agar diffusion face specific problems in the drug research and development process. The hydrophobic nature worsens the solubility of these compounds in water based media (e.g. in broth), thus the surfactants have to be added, whereas the volatility causes a risk of active substance losses by evaporation [80,81]. In addition, the transition of the vapours can affect the microplate assays results, which are not reliable as they vary significantly according to the plate design used. In case of template with single sample in each row of the plate, a simple change in volatile antimicrobials layout can lead to determination of significantly different endpoint values [82].

Antimicrobial assays can be performed in direct contact with the microorganism or by vapour phase [50]. However, in contrast to well established methods for antimicrobial susceptibility testing in liquid or solid media such as broth microdilution and disc diffusion tests, there are no standardized assays for determination of microbial sensitivity to volatile compounds in vapour phase e.g. in accordance with Clinical and Laboratory Standards Institute (CLSI) or European Committee on Antimicrobial

Susceptibility Testing (EUCAST) [83,84]. The disc volatilization test is very simple and the most frequently used method for evaluation of antimicrobial effects of plant volatiles in vapour phase [85] and its various modifications have been developed. Although the methods based on disc volatilization assay performed in Petri dishes are useful tool for simple assessment of growth-inhibitory potential of EOs in vapour phase, they are not designed for a high-throughput screening, the relatively high consumption of material and labour are the main disadvantages for most of them, because each concentration of each EO has to be tested on separate disc. The principles of some methods are described below:

Disc volatilization assay using adhesive tape

This method is prepared as usual disc volatilization assay using Petri dishes with inoculated agar and EO containing paper disc on the inside surface of the upper lid, however Petri dish is then sealed by sterile adhesive tape to prevent vapour outlet. Additionally, the atmosphere generated by the EOs in the disc was measured by placing a fully retracted solid-phase microextraction fiber into the headspace of the Petri dish to characterize correlation between vapour phase of EOs and their antimicrobial behaviour [86].

***In vitro* dressing model**

Covering the antibacterial experiment performed in Petri dish with four layers containing Gamgee, gaze, Flamazine™ and Telfa Clear™ or Jelonet™ as adherence dressings is another possible alternative of disk volatilization assay reducing the losses of active substances by evaporation. Tested EO is placed onto a small central area of either the Gamgee or the gaze layers and then finally covered with four layers of dressings. Based on optimization of this method, it was found that using Telfa Clear™ as the primary layer is the most effective [87].

Disc volatilization assay using agar sealing on the lid

Nedorostova et al. [88] modified disc volatilization method by using agar sealing when 15 ml of warm medium is poured into 90 mm Petri disc, 5 ml into its cover, and then the medium in dish part is inoculated after solidification. Agar in Petri dish cover serves as a sealing and prevented adsorption of EOs onto the plastic material of Petri dish cover.

Agar vapour-inhibiting assay

Agar vapour-inhibiting assay is technique designed by Inouye et al. [89] with aim to reduce the evaporation of tested volatiles that combines agar diffusion and agar vapour methods. The inhibition of vaporization is accomplished by sealing the paper disc containing EO with a plastic seal ring. Briefly, a paper disc with tested EO is placed on the double-layered agar medium and sealed with a plastic seal ring (15, 21 or 24 mm in diameter), one side of that is closed with a cover glass using a plastic cement. The open side of the plastic ring touches the surface of the agar medium. It was found in study of Inouye et al. [89], the inhibition remains within the sealed ring in agar vapour-inhibitory assay, thus it indicates that the inhibitory diameter in common agar diffusion assay was mostly due to the vapour activity.

Airtight box

In another method, Petri dish with inoculated medium is placed in 1.3 L airtight box. The inside of the box is covered with aluminium foil to prevent the plastic absorption of EO and to protect the wall of the container from direct contamination by EO and to protect boxes of the contamination. A paper disc (9 cm in diameter) impregnated with EOs is inserted in the top of airtight box. Advantage of this method is possibility of using inoculated material as plastic and steel to evaluate surface decontamination [90].

Fast screening method using four section Petri dishes

This test designed by Kloucek et al. [81] is performed in 90 mm Petri dishes divided into four sections. Each section, as well as the lid contain 5 ml of warm agar. After solidification, three parts of dish are inoculated with different microorganism, the fourth one is left as purity control. Then the solution of EO is placed on 85 mm round sterile filter paper disc. Finally, the paper disc is put onto the walls dividing the sections of Petri dish, which is hermetically closed with its lid containing solidified medium. This relatively fast and simple screening assay allows higher throughput and more precise results than currently used methods performed in Petri dishes.

Airtight experimental apparatus

The airtight experimental apparatus was constructed by Seo et al. [91] for simultaneous assessment of antimicrobial effect of volatile compounds at various concentrations. The apparatus consist of an upper chamber with seven wells containing inoculated agar medium, and a lower chamber with seven wells containing EO. To avoid vapours leakage, O-rings are inserted at the juncture of the upper and lower well rims and around whole set of wells, moreover the four corners and centre of the apparatus are tightly sealed with nuts and bolts. It is expected that this method would reduce the time required for evaluation of antimicrobial effect of EO gaseous phase, because it is possible to test several concentration simultaneously, however it requires the special equipment, which is not commonly available.

3 Hypothesis

In tropical regions, there is a number of plant species that are known for high content of EOs. It is assumed that EOs obtained from these plant taxa releasing volatile substances, which can effectively inhibit pathogenic microorganisms responsible for pneumonia, and they are suitable for inhalation therapy acting directly in the infection site. Although the EOs are generally considered as safe, there is potential risk of drug-induced lung toxicity when they are administered in form of inhalation therapy. Many aromatic plants still await for scientific validation and identification of their anti-infective properties, active substances content and safety. We expect that the phytochemical analysis of these plants could lead to the discovery of active compounds for affordable medicinal products used to treat respiratory diseases in developing countries. The broth microdilution assay is commonly used for determination of growth-inhibitory effects of antimicrobial agents including EOs and their constituents, nevertheless the affecting results by vapours of these volatile substances was demonstrated based of series of various experiments. Another methods for *in vitro* antibacterial activity testing of EOs in vapour phase have been developed, however they are not designed for a high-throughput screening, some of them allow evaluating only one concentration of samples, some needs special equipment, which is not commonly available, and they determine antimicrobial activity of volatile plant-derived compounds either in liquid or gaseous phase. These observations appeal to development of new appropriate method suitable for evaluation of antibacterial potential of volatile substances.

4 Objectives

Objective of this study was to develop *in vitro* assay suitable for evaluation of growth-inhibitory effect of plant volatiles and then to investigate antibacterial potential, cytotoxicity and chemical composition of less known plant species from Cambodia and Philippines.

The specific aim of this study were:

1. To optimize novel broth microdilution volatilization method for *in vitro* testing of growth-inhibitory effect of plant volatile compounds and EOs against pneumonia causing bacteria.
2. To determine *in vitro* cytotoxic effect of plant volatile compounds and EOs to human lung cells using modified MTT cytotoxicity assay.
3. To analyse chemical composition of antibacterially effective EOs using dual column/dual detector GC-MS system.

5 Development of new broth microdilution volatilization method and modification of MTT cytotoxicity assay

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

Marketa Houdkova participated in the development and optimization of broth microdilution volatilization method. She performed experiments focused on antibacterial activity of compounds tested in liquid and vapour phase. She also prepared the manuscript including its required revisions.

5.1 Introduction

Bacterial diseases of respiratory system (e.g. pneumonia, bronchitis) are leading causes of morbidity and mortality which pose the highest risk to children under five years old and older adult especially in low-income countries [1]. They are caused by bacterial pathogens such as *Haemophilus influenzae*, *Staphylococcus aureus*, and *Streptococcus pneumoniae* [2]. Besides a systematic antibiotic treatment, an inhalation therapy is possible way of the cure for such ailments. This approach based on direct delivery of the antibacterial agents to the site of infection in the respiratory system maximizes their efficacy and simultaneously restricts systemic exposure and associated toxicity [3]. Inhalation administration of therapeutics prevents the degradation of active components in the gastrointestinal tract as well [4].

Because of the wide range of chemical diversity, the natural substances including plant volatile compounds are of great potential for development of novel antimicrobial drugs [5,6]. For bacteria, it is more difficult to develop resistance to the multi-component mixtures (e.g. EOs) than to single-ingredient conventional antibiotics [7]. Oxygenated terpenoids, particularly phenolic terpenes, phenylpropanoids and alcohols are the main classes of compounds responsible for the antimicrobial activity of plant EOs [8]. Nevertheless, due to their specific physico-chemical properties such as high volatility and hydrophobicity, conventional antimicrobial susceptibility testing methods like broth dilution and agar diffusion face specific problems in the drug research and development process. The hydrophobic nature worsens the solubility of these compounds in water based media (e.g. in broth), thus the surfactants have to be added, whereas the volatility causes a risk of active substance losses by evaporation [9,10]. In addition, the transition of the vapours can affect the microplate assays results, which are not reliable as they vary significantly according to the plate design used. In case of template with single sample in each row of the plate, a simple change in volatile antimicrobials layout can lead to determination of significantly different endpoint values [11].

In the past decades, several methods for testing of the antimicrobial effects of EOs have been developed with aim to study the potential of their volatile constituent for development of novel inhalation therapeutics [12]. Among *in vitro* methods, the vapour phase tests demonstrate the antimicrobial activity of EOs and their constituents in the most appropriate way because it was confirmed that their vapours are more effective

antimicrobials than liquid phases [13]. Therefore the results of these tests can be useful to understand the antibacterial potential of EOs in the respiratory tract [14]. In contrast to well established methods for antimicrobial susceptibility testing in liquid media, there are no standardized assays for determination of microbial sensitivity to volatile compounds in vapour phase e.g. in accordance with Clinical and Laboratory Standards Institute (CLSI) or European Committee on Antimicrobial Susceptibility Testing (EUCAST) [15,16]. The disc volatilization test is the most frequently used method for evaluation of antimicrobial effects of plant volatiles in vapour phase [17]. Its various modifications using Petri dishes with dressing model [18], sterile adhesive tape [19], agar sealing on the lid [20], and four section Petri dishes [10] have also been developed. In another study, [21] used a special apparatus invented for evaluation of gaseous phase of EOs. Although all of these methods are commonly used for assessment of antimicrobial properties of volatile compounds in vapour phase, they are not designed for a high-throughput screening, some of them allow evaluating only one concentration of samples, some needs special equipment which is not commonly available, and they determine antimicrobial activity of volatile plant-derived compounds either in liquid or gaseous phase.

Although the EOs are generally considered as safe agents with beneficial properties on human health [22], there is potential risk of possible drug-induced pulmonary toxicity when they are administered in form of inhalation therapy [13]. Therefore, the development process of respiratory system therapeutic preparations should involve determination of their safety to lung tissue [12]. Thiazolyl blue tetrazolium bromide (MTT) colorimetric assay is one of the most commonly used methods for evaluation of *in vitro* cytotoxicity using microtiter plate design. This method is applicable to cytotoxicity assessment of natural products [23] including volatiles [24]. Nevertheless, the volatile substances can influence the results of biological assays using microtiter plates as it has previously been described by several authors [11,25]. For this reason, it is necessary to modify testing methodology and prevent the transmission of vapours. In the case of combinatorial effect testing of volatile compounds with antibiotics, [26] showed the significance of using ethylene vinyl acetate (EVA) capmat^{TM} as effective vapour barrier to avoid spreading of volatiles into adjoining wells.

With aim to evolve effective proof-of-concept approach which can be used in a development of a new preparations for the inhalation therapy of the respiratory diseases,

we designed a new screening method based on the broth microdilution volatilization for simple and rapid simultaneous determination of antibacterial potential of plant volatile compounds in the liquid and the vapour phase at different concentrations accompanied by MTT cytotoxicity assay modified for testing of volatiles on microtiter plates with a vapour barrier cover. In this study, we tested carvacrol, cinnamaldehyde, eugenol, 8-hydroxyquinoline, thymol, and thymoquinone (Fig. 5.1), as representatives of various classes of antimicrobially effective phytochemicals, with goal to verify appropriateness of both methods for assessment of plant-derived volatile constituents.

5.2 Material and methods

5.2.1 Chemicals

The plant volatile compounds: carvacrol (97 %, CAS 499-75-2), cinnamaldehyde (99 %, CAS 14371-10-9), eugenol (99 %, CAS 97-53-0), 8-hydroxyquinoline (99 %, CAS 148-24-3), thymol (99 %, CAS 89-83-8), and thymoquinone (99 %, CAS 490-91-5); antibiotics: amoxicillin (90 %, 26787-78-0), ampicillin (84.5 %, 69-52-3), and oxacillin (86.3 %, 7240-38-2); and other chemicals: dimethylsulfoxide (DMSO), Tween 20 %, and dye thiazolyl blue tetrazolium bromide were purchased from Sigma-Aldrich (Prague, Czech Republic).

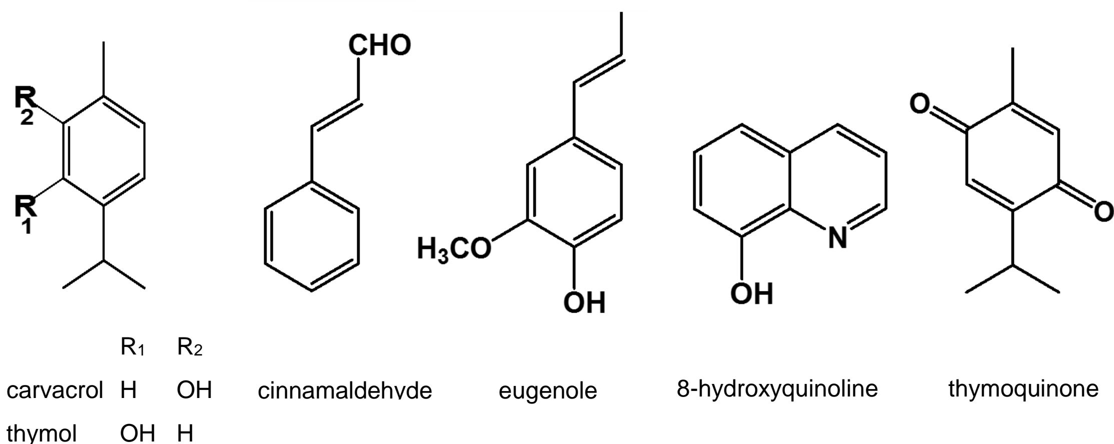

Figure 5.1 Chemical structures of volatile compounds tested

5.2.2 Bacterial strains and culture media

The following standard strains of the American Type Culture Collection (ATCC) were used: *Haemophilus influenzae* ATCC 49247, *Staphylococcus aureus* ATCC 29213, and *Streptococcus pneumoniae* ATCC 49619. The 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. pneumoniae*). The pH of broths were equilibrated to final value of 7.6 using Trizma® base (Sigma-Aldrich, Prague, Czech Republic). All microbial strains and cultivation media were purchased from Oxoid (Basingstoke, UK).

Stock cultures of bacterial strains were cultivated in appropriate medium at 37 °C for 24 h prior the testing, and then the turbidity of the bacterial suspension was adjusted to 0.5 McFarland standard using Densi-La-Meter II (Lachema, Brno, CZ) to get the final concentration of 10^7 CFU/mL. The susceptibilities of *H. influenzae*, *S. aureus* and *S. pneumoniae* to ampicillin, oxacillin and amoxicillin, respectively, were checked as positive antibiotic controls [15].

5.2.3 Antimicrobial assay

Antibacterial potential of plant volatile compounds in liquid and vapour phase was determined using newly developed broth microdilution volatilization method. The experiments were performed in standard Nunclon 96-well microtiter plates (well volume = 400 µl), covered by tight-fitting lids with flanges designed to reduce evaporation (Thermo Scientific, Roskilde, Denmark). Initially, 30 µL of agar was pipetted into every

flange on the lid (Fig. 5.2a) and inoculated with 5 µL of bacterial suspension after agar solidification. In the second part of this method, each sample of volatile compounds was dissolved in DMSO at maximum concentration 1 %, and diluted in appropriate broth medium. Seven two-fold serially diluted concentrations of samples starting from 1,024 µg/mL were prepared for all compounds, with exceptions of thymoquninone and 8-hydroxyquinoline. In case of *H. influenzae*, thymoquninone was tested at concentration range 4–256 µg/mL, whereas 8-hydroxyquinoline was assayed within ranges 4–256 µg/mL and 1–64 µg/mL for *S. pneumoniae* and *S. aureus*, respectively. The final volume in each well was 100 µL. The plates were then inoculated with bacterial suspensions. The wells containing inoculated and non-inoculated broth were prepared as growth and purity controls simultaneously. The outer most wells were left empty to prevent edge effect. Finally the clamps (Lux Tool, Prague, CZ) were used for fastening plate and lid together (Fig. 5.2b), with the handmade wooden pads (size 8.5 × 13 × 2 mm) for better fixing and the microtiter plates were incubated at 37 °C for 24 h. The minimum inhibitory concentrations (MICs) were evaluated by visual assessment of bacterial growth after colouring of metabolically active bacterial colony with MTT dye when the interface of colour change from yellow and purple (relative to that of colours in control wells) was recorded in broth and agar (Figs. 5.2c,d). The MIC values were determined as the lowest concentrations that inhibited bacterial growth compared with the compound-free control and expressed in µg/mL (in the case of vapour phase also in µg/cm^3). All experiments were carried out in triplicate in three independent experiments and results were expressed as median/modal MICs values. Schematic design of experiment is shown in Fig. 5.3. Detail of cross-sectional view of one well of microtiter plate with one flange on the lid filled with broth and agar is shown in Fig. 5.4.

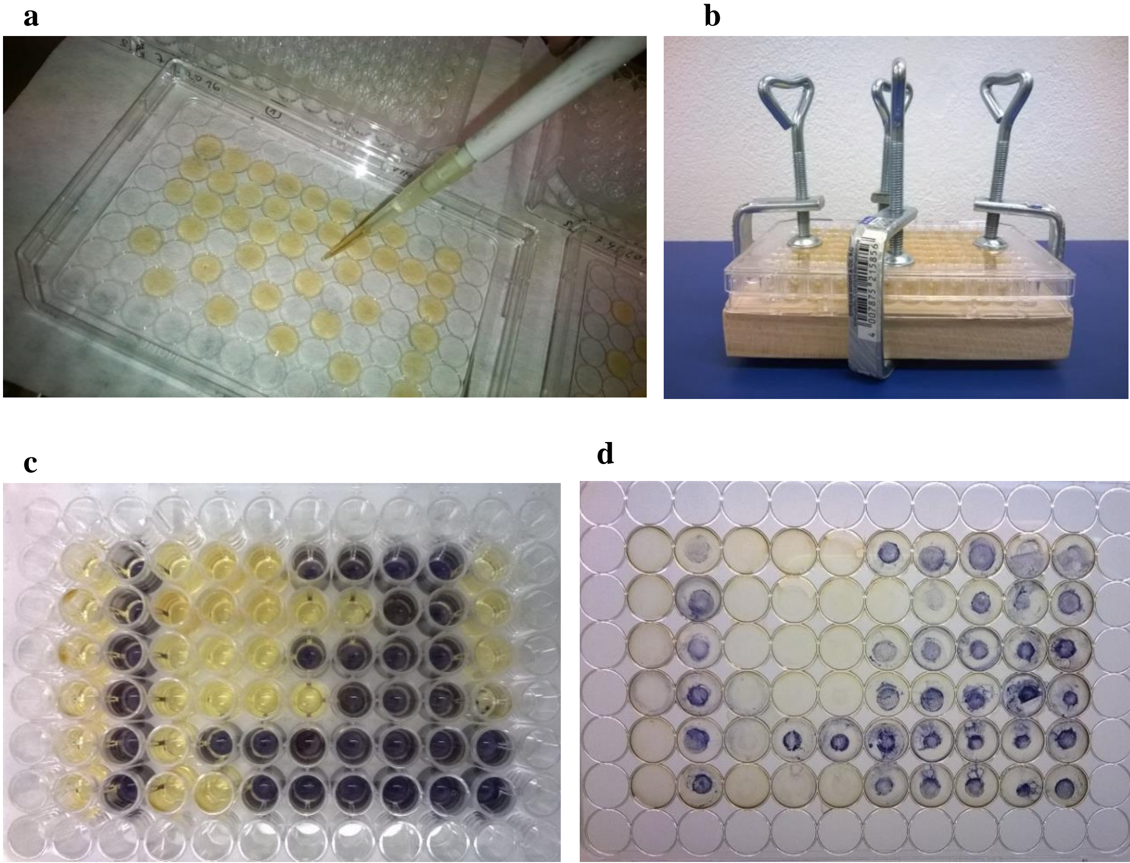

Figure 5.2 Microdilution volatilization method: **(a)** agar pipetting into every flange on the lid; **(b)** using of clamps for fastening plate and lid together; **(c)** colouring of living bacterial colony with MTT in plate (broth culture); **(d)** colouring of living bacterial colony with MTT on lid (agar culture)

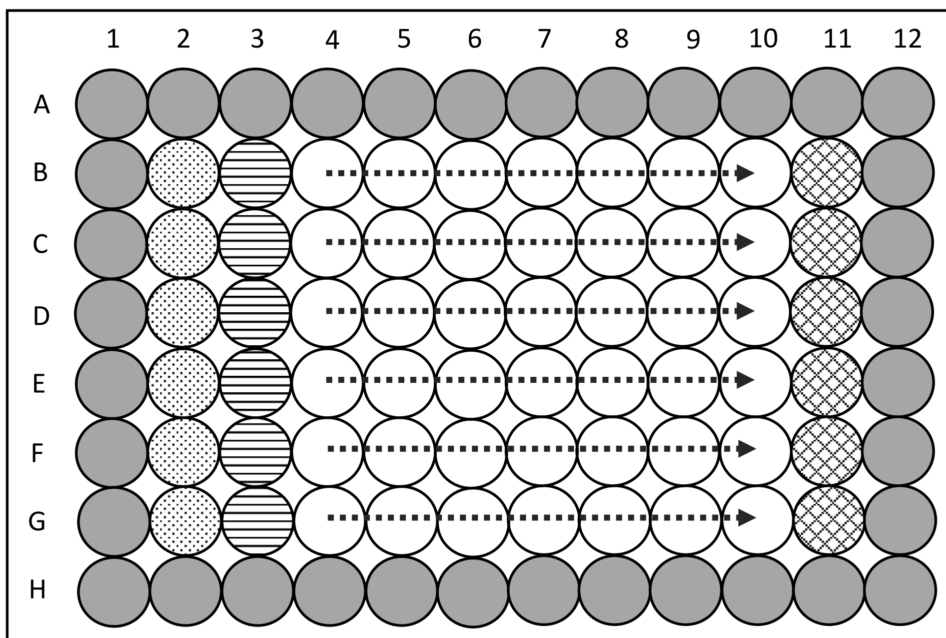

Figure 5.3 Schematic design of experiments demonstrating: template for 6 compounds with 7 serial dilutions (two-fold dilution series); ○ serial two-fold dilution of tested volatile compounds; ● empty wells, not used in data calculation (problem of evaporation); purity control (non-infected medium control; 0 % growth of bacteria); growth control (100 % growth of bacteria); serial two-fold dilution of positive antibiotic control

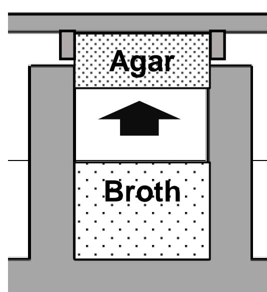

Figure 5.4 Detail of cross-sectional view of one well of microtiter plate with one flange on the lid filled with broth and agar, respectively

5.2.4 Cell culture

Primary lung fibroblast cells MRC-5, obtained from ATCC (Manassas, VA, USA), were propagated in Eagle's Minimum Essential Medium (EMEM) supplemented

with 10 % foetal bovine serum (FBS), 2 mM glutamine, 10 µL/mL non-essential amino acids, and 1 % penicillin–streptomycin solution (10,000 units/mL of penicillin and 10 mg/mL of streptomycin), all these components purchased from Sigma-Aldrich (Prague, Czech Republic). The cells were preincubated in a 96-well microtiter plates at a density of 2.5×10^3cells per well for 24 h at 37 °C in a humidified incubator in atmosphere of 5 % CO_2 in air.

5.2.5 Cytotoxicity assay

Modified method based on metabolization of MTT to blue formazan by mitochondrial dehydrogenases in living lung cells previously described by Mosmann [27] was used. The lung fibroblast cells were treated for 72 h with tested compounds dissolved in DMSO. Twelve two-fold serially diluted concentrations of these agents ranging from 512 to 0.25 µg/mL were prepared. The microtiter plates were covered by EVA capmats™ at 37 °C in humidified atmosphere of 5 % CO_2 in air, for comparison they were also set without barrier. Thereafter, MTT reagent (1 mg/mL) in EMEM solution was added to each well and plates were incubated for additional 2 h at 37 °C in humidified atmosphere of 5 % CO_2 in air. The media were removed, and the intracellular formazan product was dissolved in 100 µL of DMSO. The absorbance was measured at 555 nm and the percentage of viability was calculated when compared to untreated control. Results were expressed as half-maximal inhibitory concentration (IC_{50}) of proliferation in µg/mL.

5.3 Results and discussion

The results of *in vitro* growth-inhibitory effect of plant volatiles against bacteria associated with respiratory system infections in liquid and vapour phase using the broth microdilution volatilization method are summarized in Table 5.1. All compounds tested produced certain degree of antibacterial effect in both media; however their effectiveness varied substantially ranging from 2 to 1,024 µg/mL in broth and from 8 to 1,024 µg/mL on agar.

Table 5.1 Antibacterial activity of plant volatile compounds and antibiotics in liquid and vapour phase against pneumonia causing bacteria.

Compound	Bacterium/Growth medium/MIC (µg/mL)					
	Haemophilus influenzae		*Staphylococcus aureus*		*Streptococcus pneumoniae*	
	broth	**agar**	**broth**	**agar**	**broth**	**agar**
Carvacrol	256.0	256.0	256.0	256.0	256.0	256.0
Cinnamaldehyde	256.0	256.0	256.0	256.0	512.0	256.0
Eugenol	512.0	512.0	1,024.0	512.0	1,024.0	1,024.0
8-Hydroxyquinoline	8.0	16.0	2.0	8.0	32.0	128.0
Thymol	256.0	128.0	256.0	128.0	256.0	128.0
Thymoquinone	8.0	8.0	16.0	16.0	16.0	32.0
Positive antibiotic control						
Amoxicillin	-	-	-	-	0.1	ND
Ampicillin	1.0	ND	-	-	-	-
Oxacillin	-	-	0.1	ND	-	-

MIC: minimum inhibitory concentration, ND: not determined

In liquid phase, the lowest MIC values were observed for 8-hydroxyquinoline against *S. aureus* (2 µg/mL), and *H. influenzae* (8 µg/mL) followed by thymoquinone with MICs 8 and 16 µg/mL for *H. influenzae* and *S. pneumoniae*, respectively. Moderate antibacterial activity was shown by carvacrol, cinnamaldehyde, and thymol with MICs ranging from 256 to 512 µg/mL. Eugenol possessed only weak inhibitory effect (512 and 1,024 µg/mL). In general, the results of our assay are in correspondence with those obtained by other authors using standard broth microdilution method as it can be demonstrated for *S. aureus* ATCC 29213. In case of thymoquinone, the MIC (8 µg/mL) previously detected by Novy *et al.*[11] is within experimental error of susceptibility testing close to value determined in this study (16 µg/mL). The MICs previously observed for carvacrol (280 µg/mL), cinnamaldehyde (370 µg/mL), and thymol (250 µg/mL) by various authors [28-30] are also corresponding with our results (MICs = 256 µg/mL). MIC for 8-hydroxyquinoline against strain KCCM 11335 (10 µg/mL) reported by [31] is higher than value of MIC (2 µg/mL) detected in this study. Similarly MIC for eugenol against strain ATCC 21212 (3,343 µg/mL) observed by [32] is higher than our result (1,024 µg/mL). As it has previously been reported for number of plant-derived compounds [33,34], the differences in susceptibility of individual strains to antibacterial agents may be responsible for various results observed in our study and in the literature.

Likewise, in broth microdilution assay, thymoquinone and 8-hydroxyquinoline were the most effective antibacterial agents against *H. influenzae* and *S. aureus* in vapour phase with MIC 8 µg/mL. In addition, 8-hydroxyquinoline effectively inhibited growth of *S. pneumoniae* on agar medium at concentration 32 µg/mL. Other substances, carvacrol, cinnamaldehyde, and thymol possessed moderate antibacterial activity in vapour phase with MICs ranging 128–256 µg/mL. A low inhibitory activity was observed for eugenol (512 and 1,024 µg/mL). In contrast with a number of studies on antibacterial activity of volatile compounds in liquid media, there are only few reports on their effect in vapour phase. Various assays have previously been used for determination of antibacterial effectiveness of plant volatile compounds in vapour phase, however standardized methods (e.g. CLSI and EUCAST) [15,16] are still missing. Using a vapour diffusion method, Becerril *et al*. [29] determined inhibitory concentrations for carvacrol and cinnamaldehyde against *S. aureus* ATCC 29213 at the levels 10 and 20 µg/mL, respectively. Inouye *et al*. [35] recorded minimum inhibitory dose for thymol against *H. influenzae* ATCC 33391, penicillin-susceptible *S. pneumoniae* IP-692, penicillin-resistant *S. pneumoniae* PRC-53, and *S. aureus* FDA 209P with the same value 3.13 µg/mL for all strains by gaseous contact assay in an airtight box. Number of authors used vapour diffusion assays to study MICs of EOs, e.g. Goni *et al*. [36] detected MIC 27 µg/mL of clove EO (*Syzigium aromaticum*) against *S. aureus* ATCC 29213 with eugenol as the major components. In contrast to all above-mentioned results, values of MIC observed in our study are generally higher (128–1,024 µg/mL). The using different methods for testing of antibacterial effect in vapour phase may be responsible for the variability of results in our and previously reported studies [10,19,37,38]. The varying results may also be caused by different antibacterial efficacy of EOs and their individual constituents [35], as well as by various bacterial strains used [39].

As it can be seen in Table 5.1, some compounds showed significant differences of MICs in liquid and vapour phase. Two times lower MIC values on agar than in broth were determined in the case of thymol against all bacteria tested, and for cinnamaldehyde, and eugenol against *S. pneumoniae* and *S. aureus*, respectively. As the typical representatives of phenolic terpenoids, these compounds exhibit high volatility and low water solubility, which are properties increasing their efficacy in vapour phase and reducing their activity in liquid media [36,40,41]. Opposite results were found in the case of 8-hydroxyquinone and thymoquinone. In broth medium, antibacterial efficacy of 8-hydroxyquinone against

S. aureus and *S. pneumoniae* was four times higher than in vapour phase. Two times higher MICs were also observed for 8-hydroxyquinoline and thymoquinone against *H. influenzae* and *S. pneumoniae*, respectively. These compounds belonging to alkaloids and quinones usually exhibit limited volatility because of their low vapour pressure [42], thus they show more potent antibacterial activity in broth.

The above mentioned results demonstrate the validity of our novel broth microdilution volatilization method, which combines principles of standard broth microdilution assay with disc volatilization test. Previously developed techniques are able to evaluate antibacterial activity in liquid and vapour phases separately but the antimicrobial efficacy of the plant-derived volatile compounds differs under these conditions [17]. Therefore, the suitability for fast comparison of MIC values in both liquid and solid media is the main advantage of our method. It is applicable for testing range of concentrations in one 96-well microtiter plate; however it can be modified for microtiter plates with lower number of wells (e.g. 48) as well. In comparison with modified disc volatilization assays using Petri dishes [10,19], our method based on 96-well microtiter plate design saves material and labour consumption. Despite obvious benefits of this new antibacterial assay, it does not resolve specific drawbacks of previously developed methods caused by the physico-chemical properties of volatile compounds tested, e.g. the final concentrations of antimicrobial agents can be affected by transition between liquid and gaseous systems as well as by loses caused by their evaporation during assay processing. For this reason, the concentrations in vapour phase should be considered as indicative values only. If the distribution of volatiles is uniform in liquid and gaseous phase, the concentrations can be expressed as weight of volatile agent per volume unit of a well, whereas their real values will be 256, 128, 64, 32, 16, 8, 4 and 2 μg/cm^3 for 1024; 512; 256; 128; 64; 32; 16 and 8 μg/mL, respectively. However, the volatile compounds are usually not distributed evenly in the well. Therefore, in case of concentrations used in our experiment, they can be ranging from traces up to 341.3 μg/mL of air (for 1,024 μg/mL) depending on amount of component evaporated from the broth. If required, the exact concentrations can be determined e.g. using combination of solid phase microextraction and gas chromatography/mass spectrometry analysis [19], nevertheless the serial concentrations of broth microdilution assay can be used for indicative expressing of results of screening experiments in vapour phase.

The results of MTT test performed with the lung fibroblast cells showed certain degree of toxicity for all compounds tested, however their cytotoxicity varied substantially in ranges 0.9–295.7 µg/mL and 1.0–6.5 µg/mL for EVA capmat™ covered and non-covered microtiter plates, respectively. The IC_{50} values are summarized in Table 5.2. The levels of cytotoxic effect were classified according to the Special Programme for Research and Training in Tropical Diseases [43] as cytotoxic (IC_{50} < 2 µg/mL), moderately cytotoxic (IC_{50} 2–89µg/mL), and non-toxic (IC_{50} > 90 µg/mL).

Table 5.2 Cytotoxicity of plant volatile compounds in the lung fibroblast cells tested in covered and non-covered microtiter plates.

Compound	IC_{50} ± SD (µg/mL)	
	Non-covered plates	**EVA capmat™ covered plates**
Carvacrol	5.1 ± 1.1	122.3 ± 28.6
Cinnamaldehyde	2.5 ± 0.2	4.6 ± 1.9
Eugenol	5.5 ± 1.8	295.7 ± 61.9
8-Hydroxyquinoline	1.0 ± 0.2	0.9 ± 0.3
Thymol	6.5 ± 0.6	112.9 ± 14.7
Thymoquinone	3.0 ± 0.6	1.7 ± 0.8

IC_{50}: half maximal inhibitory concentration of proliferation in µg/mL, SD: standard deviation

In the case of non-covered microtiter plates, all tested volatiles proved to be toxic or moderately toxic. The lowest cytotoxicity was observed for thymol, followed by eugenol and carvacrol with IC_{50} values 6.5, 5.51, and 5.1 µg/mL, respectively. The highest cytotoxic potency was detected for 8-hydroxyquinoline, then for cinnamaldehyde and thymoquinone with the level of IC_{50} 1.0, 2.5, and 3.0 µg/mL, respectively. A number of assays for testing cytotoxicity of plant compounds have previously been performed on human and animal cancer cell cultures, however only few of them assessed the healthy human lung cells. With exception of thymol, which did not alter the viability of Chinese hamster lung fibroblast cells even at concentration 100 µg/mL [44], all other compounds have been observed to possess cytotoxic effect. In experiments performed with various types of human lung cancer cell lines, carvacrol, cinnamaldehyde, eugenol, and thymoquinone inhibited their proliferation with respective IC_{50} values 37.6, 11.0–40.0, 34.7–98.1, and 13.1–16.4 µg/mL [45-48]. In comparison with results obtained by other authors, our levels of cell proliferation inhibitions are generally lower. As it has

previously been described [49,50], the using of various types of lung cell cultures, including normal and cancer cell lines from the same tissue may cause the different cytotoxicity levels of plant-derived compounds.

Similarly to non-covered plates, eugenol, carvacrol and thymol exhibited the lowest cytotoxic effect in plates covered with EVA capmat™, however their IC_{50} values are up to fifty-four times higher (295.7, 122.3, and 112.9 µg/mL, respectively) than in non-covered plates and they were evaluated as non-toxic. 8-hydroxyquinoline appeared to be the most cytotoxic with IC_{50} value 0.9 µg/mL, followed by thymoquinone, and cinnamaldehyde which are toxic and moderately toxic and their IC_{50} values are about two times lower and two times higher (1.7, and 4.6 µg/mL, respectively) than in non-covered plates. Based on these results, our experiment demonstrate that the vapours of volatile compounds can significantly alter results of cytotoxicity testing, as it has previously been observed for other biological activity assays [11,25]. As in the study of Rondevaldova *et al*. [26] dealing with synergistic effect of volatile compounds with antibiotics, the EVA capmat™ was proved to be effective vapour barrier in MTT test. According to these findings, it is apparent, that the results of cytotoxicity assays of volatiles in non-covered microtiter plates might be unreliable.

Although several methods for inhalation administration of conventional antibiotics to the respiratory system has successfully been developed to deliver the drugs directly to the lung in the form of micronized droplets or solid microparticles [3,51], the application of EOs and their components is still considered as beneficial and simple approach with respect to their low price and relative safety as natural materials [14]. In this study, 8-hydroxyquinoline and thymoquinone have been determined as effective antimicrobial agents against bacteria causing diseases of respiratory system with the lowest MICs ranging from 2 to 128 µg/mL, but they also possessed high toxicity in lung cell lines with IC_{50} values 0.9–3.0 µg/mL. On the other hand, compounds evaluated as non-toxic did not show significant antibacterial activity (e.g. eugenol with IC_{50} 295.7 µg/mL and MICs 512–1,024 µg/mL). Therefore, the potential of their uses in the inhalation therapy of human respiratory diseases seems to be very limited. However, different results of cytotoxicity may be obtained *in vitro* and *in vivo* tests, as in the case of *in vivo* experiment with immunodeficiency mice that demonstrated no significant toxicity of thymoquinone [47]. Moreover, the above mentioned problems can be solved

by specific modifications of compounds, which reduce their cytotoxicity or/and enhance their antimicrobial potency. For example, thymoquinone encapsulated in nanostructured lipid carrier was found to be less toxic than pure thymoquinone in the acute toxicity study [52] and synthetic derivatives of eugenol were investigated as more efficient antibacterial agents than natural eugenol with no cytotoxic effect evaluated by MTT assay [53].

5.4 Conclusions

The effective proof-of-concept approach applicable in the development of new preparations for the inhalation therapy of the respiratory diseases has been designed in this study. According to our results, newly developed broth microdilution volatilization assay is suitable for simple and rapid simultaneous determination of antibacterial potential of plant volatile compounds in the liquid and the vapour phase at different concentrations. In addition, the use of EVA capmat^{TM} as vapour barrier cover is reliable modification of MTT assay for cytotoxicity testing of volatiles on microtiter plates. Considering the effectiveness of individual compounds tested, 8-hydroxyquinoline and thymoquinone were identified as the most active agents, whereas their antibacterial activity in vapour phase, as well as their growth-inhibitory effect in liquid media against *H. influenzae* and *S. pneumoniae*, were reported for the first time in this study. Nevertheless, their high *in vitro* toxicity towards human lung cells lines determined in this study suggests that the practical potential of their uses in the inhalation therapy of respiratory diseases will be very limited.

To summarize practical benefits of the broth microdilution volatilization method, this assay allows cost and labour effective high-throughput screening of volatile agents without the need of special apparatus. In our opinion, this assay can also potentially be used for development of various medicinal, agricultural, and food applications that are based on volatile antimicrobials such as disinfection and sterilization gases in healthcare facilities, fumigants for controlled-atmosphere storage of agricultural products, and preservation agents for active or smart packaging of food products. However, further research focused on optimization this new method for susceptibility testing of broader spectrum of microorganisms associated with above mentioned examples will be necessary.

5.5 References

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6 *In vitro* growth-inhibitory effect of Cambodian essential oils and their toxicity to lung fibroblasts

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Marketa Houdkova collected plant materials, distilled essential oils, tested antibacterial activity and analysed chemical composition. She prepared manuscript including its required revisions.

6.1 Introduction

Pneumonia belongs to the leading causes of morbidity and mortality, especially in low-income countries. The majority of severe episodes occurs in children under five years, the elderly and immuno-compromised individuals [1]. In Cambodia, about 9,100 children die from pneumonia every year [2] and according to the World Health Organization (WHO) [3] only 64.2% of children with pneumonia symptoms are taken to an appropriate healthcare provider. This acute respiratory infection of lung parenchyma is caused by bacterial pathogens such as *Haemophilus influenzae*, *Streptococcus pneumoniae*, and *Staphylococcus aureus* [4]. A timely antibiotic therapy can considerably reduce fatal cases of pneumonia [5], nevertheless many low-income countries have limited access to health services and synthetic drugs as well, whereas less than 40% of children are treated with antibiotics in Cambodia [3].

The plant EOs are of great potential for the development of novel antimicrobial preparations. They have been widely used for their diverse biological effects since the Middle Ages [6]. Since the presence of volatile compounds is characteristic for some plant taxa, chemotaxonomic research is a frequent approach to their exploration. Due to the volatility of EOs, they are suitable for inhalation therapy, which is an effective way for the healing of respiratory ailments such as pneumonia. Their vapours can act directly on the site of infection in the respiratory system and simultaneously restrict systemic exposure, degradation of active components in the gastrointestinal tract and associated toxicity [7]. In addition, EOs contain a broad spectrum of chemically diverse substances with antimicrobial effect: thus, it is more difficult for bacterial pathogens to develop resistance to these multi component mixtures than to single-ingredient conventional antibiotics [8]. During the last few years, several inhalation devices and suitable delivery systems for EOs in the treatment of respiratory infections (e.g. pocket inhaler, aromatherapy patch, decongestant on a foraminous carrier, and encapsulated EOs) have been developed and patented [9].

In Cambodia, after several decades of human destruction and the collapse of all social welfare systems during the Pol Pot regime, medicinal plants are considered as a very important factor for health security and traditional Khmer herbal medicine remains the oldest and the most accessible source of primary health care [10]. Cambodia also possesses rich natural resources and unique original ecosystems e.g. the Cardamom

Mountains, which contain a number of endemic plant taxa belonging to essential oil-bearing families such as Zingiberaceae, Lauraceae and Myrtaceae [11]. Nevertheless, scientific validation and identification of many Cambodian medicinal plants, as well as assessment of their anti-infective properties, active substances content and safety, are desirable. Recently, several *in vitro* studies have investigated biological activity and revealed some antibacterial potential of EOs derived from different parts of Cambodian plant species that are easily available in traditional markets and in wild nature [12,13]. However, no experiments determining their antibacterial potential in vapour phase against pathogens causing pneumonia had been carried out until now.

In this article, we report a detailed examination of *in vitro* growth-inhibitory effect of EOs from seven Cambodian medicinal and edible plant species against pneumonia causing bacteria in liquid and vapour phase by using a new broth microdilution volatilization method recently developed by our team [14]. This is the first practical application of this novel method in the field of EOs. Additionally, the cytotoxicity and chemical composition of tested EOs were analysed with the aim of assessing the relationship between their antimicrobial potential, chemistry and safety for treatment of pneumonia.

6.2 Materials and methods

6.2.1 Plant material

Based on chemotaxonomic criteria, seven local plant species (*Alpinia oxymitra* K. Schum., *Boesenbergia rotunda* (L.) Mansf., *Cinnamomum cambodianum* Lecomte, *Citrus lucida* (Scheff.) Mabb., *Limnophila aromatica* (Lam.) Merr., *Rhodamnia dumetorum* (DC.) Merr. & L.M.Perry, *Sindora siamensis* Miq.) were selected as phytochemically less explored representatives of taxa containing EOs. The plant material was collected between July and September 2016. *A. oxymitra*, *C. cambodianum*, *R. dumetorum*, and *S. siamensis* were collected from various districts of Cambodia (Cardamom Mountains, Elephant Mountains, Chant Saen Commune in Oudong District) from wild populations of at least three independent plants. *B. rotunda*, *C. lucida*, and *L. aromatica* were purchased in local markets (Psar Thmei, Chbar Ampov, and Cham Kar Dong in Phnom Penh). Identification of species was performed in the field by

ethnobotany expert Prof Ladislav Kokoska, currently head of the Laboratory of Ethnobotany and Ethnopharmacology of the Faculty of Tropical AgriSciences, Czech University of Life Sciences Prague. Voucher specimens were deposited in the herbarium of the 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). A detailed description of collected plant samples is summarized in Table 6.1.

6.2.2 Essential oils isolation

Essential oils were obtained by hydrodistillation of dried plant material (except *C. lucida* fruit peel which was obtained by the grating of fresh fruits using a stainless steel grater) in 1 L of distilled water for 3 h using a Clevenger-type apparatus (Merci, Brno, CZ) according to the procedures described in the *European Pharmacopoeia* [15]. The EOs were stored in sealed glass vials at 4°C. The data on yields (v/w, based on the dry plant weight) of obtained EOs are shown in Table 6.1.

6.2.3 Bacterial strains and culture media

The following standard strains of the American Type Culture Collection (ATCC) were used: *Haemophilus influenzae* ATCC 49247, *Staphylococcus aureus* ATCC 29213, and *Streptococcus pneumoniae* ATCC 49619. The 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. pneumoniae*). The pH of broths were equilibrated to a final value of 7.6 using Trizma base (Sigma-Aldrich, Prague, CZ). All microbial strains and cultivation media were purchased from Oxoid (Basingstoke, UK).

Stock cultures of bacterial strains were cultivated in appropriate medium at 37°C for 24 h prior to the testing and then the turbidity of the bacterial suspension was adjusted to 0.50 McFarland standard using Densi-La-Meter II (Lachema, Brno, CZ) to get the final concentration of 10^7 CFU/mL. The susceptibilities of *H. influenzae*, *S. aureus*, and *S. pneumoniae* to ampicillin (84.5%, CAS 69-52-3), oxacillin (86.3%, CAS 7240-38-2)

Table 6.1 Cambodian plant species selected for antibacterial and cytotoxicity testing

Scientific name	Family	Collection number	Area of collection	Part used	Weight of sample (g)	Essential oil yield % (v/w)	Essential oil colour
Alpinia oxymitra K.Schum.	Zingiberaceae	02463KBFR6	Mt Aoral	leaves	49.0	0.4	colourless
				pericarp	12.3	0.3	pale yellow
				rhizomes	125.4	0.1	colourless
				seeds	21.9	4.7	colourless
Boesenbergia rotunda (L.) Mansf.	Zingiberaceae	-	Chbar Ampov	rhizomes	109.9	0.3	colourless
Cinnamomum cambodianum Lecomte	Lauraceae	02455KBFR7	Mt Aoral	bark	44.5	0.5	pale yellow
				leaves	41.7	0.4	pale yellow
Citrus lucida (Scheff.) Mabb.	Rutaceae	02476KBFRA	Cham Kar Dong	fruit peel	74.5	0.4	pale yellow
Limnophila aromatica (Lam.) Merr.	Plantaginaceae	02469KBFRC	Psar Thmei	aerial part	13.8	1.2	slightly yellow
Rhodamnia dumetorum (DC.) Merr. & L.M.Perry	Myrtaceae	02458KBFRA	Oudong	leaves	164.8	0.2	bright yellow
Sindora siamensis Miq.	Leguminosae	02481KBFR6	Angkor Chey	fruit husk	44.0	0.5	pale yellow

and amoxicillin (90%, CAS 26787-78-0), respectively, purchased from Sigma-Aldrich (Prague, CZ), were checked as positive antibiotic controls [16].

6.2.4 Antimicrobial assay

The antibacterial potential of plant EOs in liquid and vapour phase was determined using a broth microdilution volatilization method [14]. The experiments were performed in standard Nunclon 96-well microtiter plates (well volume = 400 µL), covered by tight-fitting lids with flanges designed to reduce evaporation (Thermo Scientific, Roskilde, DK). Initially, 30 µL of agar was pipetted into every flange on the lid except the outermost flanges and inoculated with 5 µL of bacterial suspension after agar solidification. In the second part of this method, each sample of volatile oils was dissolved in dimethylsulfoxide (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 1,024.0 µg/mL were prepared for all EOs. The final volume in each well was 100 µL. The plates were then inoculated with bacterial suspension using a 96-pin multi-blot replicator (National Institute of Public Health, Prague, CZ). The wells containing inoculated and non-inoculated broth were prepared as growth and purity controls simultaneously. The outermost wells were left empty to prevent edge effect. Finally, clamps (Lux Tool, Prague, CZ) were used for fastening the plate and lid together, with the handmade wooden pads (size 8.5 × 13 × 2 mm) for better fixing and the microtiter plates were incubated at 37°C for 24 h. The minimum inhibitory concentrations (MICs) were evaluated by visual assessment of bacterial growth after colouring of a metabolically active bacterial colony with thiazolyl blue tetrazolium bromide dye (MTT) in a concentration of 600 µg/mL (Sigma-Aldrich, Prague, CZ) when the interface of colour change from yellow to purple (relative to that of colours in control wells) was recorded in broth and agar. The MIC values were determined as the lowest concentrations that inhibited bacterial growth compared with the compound-free control and expressed in µg/mL (in the case of vapour phase also in µg/cm^3, where 256, 128, 64, 32 16, 8, 4, and 2 µg/cm^3 are real values for 1024, 512, 256, 128, 64, 32, 16, and 8 µg/mL, respectively). The DMSO assayed as the negative control at concentration of 1% did not inhibit any of the strains tested either in broth or agar media. All experiments were carried out in triplicate in three independent experiments and results were expressed as median/modal MICs values.

6.2.5 Cell culture

Primarily, lung fibroblast cells MRC-5 obtained from ATCC (Manassas, VA, USA), were propagated in Eagle's Minimum Essential Medium (EMEM) supplemented with 10% foetal bovine serum (FBS), 2 mM glutamine, 10 µL/mL non-essential amino acids, and 1% penicillin–streptomycin solution (10,000 units/mL of penicillin and 10 mg/mL of streptomycin), all these components purchased from Sigma-Aldrich (Prague, CZ). The cells were preincubated in 96-well microtiter plates at a density of 2.5 $\times 10^3$ cells per well for 24 h at 37°C in a humidified incubator in an atmosphere of 5% CO_2 in air.

6.2.6 Cytotoxicity assay

The modified method for toxicity assessment of volatile agents [14] based on metabolization of MTT to blue formazan by mitochondrial dehydrogenases in living lung cells previously described by Mosmann [17] was used. At first, twelve two-fold serially diluted concentrations ranging from 512 to 0.25 µg/mL of EOs dissolved in DMSO were prepared. The microtiter plates were covered by EVA capmats™ at 37°C in a humidified atmosphere of 5% CO_2 in air and cultivated for 72 h. Thereafter, MTT reagent (1 mg/mL) in EMEM solution was added to each well and plates were incubated for an additional 2 h under the same conditions. The media were removed and the intracellular formazan product was dissolved in 100 µL of DMSO. The solvent used did not affect the viability of the lung cells at the concentrations tested. The absorbance was measured at 555 nm and the percentage of viability was calculated when compared to an untreated control. The results of the cytotoxicity effect were expressed as half maximal inhibitory concentration (IC_{50}) in µg/mL. The levels of cytotoxic effect were classified according to the Special Programme for Research and Training in Tropical Diseases [18] as cytotoxic ($IC_{50} < 2$ µg/mL), moderately cytotoxic (IC_{50} 2–89 µg/mL), and non-toxic ($IC_{50} > 90$ µg/mL). Furthermore, 80% inhibitory concentration of proliferation (IC_{80}) was calculated as equivalent to MIC endpoint usually defined as 80% bacterial growth inhibition [19] and therapeutic indices (TI), defined as the ratio of IC_{50} or IC_{80} and MIC values, were determined with the aim of comparing the amount of effective antibacterial agents with the quantity causing toxicity [20].

6.2.7 GC-MS analysis

For determination of the main components of EOs tested, GC-MS analysis was carried out using the dual-column/dual-detector gas chromatograph system Agilent GC-7890B equipped with autosampler Agilent 7693, two columns, a fused-silica HP-5MS column (30 m × 0.25 mm, film thickness 0.25 µm, Agilent 19091s-433) and a DB-17MS column (30 m × 0.25 mm, film thickness 0.25 µm, Agilent 122-473), and a flame ionization detector (FID) coupled with single quadrupole mass selective detector Agilent MSD-5977B (Agilent Technologies, Santa Clara, CA, USA). The operational parameters were: helium as carrier gas at 1 mL/min, injector temperature 250 and 200°C for HP-5MS and DB-17MS, respectively. The oven temperature was raised from 50 to 300°C for HP-5MS and from 50 to 280°C for DB-17MS. Samples of EOs diluted in n-hexane for GC/MS (Merck KGaA, Darmstadt, DE) at concentration 1 µg/mL and 1 µL of solution was injected in splitless mode. The mass detector was set to following conditions: ionization energy 70 eV, ion source temperature 200°C, scan time 1 s, mass range 30–600 m/z.

The identification of constituents was based on comparison of their retention indices (RI) and retention times (RT) with the National Institute of Standards and Technology Library ver. 2.0.f (NIST, USA), as well as authentic standards (Sigma-Aldrich, Prague, CZ) and literature [21]. The RI were calculated for compounds separated by the HP-5MS column using the retention times of *n*-alkanes series ranging from C9 to C29 (Sigma-Aldrich, Prague, CZ). For each EO analysed, the final number of compounds was calculated as the sum of components simultaneously identified using both columns and the remaining constituents identified by individual columns only. The relative percentage contents of EO components were determined by FID and indicated for both columns.

6.3 Results

In this study, eleven EOs derived from different parts of seven Cambodian medicinal and edible plant species were obtained in yields ranging from 0.1 to 1.2% (v/w). All EOs exhibited a certain degree of antibacterial activity in liquid and vapour phase against at least one bacteria associated with respiratory system infections. The results of

their *in vitro* growth-inhibitory effect against three bacterial strains using the broth microdilution volatilization method are summarised in Table 6.2. The MTT assay performed with the lung fibroblast cells showed the potential safety of certain EOs. The results of cytotoxicity assay are listed in Table 6.3. Based on GC-MS analysis, it was found that monoterpenoids and sesquiterpenoids were the leading compounds present in the EOs tested. The complete chemical composition is provided in Tables 6.4.

6.3.1 Antibacterial activity

All essential oils tested in this study exposed some antibacterial efficacy; however, only the EO from *A. oxymitra* rhizomes was active against all bacteria tested. In general, the effectiveness of EOs varied substantially ranging from 64 to 1,024 µg/mL in broth and from 32 to 1,024 µg/mL on agar.

In liquid phase, the lowest MIC value was observed for *A. oxymitra* pericarp (64 µg/mL) against *H. influenzae*, followed by *A. oxymitra* rhizomes with MIC 128 µg/mL against *H. influenzae* and *S. pneumoniae*. Moderate antibacterial activity was produced by *A. oxymitra* leaves and seeds, *C. lucida*, and *S. siamensis* with MICs ranging from 256 to 512 µg/mL. *C. cambodianum* leaves and *R. dumetorum* possessed only weak inhibitory effect (1,024 µg/mL). No antibacterial activity in broth medium was determined for *B. rotunda*, *C. cambodianum* bark, and *L. aromatica*.

As well as in broth, *A. oxymitra* pericarp was the most effective antibacterial agent against *H. influenzae* in gaseous phase with MIC 32 µg/mL. In addition, *A. oxymitra* rhizomes effectively inhibited growth of *H. influenzae* on agar medium at concentration 64 µg/mL. Other EOs, *A. oxymitra* leaves and seeds, *C. cambodianum* leaves, *C. lucida*, and *S. siamensis* possessed moderate antibacterial activity in vapour phase with MICs ranging from 256 to 512 µg/mL. A low inhibitory activity was observed for *B. rotunda*, *C. cambodianum* bark, *L. aromatica* and *R. dumetorum* against *H. influenzae* (1,024 µg/mL). All EOs affected growth of *H. influenzae* on agar, whereas *S. aureus* and *S. pneumoniae* were inhibited only by *A. oxymitra* seeds and *C. lucida* EOs, respectively.

In Table 6.2, it can be seen that some EOs showed significant differences in liquid and vapour phase MICs. Their two times lower values on agar than in broth were

Table 6.2 Antibacterial activity of Cambodian essential oils and antibiotics in liquid and vapour phase against pneumonia causing bacteria

		Bacteria/grown medium/MIC								
		Haemophilus influenzae			***Staphylococcus aureus***			***Streptococcus pneumoniae***		
		broth	**agar**		**broth**	**agar**		**broth**	**agar**	
Essential oils	**part used**	**(µg/mL)**	**(µg/mL)**	**(µg/cm³)**	**(µg/mL)**	**(µg/mL)**	**(µg/cm³)**	**(µg/mL)**	**(µg/mL)**	**(µg/cm³)**
Alpinia oxymitra	leaves	512.0	512.0	128.0	1,024.0	>1,024.0	>256.0	>1,024.0	>1,024.0	>256.0
	pericarp	64.0	32.0	8.0	512.0	>1,024.0	>256.0	>1,024.0	>1,024.0	>256.0
	rhizomes	128.0	64.0	16.0	256.0	>1,024.0	>256.0	128.0	>1,024.0	>256.0
	seeds	512.0	256.0	64.0	1,024.0	1,024.0	256.0	>1,024.0	>1,024.0	>256.0
Boesenbergia rotunda	rhizomes	>1,024.0	1,024.0	256.0	>1,024.0	>1,024.0	>256.0	>1,024.0	>1,024.0	>256.0
Cinnamomum cambodianum	bark	>1,024.0	1,024.0	256.0	>1,024.0	>1,024.0	>256.0	>1,024.0	>1,024.0	>256.0
	leaves	1,024.0	512.0	128.0	>1,024.0	>1,024.0	>256.0	>1,024.0	>1,024.0	>256.0
Citrus lucida	fruit peel	512.0	256.0	64.0	>1,024.0	>1,024.0	>256.0	>1,024.0	1,024.0	256.0
Limnophila aromatica	aerial part	>1,024.0	1,024.0	256.0	>1,024.0	>1,024.0	>256.0	>1,024.0	>1,024.0	>256.0
Rhodamnia dumetorum	leaves	1,024.0	1,024.0	256.0	>1,024.0	>1,024.0	>256.0	>1,024.0	>1,024.0	>256.0
Sindora siamensis	fruit	256.0	256.0	64.0	1,024.0	>1,024.0	>256.0	>1,024.0	>1,024.0	>256.0
Positive antibiotic control										
Amoxicillin		-	-	-	-	-	-	0.3	ND	ND
Ampicillin		0.5	0.3	0.1	-	-	-	-	-	-
Oxacillin		-	-	-	0.3	ND	ND	-	-	-

MIC: minimum inhibitory concentration; ND: not determined; -: not tested

determined for EOs from *A. oxymitra* pericarp, rhizomes and seeds, *C. cambodianum* leaves, and *C. lucida* fruit peel against *H. influenzae*. In addition, *B. rotunda*, *C. cambodianum* bark, and *L. aromatica* EOs were effective against *H. influenzae* in vapour phase, whereas no activity has been observed in broth. Similarly, *S. pneumoniae* was susceptible to *C. lucida* EO in vapour phase only. Opposite results were found in the case of volatile oils from *A. oxymitra* leaves, pericarp, rhizomes, and *S. siamensis*, which inhibited growth of *S. aureus* and *S. pneumoniae* in broth medium without effect in vapour phase.

6.3.2 Cytotoxicity

Values of IC_{50} and IC_{80} for lung fibroblasts varied substantially in ranges 2.0–225.4 µg/mL and 10.9–395.6 µg/mL, respectively. In the case of *A. oxymitra* pericarp EO, IC_{80} value was not detected ($IC_{80} > 512$ µg/mL). The comparison of MIC and IC_{80} values suggests *A. oxymitra* pericarp EO as an effective antimicrobial agent that is non-toxic to lung fibroblasts.

A. oxymitra seeds, *C. lucida*, *C. cambodianum* bark and leaves were found to be non-toxic to lung cells with respective IC_{50} values 225.4, 182.5, 133.9, and 112.2 µg/mL. Moderately toxic were EOs of other parts of *A. oxymitra*, *B. rotunda*, *L. aromatica*, and *S. siamensis* (IC_{50} value ranging from 6.0 to 30.0 µg/mL). *R. dumetorum* appeared to be the most cytotoxic with IC_{50} value 2.0 µg/mL.

Similarly to IC_{50}, in the case of IC_{80} the lowest cytotoxicity was observed for EOs of *A. oxymitra* pericarp, *C. cambodianum* bark, *A. oxymitra* seeds, *C. lucida*, *C. cambodianum* leaves, and *B. rotunda* with IC_{80} values > 512.0, 395.9, 391.6, 380.3, 364.3, and 106.34 µg/mL, respectively. Moderate toxicity was recorded for *L. aromatica*, *A. oxymitra* leaves and rhizomes, *S. siamensis*, and *R. dumetorum* (IC_{80} value range 10.9–50.8 µg/mL).

Table 6.3 Cytotoxicity of Cambodian essential oils in the lung fibroblast cells

Essential oils	part used	IC_{50} ± SD (µg/mL)	IC_{80} ± SD (µg/mL)
Alpinia oxymitra	leaves	7.1 ± 1.3	41.5 ± 11.7
	pericarp	30.0 ± 0.0	> 512.0
	rhizomes	7.1 ± 0.9	16.2 ± 4.6
	seeds	225.4 ± 37.4	391.6 ± 13.4
Boesenbergia rotunda	rhizomes	22.1 ± 6.9	106.3 ± 13.4
Cinnamomum cambodianum	bark	133.9 ± 12.9	395.6 ± 14.5
	leaves	112.2 ± 59.0	364.3 ± 27.4
Citrus lucida	fruit peel	182.5 ± 2.0	380.3 ± 1.4
Limnophila aromatica	aerial part	16.6 ± 5.6	50.8 ± 2.5
Rhodamnia dumetorum	leaves	2.0 ± 1.2	10.9 ± 0.3
Sindora siamensis	fruit	6.0 ± 2.7	16.0 ± 5.7

IC_{50}: half-maximal inhibitory concentration of proliferation in µg/ml; IC_{80}: 80% inhibitory concentration of proliferation in µg/mL; SD: standard deviation

6.3.3 GC-MS analysis

In the essential oils of *A. oxymitra* leaves, pericarp, rhizomes, and seeds, a total of 56, 77, 52, and 62 compounds were identified using both HP-5MS/DB-17MS columns, representing 93.9/92.8, 88.5/91.2, 91.6/91.2, and 95.2/93.6% of their total contents, respectively. The analyses showed that the major constituents of *A. oxymitra* leaves, pericarp, and rhizomes EOs were monoterpenoids and sesquiterpenoids in respective total contents 76.3/76.0, 55.6/58.5, 84.5/88.2, and 17.4/16.7, 27.6/29.2, 2.5/2.7%, whereas in *A. oxymitra* seeds essential oil, sesquiterpenoids were the most dominant components (88.8/85.1%). In *A. oxymitra* leaves, *β*-pinene (58.4/59.1%) was the main compound followed by caryophyllene epoxide (6.1/5.6%), *α*-pinene (4.8/5.0%), caryophyllene (3.0/3.0%), and myrtenol (2.7/2.5%). Furthermore, a relatively high amount of sabinene (2.6/3.4%) was detected. Similarly, the EO from *A. oxymitra* pericarp consisted chiefly of *β*-pinene (29.8/31.3%), caryophyllene epoxide (20.2/19.7%), myrtenol (5.5/5.2%), *trans*-pinocarveol (4.7/3.6%), and pinocarvone (3.7/4.4%). Then perillol was one of the main compounds found only by DB-17MS column (5.0%). In *A. oxymitra* rhizomes EO, *β*-pinene (50.2/53.2%) prevailed as well, followed by *o*-cymene (8.8/20.0%), *α*-pinene (5.1/5.6%), and myrtenol (3.3/1.2%). 1,8-cineole was detected present also in a significant amount by HP-5MS column only (5.2%). In contrast to EOs derived from other parts of *A. oxymitra*, sesquiterpenoids shyobunol (21.7/4.2%), germacrene D (16.5/16.3%), cubebol (16.2/16.3%), 6-*epi*-shyobunol (9.8/12.4%), and germacrene-D-4-

ol (8.4/7.5%) were the main components of seed volatile oil. Moreover, the presence of aromadendrene (4.7%) was found by DB-17MS column.

The analysis of *C. cambodianum* revealed the same number of components 76 for both bark and leaf EOs, representing 96.5/92.0 and 90.8/93.0% of total volatiles content, respectively. The EOs mainly contained monoterpenoids (80.7/70.3 and 59.7/63.0%) followed by sesquiterpenoids (15.7/21.1, 30.3/29.2%). 1,8-cineole was the major compound in both bark and leaf EOs (31.8/33.5, 30.5/33.1%). Other components of *C. cambodianum* bark EO were *α*-terpineol (9.5/8.0%), *α*-pinene (8.8/6.0%), borneol (5.7/5.0%), terpinen-4-ol (5.4/2.5%), and *β*-spathulenol (5.3% on DB-17MS column). The EO of *C. cambodianum* leaves was also rich in espathulenol (7.6/8.2%), *α*-terpineol (6.5/6.4%), *β*-spathulenol (5.8/3.6%), and linalool (4.7/4.2%).

In *B. rotunda* and *L. aromatica* EOs, 36 and 47 compounds were identified that represent 98.8/98.4 and 97.4/96.1% of their total respective contents. The EO of *B. rotunda* was composed especially of monoterpenoids that were present in the amount of 96.7/96.2%. The most abundant component was ocimene (28.0/27.6%), followed by geraniol (24.4/24.0%), camphor (18.6/19.1%), 1,8-cineole (5.5/5.6%), and camphene (5.4/5.5%). Similarly, monoterpenoids was the predominant class of compounds in *L. aromatica* EO with total contents 85.3/81.8%. Sesquiterpenoids were also represented in significant amounts (10.5/13.1%) with the main component being limonene (48.3/48.1%), followed by 3-*p*-menthen-7-al (20.1/20.3), myrtanol acetate (7.7/3.0%), *α*-humulene (2.6/5.7%), and perillyl acetate (2.8/2.8%).

When the EOs of *C. lucida*, *R. dumetorum*, and *S. siamensis* were analysed, 49, 70, and 72 compounds were identified, representing 98.3/96.6, 90.0/92.2, and 91.8/90.3% of their total contents, respectively. The EO of *R. dumetorum* was rich in sesquiterpenoids (51.3/6.8%), and monoterpenoids (36.7/85.3%) with the major compounds being caryophyllene epoxide (33.3/4.5%), *α*-pinene (26.1/73.5%), humulene-1,2-epoxide (2.5/0.4), and caryophyllene (2.4/0.6%). Moreover based on the analysis with DB-17MS column, limonene (1.4/1.7%), *trans*-verbenol (1.2/1.6%), and *α*-pinene epoxide (0.5/1.2) were present in a relatively high amounts. Similarly, *S. siamensis* EO consisted mainly of sesquiterpenoids (76.0/73.7%) and monoterpenoids (15.5/16.%) with the main constituents being *β*-bourbonene (27.5/28.5%), caryophyllene epoxide (14.5/12.4%), *α*-pinene (6.7/7.4%), espathulenol (4.48/3.74%), and *β*-pinene (4.4/4.7%). On DB-17MS

column, a significant amount of *trans-α*-bergamotene (3.2/4.2%) was detected. In contrast to others EOs analysed in this study, the major constituents of *C. lucida* EO were esters (59.1/37.5%), predominated by decyl acetate (49.4/27.6%) and dodecenyl acetate (9.3/9.0%), followed by carbonylic compounds (15.6/34.9%) and sesquiterpenoids (14.8/14.0%). Aliphatic alcohols (7.7/7.8%) were also present in significant amounts with the main components being decanal (13.6/12.4%), caryophyllene epoxide (5.8/6.4%), and decan-1-ol (5.3/5.4%).

6.4 Discussion

From a chemotaxonomic point of view, Lauraceae, Myrtaceae, Rutaceae, and Zingiberaceae are typical families containing EOs with antimicrobial properties [22-24]. In correspondence with this, the EOs from *A. oxymitra* and *B. rotunda* (Zingiberaceae), *C. cambodianum* (Lauraceae), *C. lucida* (Rutaceae), *L. aromatica* (Plantaginaceae), *R. dumetorum* (Myrtaceae), and *S. siamensis* (Leguminosae) were obtained using hydrodistillation, whereas the EOs from *A. oxymitra* leaves, pericarp and seeds, *C. lucida* fruit peel, *R. dumetorum* leaves, and *S. siamensis* fruit husk were isolated for the first time.

As far as antibacterial activity of plant species tested in this study is considered, *B. rotunda* EO is the only one previously tested for its antimicrobial effect. It exhibited growth-inhibitory activity with MIC value 12,500 µg/mL against *S. aureus* [12]. In the case of *L. aromatica*, our results can be supported by studies of Visutthi [25] who described growth-inhibitory action of its methanolic/ethanolic extracts against various strains of *S. aureus* (MIC values ranging 2,500–2,600 µg/mL). Similarly, as in our previous research [14], different MIC values of EOs were recorded in broth and agar medium, which could be caused by the diverse volatility of samples tested [26], as well as by varying levels of bacterial sensitivity in liquid and solid medium [27]. According to our best knowledge, this is the first report on antibacterial activity of *A. oxymitra*, *C. cambodianum*, *C. lucida*, *L. aromatica*, *R. dumetorum*, and *S. siamensis* EOs. Moreover, the growth-inhibitory effects of EOs from all seven species were assessed for the first time in vapour phase in this study.

Although there is a lack of data on antibacterial activity of the above-mentioned EOs, several studies reporting the antibacterial potential of their main components have recently been published. For example, a significant growth-inhibitory effect of *α*- and *β*-pinenes, constituents of *R. dumetorum* and *A. oxymitra* leaves, pericarp, and rhizomes, has previously been reported against methicillin-resistant *S. aureus* with respective MIC values 4.15 and 6.25 µg/mL [28]. Ocimene, geraniol, and camphor, dominant monoterpenoids of *B. rotunda* EO, also exhibited growth-inhibitory effect against various bacterial pathogens [29,30]. Moreover, the antibacterial potential of geraniol and camphor vapours was determined as well [31]. A certain degree of anti-*S. aureus* effect has previously been attributed to 1,8-cineole and terpinen-4-ol, which were detected as the major constituents of both *C. cambodianum* bark and leaf EOs [30,32]. According to the previous studies [33,34], decanal and *β*-caryophyllene, abundant compounds of EO from *C. lucida*, produced antibacterial effect against clinical strains of *S. aureus* and other bacterial pathogens with respective MIC values 62.5–125.0 µg/mL and 0.61–2.86 µg/mL. Antibacterial properties were also described for caryophyllene epoxide [35] and limonene [30,32], which are present in *L. aromatica* and *R. dumetorum* EOs respectively. Therefore, it is possible to suppose that the above-mentioned constituents can significantly contribute to the antibacterial effects of EOs analysed in this study when they lead to the disintegration of pathogen cell walls and membranes, deformation of cells and reduction in nucleus cytoplasm [36].

Several studies have previously evaluated the cytotoxic effect of extracts and their compounds isolated from *B. rotunda* and *L. aromatica* [37] against carcinogenic lung cell lines; however, the data on inhalation toxicity of the EOs tested in this study and their safety for healthy lung tissues are missing. Nevertheless, a number of studies concerning the toxic properties of constituents present in EOs from our samples have previously been published. Nielsen *et al.* [38] investigated the acute inhalation effect of *α*-pinene vapours on the respiratory system in mice, where no animal died at the maximum exposure of concentration 5,213 ppm. Another *in vivo* test of acute toxicity in rodents showed that 1,8-cineole was well tolerated up to a dose of 1,500 mg/kg [39] and an LD_{50} of *α*-terpineol was determined to be 2,900 mg/kg [40] both being administered by oral route. Few reports have determined *in vitro* cytotoxic levels for volatiles to various cell cultures where *α*-pinene and *β*-caryophyllene (detected e.g. in *A. oxymitra*, *C. cambodianum*, *R. dumetorum*, and *S. siamensis* EOs) exhibited a good safety profile for human

lymphocytes [41]. In contrast, caryophyllene epoxide, a dominant compound of *R. dumetorum* EO, was responsible for the strong toxicity of a number of EOs previously reviewed by Judzentiene *et al.* [42]. Although, borneol (found in *C. cambodianum* bark EO) was evaluated as toxic to human fibroblasts (IC_{50} = 1.5 µg/mL) [43], *C. cambodianum* bark EO was evaluated as non-toxic in our study.

When comparing the bacterial growth-inhibitory and cytotoxic effects of samples in this study, *A. oxymitra* pericarp EO has been determined as the most effective antimicrobial agent against *H. influenzae* with the MIC value 32 µg/mL. According to the results of toxicological evaluation, this EO is safe to lung cell lines at the IC_{80} level (> 512 µg/mL) and a calculated TI value (> 16) suggests that *A. oxymitra* pericarp EO could be considered as a potential antibacterial agent for inhalation treatment of respiratory infections [44]. However, TI calculated from IC_{50} value (30.0 µg/mL) indicated certain toxicological risk to the human lung cells (TI = 0.9). Therefore, more detailed toxicological assessments (especially *in vivo*) will be necessary for determination of its safety profile.

The chemical composition of EOs from various parts of *B. rotunda*, *L. aromatica*, *C. cambodianum*, and *C. lucida* has previously been described; however, literature about chemical analysis of *A. oxymitra*, *C. cambodianum* bark, *C. lucida* fruit peel, *R. dumetorum*, and *S. siamensis* EOs is not available. When comparing analytical data from this study with previously published works on *B. rotunda* rhizome EO, its chemical composition corresponds to results of Phanthong *et al.* [13], who detected ocimene, geraniol, camphor, 1,8-cineole, and camphene as its main constituents. The general prevalence of monoterpene compounds in *L. aromatica* EOs resembles the chemistry of those previously isolated from plants collected in Bangladesh and Vietnam [45,46]. Nevertheless, there were both quantitative and qualitative variations with respect to their major components. While we found limonene (> 48.0%) as the most abundant compound, its amount described in literature was equal to or lower than 20.0%. On the other hand methyl benzoate, pulegone, camphor, ocimene, and terpinolene, the main constituents of previous analysis, were absent or detected in noticeably lower amounts in our sample. Although linalool (33.10%) and terpinen-4-ol (12.3%) were previously identified as the main constituents of leaf EO from *C. cambodianum* grown in Vietnam [47], we detected these components in seven and four times lower quantities, respectively. The chemical

composition of previously analysed EO from *C. lucida* leaves [48] has a similar pattern of constituents to fruit peel investigated in this study, except for leaf EO's major component *β*-caryophyllene (26.60%) which was detected in a lower amount (< 5.0%) in our sample. Decyl acetate, a predominated compound of fruit peel EO, was identified in leaves at a four times lower amount. Chemical profiles of 17 various *Rhodamnia* species have previously been examined [49], whereas *R. argentea*, *R. australis*, and *R. whiteana* EOs obtained by steam distillation were chemically similar to our sample of *R. dumetorum*. Nevertheless, none of these species contained as high a quantity of caryophyllene epoxide as *R. dumetorum* did. The differences in qualitative and quantitative compositions of our, and previously analysed, EOs can be caused by genetic and environmental factors including geographical origin, as well as by using various methods for their isolation [50,51].

With the aim of achieving a higher quality identification of detected constituents, the volatile oils analyses were conducted on two columns with different polarities, non-polar HP-5MS and more polar DB-17MS. In recent studies, it has been well described that the use of a simultaneous dual-column /dual-detector system increases the resolution of the analysis leading to the improved quantitation and identification of EO components [52,53]. Based on these principles, four, eighteen, thirteen, and fourteen additional compounds have been identified using the second column DB-17MS in EOs of *A. oxymitra* leaves, pericarp, rhizomes, and seeds, respectively. Similarly, five additional constituents were found in EO of *B. rotunda*, nineteen and twenty-two in EOs of *C. cambodianum* bark and leaves, eight in *C. lucida*, three in EOs of *L. aromatica*, seven in *R. dumetorum*, and eight components in EO of *S. siamensis*. Some compounds detected by one column appeared as two peaks on the second column, e.g. it was observed in the case of *C. lucida* essential EO, when the main constituent decyl acetate, (49.4%) found on the HP-5MS column, was separated into two different substances dodecyl acetate (27.6 %) and dodecanal (21.0%) on the DB-17MS column. It was explained by Liu and Hu [54] that compounds, which have similar retention times on one column, usually have quite different retention times on the column with different polarity.

6.5 Conclusion

In this study, we reported the *in vitro* growth-inhibitory potential of eleven EOs obtained by hydrodistillation from seven Cambodian plant species (*A. oxymitra*, *B. rotunda*, *C. cambodianum*, *C. lucida*, *L. aromatica*, *R. dumetorum*, and *S. siamensis*) against pneumonia causing bacteria in liquid and vapour phase and their effect on the proliferation of lung fibroblasts. All samples exhibited a certain degree of antibacterial activity against at least one bacteria associated with respiratory system infections, whereas cytotoxicity assay showed the relative safety of some EOs tested. As a result of this research, *A. oxymitra* pericarp EO was found to be safe to human lung cell lines and at the same time, effective against *H. influenzae* in liquid and solid medium. Additionally, we analysed the chemical composition of volatile oils by GC-MS using two capillary columns of different polarity. Monoterpenoids and sesquiterpenoids were the predominant classes identified in all EOs analysed except *C. lucida* EO, where the major constituents were esters. This analysis also identified the presence of specific antimicrobial constituents such as *β*-caryophyllene, caryophyllene epoxide, 1,8-cineole, decanal, *α*-pinene, *β*-pinene, and terpinen-4-ol in the most effective EOs, which disturb cell membrane structures of pathogens and thus contribute to their mode of antibacterial action. Our results suggest a potential of certain Cambodian EOs (e.g. *A. oxymitra* pericarp EO) for application in the inhalation therapy against respiratory infections; however, further research focused on *in vivo* evaluation will be necessary to be carried out in order to verify its potential practical use.

Table 6.4 Chemical composition of Cambodian essential oils tested

Component	RI		Plant species/Column/Relative contents [%]																						Identif[c]
	Pub[a]	Obs[b]	Aol		Aop		Aor		Aos		Br		Ccb		Ccl		Cl		La		Rd		Ss		
			5	17	5	17	5	17	5	17	5	17	5	17	5	17	5	17	5	17	5	17	5	17	
Hydrocarbons																									
Nonane	900	[f]	-[e]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	tr[h]	-	-	-	-	-	-	GC-MS, Std
1,5,5-Trimethyl-6-methylene-cyclohexene	[d]	1340	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	tr	-	-	-	-	-	GC-MS, RI
Tetradeca-1,13-diene	[d]	1347	-	-	0.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	GC-MS, RI
Cyclohexadecane	1881	1897	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.2	-	-	-	GC-MS, RI
Heneicosane	2100	2186	-	-	-	-	1.5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	GC-MS, RI, Std
Tricosane	2300	2300	-	-	0.7	0.7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	GC-MS, RI, Std
Pentacosane	2500	2500	-	-	0.7	0.6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	GC-MS, RI, Std
Group sum [%]			**0.0**	**0.0**	**1.6**	**1.3**	**1.5**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.2**	**0.0**	**0.0**	**0.0**	
Carbonylic compounds																									
2-Hexanone	788	[f]	-	-	-	-	-	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	GC-MS
Octanal	998	1004	-	-	tr	-	-	-	0.3	0.4	-	-	-	-	-	-	tr	-	-	-	-	-	-	-	GC-MS, RI
Nonanal	1100	1104	-	-	0.1	0.1	-	-	-	-	-	-	-	-	-	-	0.4	0.4	-	-	-	-	-	-	GC-MS, RI
3-Methylacetophenone	1182	[f]	-	-	-	-	-	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	GC-MS
Decanal	1201	1207	-	-	-	-	-	-	tr	-	-	-	-	-	-	-	13.6	12.4	-	-	-	-	-	-	GC-MS, RI
Benzylacetone	1218	1249	-	-	-	-	-	-	-	-	0.1	0.1	-	-	-	-	-	-	-	-	-	-	-	-	GC-MS, RI
trans-2-Decenal	1255	1264	-	-	-	-	-	-	tr	-	-	-	-	-	-	-	-	0.1	-	-	-	-	-	-	GC-MS, RI
Undecanal	1306	1309	-	-	-	-	-	-	-	-	-	-	-	-	0.1	-	0.9	0.8	-	-	-	-	-	-	GC-MS, RI
Dodecanal	1408	1410	-	-	-	-	-	-	-	-	-	-	0.1	0.6	0.7	0.8	-	21.0	-	-	-	-	-	-	GC-MS, RI
Tridecanal	1510	1504	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.4	-	-	-	-	-	-	-	GC-MS, RI
Flavesone	1547	1552	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.1	-	-	-	GC-MS, RI
Tetradecanal	1612	1619	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.3	0.2	-	-	-	-	-	-	GC-MS, RI
Leptospermone	1630	1628	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.7	-	-	-	GC-MS, RI
Group sum [%]			**0.0**	**0.0**	**0.1**	**0.1**	**0.0**	**0.2**	**0.3**	**0.4**	**0.1**	**0.1**	**0.1**	**0.6**	**0.8**	**0.8**	**15.6**	**34.9**	**0.0**	**0.0**	**0.8**	**0.0**	**0.0**	**0.0**	

Component	RI		Plant species/Column/Relative contents [%]																						Identif[c]
	Pub[a]	Obs[b]	Aol		Aop		Aor		Aos		Br		Ccb		Ccl		Cl		La		Rd		Ss		
			5	17	5	17	5	17	5	17	5	17	5	17	5	17	5	17	5	17	5	17	5	17	
Aliphatic alcohols																									
Hexan-1-ol	870	[g]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.1	0.1	-	-	-	-	-	-	
1-Octen-3-ol	978	978	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.2	0.2	-	-	-	-	GC-MS, RI, Std
Octan-1-ol	1068	1070	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.2	0.3	-	-	-	-	-	-	GC-MS, RI
Nonan-1-ol	1169	1173	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.4	0.4	-	-	-	-	-	-	GC-MS, RI
Decan-1-ol	1269	1277	-	-	-	-	-	-	-	-	-	-	-	-	-	-	5.3	5.4	-	-	-	-	-	-	GC-MS, RI
Undecan-1-ol	1370	1379	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.5	0.5	-	-	-	-	-	-	GC-MS, RI
Dodecan-1-ol	1470	1477	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.2	1.1	-	-	-	-	-	-	GC-MS, RI
Hexadecan-1-ol	1875	1885	-	-	0.5	0.5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	GC-MS, RI
Octadecen-1-ol	[d]	2065	-	-	-	-	-	-	-	-	0.3	0.2	-	-	-	-	-	-	-	-	-	-	-	-	GC-MS, RI
Group sum [%]			**0.0**	**0.0**	**0.5**	**0.5**	**0.0**	**0.0**	**0.0**	**0.0**	**0.3**	**0.2**	**0.0**	**0.0**	**0.0**	**0.0**	**7.7**	**7.8**	**0.2**	**0.2**	**0.0**	**0.0**	**0.0**	**0.0**	
Fatty acids																									
Decanoic acid	[d]	1392	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.2	0.3	-	-	-	-	-	-	GC-MS, RI
Dodecanoic acid	1568	1577	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.5	1.0	-	-	-	-	-	-	GC-MS, RI
Hexadecanoic acid	1984	1969	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.1	0.6	-	-	-	-	-	-	GC-MS, RI
Group sum [%]			**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.8**	**1.9**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	
Esters																									
Ethyl hexanoate	[d]	1000	-	-	-	-	-	-	-	-	-	-	-	-	-	-	tr	0.2	-	-	-	-	-	-	GC-MS, RI
Ethyl octanoate	1193	1198	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.3	0.2	-	-	-	-	-	-	GC-MS, RI
Octyl acetate	[d]	1212	-	-	-	-	-	-	tr	-	-	-	-	-	-	-	-	0.7	-	-	-	-	-	-	GC-MS, RI
Benzyl isobutyrate	[d]	1301	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.2	-	-	-	GC-MS, RI
Benzyl 2-methylbutyrate	[d]	1391	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.6	0.1	-	-	GC-MS, RI
trans-Methyl cinnamate	[d]	1391	-	-	-	-	-	-	-	-	1.5	1.7	-	-	-	-	-	-	-	-	-	-	-	-	GC-MS, RI
Decyl acetate	1408	1424	-	-	-	-	-	-	-	-	-	-	-	-	-	-	49.4	27.6	-	-	-	-	-	-	GC-MS, RI
Dodecenyl acetate	[d]	1617	-	-	-	-	-	-	-	-	-	-	-	-	-	-	9.3	8.9	-	-	-	-	-	-	GC-MS, RI
Tetradecenyl acetate	[d]	1812	-	-	-	-	-	-	-	-	-	-	-	-	-	-	tr	-	-	-	-	-	-	-	GC-MS, RI
Dibutyl phthalate	1970	1982	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.1	-	-	-	GC-MS, RI
Methyl tetradecanoate	[d]	[f]	-	-	-	0.3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	GC-MS
Group sum [%]			**0.0**	**0.0**	**0.0**	**0.3**	**0.0**	**0.0**	**0.0**	**0.0**	**1.5**	**1.7**	**0.0**	**0.0**	**0.0**	**0.0**	**59.0**	**37.6**	**0.0**	**0.0**	**0.9**	**0.1**	**0.0**	**0.0**	
Monoterpenoids																									
Tricyclene	926	925	-	-	-	-	-	-	-	-	0.2	0.2	0.1	-	-	-	-	-	-	-	-	-	-	-	GC-MS, RI
α-Thujene	930	929	0.2	0.2	tr	tr	0.2	0.2	-	-	-	-	0.6	0.2	0.4	0.4	-	-	0.2	0.1	0.1	0.2	tr	-	GC-MS, RI

Component	RI		Plant species/Column/Relative contents [%]																						Identif[c]
	Pub[a]	Obs[b]	Aol		Aop		Aor		Aos		Br		Ccb		Ccl		Cl		La		Rd		Ss		
			5	17	5	17	5	17	5	17	5	17	5	17	5	17	5	17	5	17	5	17	5	17	
α-Pinene	939	937	4.8	5.0	2.4	2.6	5.1	5.6	0.4	0.5	0.9	0.9	8.8	5.9	3.1	3.4	tr	-	1.7	1.7	26.1	73.5	6.7	7.4	GC-MS, RI, Std
α-Fenchene	952	[f]	-	-	-	-	-	tr	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	GC-MS
Rosefuran	[d]	[f]	-	-	-	-	-	-	-	-	-	0.1	-	-	-	-	-	-	-	-	-	-	-	-	GC-MS
Cosmene	[d]	[f]	-	-	-	-	-	-	-	-	-	0.1	-	-	-	-	-	-	-	-	-	0.1	-	-	GC-MS
Camphene	954	952	0.1	0.1	0.2	0.1	0.2	0.2	-	-	5.4	5.5	4.6	0.8	0.7	0.7	-	-	tr	-	tr	0.1	0.2	0.2	GC-MS, RI, Std
Thuja-2,4(10)-diene	957	960	-	-	-	-	-	tr	-	-	-	-	-	-	-	-	-	-	-	-	0.1	0.5	-	0.3	GC-MS, RI
Sabinene	975	978	2.6	3.4	0.5	0.8	-	1.1	-	-	-	-	0.8	0.9	1.6	1.6	-	-	0.1	0.1	-	-	0.1	0.2	GC-MS, RI
β-Pinene	979	985	58.4	59.1	29.8	31.3	50.2	53.2	-	-	0.2	-	4.4	2.3	2.2	2.9	0.1	0.1	0.2	-	0.4	0.9	4.4	4.7	GC-MS, RI, Std
β-Myrcene	990	992	0.3	-	tr	-	0.3	-	2.4	3.2	1.1	1.5	1.3	0.4	0.4	-	-	-	0.2	0.4	-	-	0.1	-	GC-MS, RI, Std
2,3-Dehydro-1,8-cineole	991	994	-	-	-	-	-	-	-	-	-	-	-	-	0.2	tr	-	-	-	-	-	-	-	-	GC-MS, RI
Pseudolimonene	1004	1007	-	-	tr	-	-	tr	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	GC-MS, RI
α-Phellandrene	1002	1008	-	-	-	-	-	-	0.1	0.2	-	-	0.1	0.6	-	0.2	-	-	-	-	-	-	-	-	GC-MS, RI, Std
p-Mentha-1,5,8-triene	1135	1008	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.1	-	-	GC-MS, RI
δ-2-Carene	1002	[f]	-	-	-	-	-	-	-	-	-	-	-	0.3	-	-	-	-	-	-	-	-	-	-	GC-MS
o-Cymenene	[d]	[f]	-	-	-	tr	-	-	-	-	-	-	-	0.1	-	-	-	-	-	-	-	-	-	-	GC-MS
α-Terpinene	1017	1020	-	-	-	-	-	-	-	-	-	-	0.9	0.4	0.4	0.5	-	-	-	-	-	-	-	-	GC-MS, RI, Std
p-Cymene	1024	1027	-	-	1.2	1.5	-	-	tr	0.1	-	-	1.8	-	1.9	-	tr	tr	-	-	0.5	0.4	0.4	0.6	GC-MS, RI
o-Cymene	1026	1028	0.6	0.8	-	-	8.8	15.0	-	-	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-	GC-MS, RI
Limonene	1029	1032	0.9	1.0	0.7	0.8	1.6	1.9	-	0.4	1.6	2.0	-	1.1	-	1.0	-	-	48.3	48.1	1.4	1.7	0.6	0.7	GC-MS, RI
β-Phellandrene	1029	[f]	-	0.1	-	-	-	tr	-	2.9	-	-	-	0.4	-	0.6	-	-	-	-	-	-	-	-	GC-MS
β-Terpinene	1071	1034	-	-	-	-	-	-	2.5	-	-	-	-	2.7	-	-	-	-	-	-	-	-	-	-	GC-MS, RI
1,8-Cineole	1031	1035	0.1	-	0.2	-	5.2	-	-	-	5.5	5.6	31.8	33.5	30.5	33.1	-	-	-	-	-	-	-	-	GC-MS, RI, Std
trans-β-Ocimene	1050	1039	-	-	-	-	-	-	-	-	3.3	2.9	-	-	-	-	-	-	-	-	-	0.1	-	-	GC-MS, RI
Ocimene	1050	1049	-	-	-	-	-	-	0.4	0.5	28.0	27.6	-	-	0.4	0.5	-	-	0.2	0.2	-	-	-	-	GC-MS, RI
γ-Terpinene	1059	[f]	-	-	-	-	-	-	-	-	-	0.1	1.1	0.6	0.8	1.0	-	-	-	-	-	-	-	-	GC-MS, Std
trans-Sabinene hydrate	1070	1070	0.2	0.1	-	tr	-	-	-	-	-	-	-	-	0.3	0.2	-	-	-	-	-	-	-	-	GC-MS, RI
cis-Sabinene hydrate	1070	1071	-	-	0.1	-	-	-	-	-	-	-	0.1	0.1	-	-	-	-	-	-	-	-	0.1	-	GC-MS, RI
cis-Linalool oxide (furanoid)	1072	1075	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	tr	-	-	-	GC-MS, RI
L-Menthol	1172	[f]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.1	GC-MS, Std
Camphenilone	1082	1089	-	-	tr	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	GC-MS, RI
Terpinolene	1088	1092	-	-	-	-	-	-	-	-	0.2	0.2	0.6	0.5	0.3	0.4	-	-	tr	-	-	-	-	-	GC-MS, RI, Std
Linalool	1096	1102	-	0.1	0.2	0.1	-	-	0.2	0.2	1.6	1.5	0.3	2.3	4.7	4.2	0.2	0.1	0.3	0.3	0.5	0.9	-	-	GC-MS, RI, Std
4-Thujanol	[d]	1102	0.3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	GC-MS, RI

Component	RI		Plant species/Column/Relative contents [%]																						Identif[c]
	Pub[a]	Obs[b]	Aol		Aop		Aor		Aos		Br		Ccb		Ccl		Cl		La		Rd		Ss		
			5	17	5	17	5	17	5	17	5	17	5	17	5	17	5	17	5	17	5	17	5	17	
α-Pinene epoxide	1099	1103	-	0.1	-	-	0.1	-	-	-	0.2	0.2	-	-	-	-	-	-	-	0.1	0.5	1.2	0.3	0.2	GC-MS, RI
Hotrienol	1101	1107	-	-	-	-	-	-	-	-	-	-	-	-	0.2	0.2	-	-	-	-	-	-	-	-	GC-MS, RI
cis-Verbenol	1141	1111	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.4	1.1	-	0.1	GC-MS, RI
Fenchol	[d]	1119	-	-	0.2	-	0.2	0.2	-	-	-	-	0.1	0.1	-	-	-	-	-	-	-	-	-	-	GC-MS, RI
trans-p-Menth-2,8-dien-1-ol	[d]	1125	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.1	-	-	-	-	-	GC-MS, RI
cis-2-Menthenol	1121	1126	0.1	-	0.3	-	0.1	-	tr	-	-	-	0.2	0.1	0.2	0.2	-	-	-	-	-	-	-	-	GC-MS, RI
α-Campholenal	1126	1131	-	-	0.3	0.3	0.2	0.2	-	-	-	-	-	-	-	0.1	-	-	-	-	0.5	0.4	0.1	0.1	GC-MS, RI
Allo-Ocimene	1132	[f]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.2	-	-	GC-MS
Terpinen-1-ol	1133	[f]	-	-	-	-	-	-	-	-	-	-	-	0.1	-	-	-	-	-	-	-	-	-	-	GC-MS
trans-2-Menthenol	1140	[f]	-	-	-	-	-	-	-	-	-	-	0.1	0.1	-	0.2	-	-	-	-	-	-	-	-	GC-MS
Neo-allo-ocimene	1144	1132	-	-	-	-	-	-	-	-	0.6	0.7	-	-	-	-	-	-	-	-	-	-	-	-	GC-MS, RI
Limonen-1,2-epoxide	[d]	1138	-	-	-	tr	-	-	-	-	-	-	-	-	-	-	-	-	1.0	1.0	-	-	-	-	GC-MS, RI
trans-Limonene epoxide	1142	1142	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.5	0.5	-	-	-	-	GC-MS, RI
trans-Pinocarveol	1139	1145	2.0	1.7	4.7	3.6	2.0	1.5	-	-	-	-	-	0.2	-	0.3	-	-	-	-	-	-	-	-	GC-MS, RI
Sabinol	1142	1145	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.6	0.7	GC-MS, RI
Myroxide	1145	1147	-	-	-	-	-	-	-	-	0.1	0.1	-	-	-	-	-	-	-	-	-	-	-	-	GC-MS, RI
trans-Verbenol	1144	1149	0.1	-	0.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.2	1.6	0.4	-	GC-MS, RI
Camphene hydrate	1149	1155	-	-	0.1	-	0.1	0.1	-	-	0.6	0.7	0.2	0.1	-	-	-	-	-	-	-	-	-	-	GC-MS, RI
Camphor	1146	1156	-	-	-	-	-	-	-	-	18.6	19.1	1.7	0.8	-	-	-	-	-	-	-	-	-	-	GC-MS, RI, Std
β-Pinene epoxide	1159	[f]	-	-	-	-	-	0.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	GC-MS
β-Terpineol	1159	[f]	-	-	-	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	GC-MS
trans-β-Terpineol	1163	1168	-	-	-	-	-	-	tr	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	GC-MS, RI
Pinocarvone	1164	1169	0.9	0.9	3.7	4.4	2.3	2.4	-	-	-	-	-	-	-	0.1	-	-	-	-	0.2	0.2	0.3	0.2	GC-MS, RI
Borneol	1169	1172	0.1	-	0.3	-	0.3	-	-	-	0.4	-	5.7	5.0	1.4	-	-	-	0.2	0.2	0.1	-	-	-	GC-MS, RI, Std
L-Menthol	1172	[f]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.1	GC-MS, Std
Terpinen-4-ol	1177	1183	0.5	0.6	0.7	0.8	0.9	1.1	tr	-	0.3	-	5.4	2.5	3.0	4.4	-	-	tr	-	0.3	0.1	0.1	0.1	GC-MS, RI
p-Cymene-8-ol	1182	1190	-	-	0.4	-	0.4	0.2	-	-	-	-	-	-	-	-	-	-	-	-	0.6	0.1	-	-	GC-MS, RI
Isopinocarveol	[d]	1192	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	GC-MS, RI
Cinerone	1183	[f]	-	-	-	-	-	-	-	-	-	0.1	-	-	-	-	-	-	-	-	-	-	-	-	GC-MS
Cryptone	1185	1193	-	-	-	-	-	-	0.1	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	GC-MS, RI
Methyl salicylate	1191	[f]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.1	-	-	-	-	GC-MS
Myrtenal	1195	[f]	-	-	-	-	-	1.6	-	-	-	-	-	0.1	-	0.2	-	-	-	-	-	0.2	-	0.3	GC-MS
α-Terpineol	1188	1196	0.3	0.3	1.2	1.1	1.1	1.2	-	-	0.6	0.6	9.5	7.9	6.5	6.4	-	0.2	tr	0.1	0.7	0.7	0.1	0.1	GC-MS, RI
cis-Dihydrocarvone	[d]	[f]	-	-	-	0.4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	GC-MS, Std
Myrtenol	1195	1203	2.7	2.5	5.5	5.2	3.3	1.2	-	-	-	-	-	tr	0.3	tr	-	-	-	-	0.4	0.3	0.6	0.3	GC-MS, RI
cis-Piperitol	1196	1213	-	-	-	-	-	-	-	-	-	-	0.1	-	-	-	-	-	-	-	-	-	-	-	GC-MS, RI

Component	RI		Plant species/Column/Relative contents [%]																						Identif[c]
	Pub[a]	Obs[b]	Aol		Aop		Aor		Aos		Br		Ccb		Ccl		Cl		La		Rd		Ss		
			5	17	5	17	5	17	5	17	5	17	5	17	5	17	5	17	5	17	5	17	5	17	
Cosmen-2-ol	[d]	1210	-	-	-	-	-	-	-	-	0.2	0.1	-	-	-	-	-	-	-	-	-	-	-	-	GC-MS, RI
trans-Carveol	1216	1214	-	-	0.1	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	GC-MS, RI
3-p-Menthen-7-al	[d]	1215	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	20.1	20.3	-	-	-	-	GC-MS, RI
Verbenone	1205	1217	-	-	0.2	0.1	-	tr	-	-	-	0.1	-	-	-	-	-	-	-	-	0.6	0.6	0.2	0.2	GC-MS, RI
cis-Carveol	1229	1224	-	-	-	tr	-	-	-	-	-	-	-	-	-	-	-	-	0.1	-	0.3	0.1	0.1	0.1	GC-MS, RI
2-Hydroxycineole	1219	1229	-	-	0.1	-	0.1	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	GC-MS, RI
Citronellol	1225	1230	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.1	-	GC-MS, RI, Std
Nerol	1229	1257	-	-	-	-	-	-	tr	-	0.2	0.1	-	-	0.1	0.1	-	-	-	-	-	-	-	-	GC-MS, RI
Isobornyl formate	1239	1235	-	-	tr	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	GC-MS, RI
Neral	1238	1245	-	-	-	-	-	-	-	-	0.4	0.4	-	-	-	-	-	-	-	-	-	-	-	-	GC-MS, RI
Cuminaldehyde	1241	1247	-	-	0.2	-	-	tr	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	GC-MS, RI
Carvacrol methyl ether	1244	1248	-	-	-	-	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	GC-MS, RI
Carvone	1243	1250	-	-	0.1	-	tr	-	-	-	-	-	-	-	-	-	-	-	0.1	0.1	0.1	-	-	-	GC-MS, RI, Std
Piperitone	1252	1261	-	-	-	-	-	-	tr	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	GC-MS, RI
Geraniol	1252	1265	-	-	-	-	-	-	-	-	24.4	24.0	-	-	-	-	-	-	-	-	-	-	-	-	GC-MS, RI, Std
Citral	1240	1274	-	-	-	-	-	-	tr	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	GC-MS, RI, Std
Geranial	1267	1277	-	-	-	-	-	-	-	-	2.0	1.8	-	-	-	-	-	-	-	-	-	-	-	-	GC-MS, RI
p-Menth-2-en-1,4-diol	1269	1277	-	-	0.1	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	GC-MS, RI
trans-Shisool	[d]	1277	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.5	-	-	-	-	GC-MS, RI
Perillal	1271	1283	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2.2	2.8	-	-	-	-	GC-MS, RI
Bornyl acetate	[d]	1292	tr	-	-	-	-	tr	tr	-	-	-	0.3	0.2	0.1	0.1	-	-	-	-	-	-	-	-	GC-MS, RI, Std
p-Menth-1-en-9-ol	[d]	1293	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2.1	2.2	-	-	-	-	GC-MS, RI
Safrole	1287	1294	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	tr	-	-	-	GC-MS, RI
p-Cymen-7-ol	1290	[f]	-	-	-	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	GC-MS
Perillol	[d]	[f]	-	-	-	5.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	GC-MS
Methyl myrtenate	1294	1303	0.3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	GC-MS, RI
trans-Pinocarvyl acetate	1298	1304	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.5	-	-	-	GC-MS, RI
Carvacrol	1299	1304	-	-	-	-	-	-	-	-	-	-	0.1	-	-	-	-	-	-	-	-	-	-	-	GC-MS, RI, Std
δ-Terpinyl acetate	1317	1323	-	-	-	-	tr	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	GC-MS, RI
p-Mentha-1,4-dien-7-ol	[d]	1328	0.4	-	1.7	-	0.9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	GC-MS, RI
Myrtenyl acetate	[d]	1331	0.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	tr	-	-	-	GC-MS, RI
Terpinyl acetate	1349	1355	-	-	-	-	0.5	1.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	GC-MS, RI
Neryl acetate	1361	1366	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	tr	-	-	-	-	-	GC-MS, RI
Sobrerol	[d]	1387	-	-	0.2	-	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-	0.1	-	-	-	GC-MS, RI
Myrtanol acetate	1381	1424	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	7.7	3.0	-	-	-	-	GC-MS, RI

Component	RI		Plant species/Column/Relative contents [%]																						Identif[c]
	Pub[a]	Obs[b]	Aol		Aop		Aor		Aos		Br		Ccb		Ccl		Cl		La		Rd		Ss		
			5	17	5	17	5	17	5	17	5	17	5	17	5	17	5	17	5	17	5	17	5	17	
Methyleugenol	1403	1406	-	-	-	-	-	-	-	-	-	-	tr	-	-	-	-	-	-	-	-	-	-	-	GC-MS, RI
α-Ionone	1430	1435	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	GC-MS, RI
Geranyl acetone	1455	1456	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.1	-	-	-	GC-MS, RI
(1S,2S,4S)-Trihydroxy-p-menthane	[d]	1487	-	-	-	-	0.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	GC-MS, RI
Group sum [%]			**76.3**	**76.0**	**55.6**	**58.5**	**84.5**	**88.2**	**6.1**	**8.1**	**96.7**	**96.2**	**80.7**	**70.3**	**59.7**	**63.0**	**0.3**	**0.4**	**85.3**	**81.8**	**36.7**	**85.3**	**15.5**	**16.7**	
Sesquiterpenoids																									
δ-Elemene	1338	1344	-	-	-	-	-	-	-	-	-	-	0.1	0.1	0.1	-	-	-	-	-	0.1	-	-	-	GC-MS, RI
Elemene isomer	[d]	1345	-	-	-	-	-	-	0.1	-	-	-	-	-	0.8	0.5	-	-	-	-	-	-	-	-	GC-MS, RI
α-Cubebene	1388	1357	-	-	-	-	-	-	tr	-	-	-	0.2	-	-	-	-	-	-	-	-	-	0.3	-	GC-MS, RI
α-Longipinene	1352	1363	-	-	-	-	-	-	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	GC-MS, RI
Cyclosativene	1371	[f]	-	-	-	-	-	-	-	-	-	-	-	0.2	0.1	-	-	-	-	-	-	-	-	-	GC-MS
α-Copaene	1374	1385	0.1	0.2	-	-	-	-	1.2	1.7	-	-	0.9	0.7	0.5	0.5	-	-	0.2	-	0.2	-	2.4	3.5	GC-MS, RI
α-Ylangene	1375	1381	-	-	-	-	-	-	-	0.2	-	-	-	-	-	-	-	-	-	-	-	-	0.1	-	GC-MS, RI
Isoledene	1376	[f]	-	-	-	-	-	-	-	-	-	-	0.2	0.4	0.1	0.1	-	-	-	-	-	-	-	-	GC-MS
β-Bourbonene	1388	1395	0.1	0.1	0.2	-	-	-	-	-	-	-	-	-	-	-	0.1	-	-	-	-	-	27.5	28.5	GC-MS, RI
β-Elemene	1390	1399	0.4	0.7	-	-	-	-	0.7	0.6	-	-	0.3	0.2	0.1	0.1	-	-	-	-	0.5	-	-	-	GC-MS, RI
α-Bourbonene	[d]	1407	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.4	0.2	GC-MS, RI
β-Maaliene	[d]	1420	-	-	-	-	-	-	-	-	-	-	0.1	0.1	-	-	-	-	-	-	-	-	-	-	GC-MS, RI
α-Gurjunene	1409	1421	-	-	-	-	-	-	tr	tr	-	-	-	-	0.2	0.1	-	-	-	-	-	-	-	-	GC-MS, RI
α-Cedrene	1411	1422	-	-	-	-	-	-	-	-	-	-	0.1	-	-	0.6	-	-	-	-	-	-	0.7	0.3	GC-MS, RI
cis-α-Bergamotene	1412	1423	-	-	-	-	-	-	-	-	-	-	-	-	0.5	-	-	-	-	-	-	-	0.3	-	GC-MS, RI
β-Funebrene	1414	[f]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.5	GC-MS
β-Caryophyllene	1419	1431	3.0	3.0	-	tr	-	-	1.7	2.6	-	-	-	0.8	1.4	1.3	5.0	3.7	1.5	1.5	2.4	0.6	0.7	0.7	GC-MS, RI, Std
Selina-5,11-diene	[d]	[f]	-	-	-	-	-	-	-	-	-	-	-	0.1	tr	0.2	-	-	-	-	-	-	-	-	GC-MS
β-Cedrene	1420	[f]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.1	-	GC-MS
β-Ylangene	1420	[f]	-	-	-	-	-	-	-	2.0	-	-	-	-	-	-	-	-	-	-	-	-	2.1	1.3	GC-MS
α-Guaiene	1439	[f]	-	-	-	-	-	-	-	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	GC-MS
β-Cubebene	1388	1440	-	0.1	0.1	-	-	-	-	0.3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	GC-MS, RI
β-Copaene	1432	1441	tr	-	-	-	-	-	0.5	1.4	-	-	-	-	-	-	-	-	-	-	-	-	2.0	1.8	GC-MS, RI
Perillyl acetate	[d]	1443	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2.8	2.8	-	-	-	-	GC-MS, RI
trans-α-Bergamotene	1434	1444	-	-	-	-	-	-	-	-	-	0.1	-	-	-	-	0.4	-	-	-	-	-	3.2	4.2	GC-MS, RI
Cadina-3,5-diene	1458	[f]	-	-	-	-	-	-	-	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	GC-MS
Aromadendrene	1441	1451	-	-	-	0.2	-	-	-	4.7	-	-	0.8	1.1	0.6	1.1	-	-	-	-	-	0.1	-	-	GC-MS, RI
Isogermacrene D	[d]	1457	-	-	-	-	-	-	0.2	-	-	-	-	-	-	-	-	-	-	-	-	-	1.9	1.8	GC-MS, RI
α-Himachalene	1451	1463	-	-	-	-	-	-	0.1	-	-	-	-	-	-	-	-	-	-	-	0.8	-	0.6	0.6	GC-MS, RI

Component	RI		Plant species/Column/Relative contents [%]																						Identif[c]
	Pub[a]	Obs[b]	Aol		Aop		Aor		Aos		Br		Ccb		Ccl		Cl		La		Rd		Ss		
			5	17	5	17	5	17	5	17	5	17	5	17	5	17	5	17	5	17	5	17	5	17	
Sesquisabinene	1459	1465	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.1	0.4	-	-	-	-	2.6	2.3	GC-MS, RI
trans-β-Farnesene	1456	1461	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.1	0.9	-	-	-	-	GC-MS, RI
α-Humulene	1454	1466	0.7	0.7	-	-	-	-	1.0	1.7	-	-	-	-	-	-	0.6	0.4	2.6	5.7	0.5	0.1	0.4	-	GC-MS, RI, Std
Alloaromadendrene	1460	1473	0.4	0.4	-	-	tr	-	3.5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	GC-MS, RI
dehydro-Aromadendrene	1462	1463	-	-	-	-	-	-	-	-	-	-	-	-	0.1	0.4	-	-	-	-	-	-	-	-	GC-MS, RI
Eudesma-1,4(15),11-triene	[d]	1471	-	-	-	-	-	-	-	-	-	-	-	-	0.2	0.2	-	-	-	-	-	-	-	-	GC-MS, RI
9-epi-(E)-Caryophyllene	1466	1473	-	-	0.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	GC-MS, RI
γ-Gurjunene	1477	1483	-	-	-	-	-	-	-	-	-	-	0.1	0.1	0.1	0.2	-	-	-	-	0.1	-	0.4	0.7	GC-MS, RI
γ-Muurolene	1479	1486	0.2	0.2	0.1	0.1	-	-	-	0.4	-	-	0.7	0.3	-	-	-	-	tr	-	0.3	-	0.6	0.7	GC-MS, RI
α-Curcumene	1480	1489	-	-	-	-	-	-	-	-	-	-	0.1	-	0.2	-	tr	-	-	-	-	-	0.6	0.9	GC-MS, RI
α-Amorphene	1484	[f]	-	-	-	-	-	-	-	-	-	-	-	-	-	0.1	-	-	-	-	0.7	0.1	-	-	GC-MS
Isocaryophyllene	[d]	[f]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.1	GC-MS
β-Selinene	1490	1498	0.3	0.5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	GC-MS, RI
Bicyclosesquiphellandrene	1490	[f]	-	-	-	-	-	-	-	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	GC-MS
β-Guaiene	1490	[f]	-	-	-	-	-	-	-	-	-	-	-	tr	-	-	-	-	-	-	-	-	-	0.4	GC-MS
trans-β-Bergamotene	[d]	1495	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.4	-	-	-	-	-	0.4	-	GC-MS, RI
Eremophilene	[d]	1499	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	tr	-	0.2	-	0.2	-	GC-MS, RI
Germacrene D	1485	1501	-	-	-	-	-	-	16.5	16.3	-	-	-	-	-	-	-	-	-	-	-	-	0.3	-	GC-MS, RI
Bicyclogermacrene	1494	[f]	-	-	-	-	-	-	-	-	-	-	1.2	1.0	3.6	2.2	-	-	-	-	-	-	-	-	GC-MS
Epicubebol	1494	1505	-	-	-	-	-	-	0.7	1.7	-	-	-	-	-	-	-	-	-	-	-	-	0.3	0.7	GC-MS, RI
γ-Amorphene	1495	[f]	-	-	-	-	-	-	-	0.6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	GC-MS
Cubebol	1515	1505	0.1	0.4	-	-	-	-	16.2	20.2	-	-	-	-	-	-	-	-	-	-	-	-	0.5	0.6	GC-MS, RI
Viridiflorene	1496	1507	-	-	-	-	-	-	-	-	-	-	1.2	1.0	-	1.3	-	-	-	-	-	-	-	-	GC-MS, RI
Aciphyllene	1501	1507	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	tr	-	0.8	-	-	-	GC-MS, RI
Epishyobunone	[d]	1505	-	-	-	-	-	-	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	GC-MS, RI
α-Muurolene	1499	1509	0.2	0.2	0.1	0.2	-	-	0.7	1.5	-	-	0.4	0.2	-	-	-	-	-	-	-	-	0.1	-	GC-MS, RI
Cuparene	1504	[f]	-	-	-	-	-	-	-	-	-	-	-	-	-	0.3	-	-	-	-	-	-	-	-	GC-MS
α-Farnesene	1505	1511	-	-	-	-	-	-	0.5	0.5	0.1	-	-	-	-	-	-	-	0.1	0.1	-	-	-	-	GC-MS, RI, Std
β-Bisabolene	1505	[f]	-	-	-	-	-	-	-	-	-	-	-	-	-	0.1	1.1	1.7	-	-	-	-	0.2	0.3	GC-MS
cis-γ-Bisabolene	1507	1516	-	-	-	-	-	-	-	-	-	-	-	-	0.2	-	-	-	-	-	-	-	-	-	GC-MS, RI
β-Curcumene	1515	1518	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	tr	-	GC-MS, RI
Sesquicineole	1516	1522	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.2	0.4	GC-MS, RI
γ-Cadinene	1513	1525	-	-	0.2	0.3	-	-	-	-	-	-	0.4	-	-	-	-	-	0.1	-	0.2	-	-	-	GC-MS, RI
δ-Cadinene	1523	1525	0.4	-	-	-	-	-	-	-	-	-	-	-	0.3	0.9	-	-	0.1	-	-	-	0.3	-	GC-MS, RI
β-Sesquiphellandrene	1522	1532	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	GC-MS, RI
β-Cadinene	1472	1534	-	-	-	-	-	-	-	-	-	-	2.3	0.7	-	-	-	-	-	-	-	-	-	-	GC-MS, RI

Component	RI		Plant species/Column/Relative contents [%]																						Identif[c]
	Pub[a]	Obs[b]	Aol		Aop		Aor		Aos		Br		Ccb		Ccl		Cl		La		Rd		Ss		
			5	17	5	17	5	17	5	17	5	17	5	17	5	17	5	17	5	17	5	17	5	17	
cis-Calamenene	1529	1534	0.3	0.3	-	-	-	-	-	-	-	-	-	0.4	-	0.1	-	-	-	-	0.2	-	0.1	0.2	GC-MS, RI
Cadina-1,4-diene	1534	1543	-	-	-	-	-	-	-	-	-	-	0.1	-	-	-	-	-	-	-	-	-	-	-	GC-MS, RI
6-epi-shyobunol	[d]	1548	-	-	-	-	-	-	9.8	12.4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	GC-MS, RI
trans-α-Bisabolene	1549	1550	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.5	0.4	-	-	-	-	-	-	GC-MS, RI
α-Cadinene	1538	1552	-	-	-	-	-	-	0.1	1.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	GC-MS, RI
α-Calacorene	1545	1555	tr	-	-	-	-	-	-	-	-	-	0.3	0.1	-	tr	-	-	-	-	-	-	-	-	GC-MS, RI
Cadala-1(10),3,8-triene	[d]	1555	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.1	-	-	-	GC-MS, RI
Elemol	1549	1560	-	-	-	-	0.1	-	0.3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	GC-MS, RI
cis-Sesquisabinene hydrate	1544	1563	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.8	0.9	GC-MS, RI
β-Vetivenene	1555	[f]	-	-	-	-	-	-	-	-	-	-	-	-	-	tr	-	-	-	-	-	-	-	-	GC-MS
β-Spathulenol	1578	1567	-	-	0.9	0.8	-	-	-	-	-	-	0.5	5.3	5.8	3.6	-	-	-	-	0.5	0.4	-	-	GC-MS, RI
trans-Nerolidol	1563	1569	0.1	0.2	-	-	-	-	0.1	-	0.1	0.1	-	0.2	0.4	0.3	-	-	0.3	0.3	-	-	-	-	GC-MS, RI
Palustrol	1568	[f]	-	-	-	-	-	-	-	-	-	-	-	-	-	0.3	-	-	-	-	-	-	-	-	GC-MS
1,5-Epoxysalvial-4(14)-ene	[d]	1575	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.8	1.8	GC-MS, RI
Norbourbonone	1571	1576	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.3	-	GC-MS, RI
Globulol	1590	1582	-	-	-	-	-	-	0.6	-	-	-	0.2	0.3	0.5	1.5	-	-	-	-	0.1	-	-	-	GC-MS, RI
Espathulenol	[d]	1594	-	-	-	-	-	-	-	-	-	-	-	-	7.6	8.2	-	-	-	-	-	-	4.5	3.7	GC-MS, RI
Germacrene-D-4-ol	1575	1596	-	-	-	-	-	-	8.4	7.5	-	-	-	-	-	-	-	-	-	-	-	-	-	0.2	GC-MS, RI
Viridiflorol	1592	1599	-	-	-	-	-	-	-	-	-	-	1.6	1.3	-	-	-	-	-	-	-	-	-	-	GC-MS, RI
Caryophyllene epoxide	1583	1600	6.1	5.6	20.2	19.7	1.2	1.2	-	-	-	-	-	-	-	-	5.8	6.4	0.1	0.7	33.3	4.5	14.5	12.4	GC-MS, RI
Isoaromadendrene epoxide	1579	1603	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.5	0.2	0.5	-	0.3	1.0	GC-MS, RI
Gleenol	1587	[f]	-	-	-	-	-	-	-	-	-	-	-	0.1	-	-	-	-	-	-	-	-	-	-	GC-MS
Cubeban-11-ol	1595	1607	-	-	-	-	-	-	-	-	-	-	0.5	-	-	-	-	-	-	-	-	-	-	-	GC-MS, RI
Salvialenone	1594	1609	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.2	0.5	GC-MS, RI
Guaiol	1600	1610	-	-	-	-	-	-	-	-	-	-	-	-	1.1	-	-	-	-	-	-	-	-	-	GC-MS, RI
Rosifoliol	1600	1615	-	-	-	-	-	-	-	-	-	-	0.4	2.2	1.3	1.7	-	-	-	-	-	-	-	-	GC-MS, RI
Epiglobulol	1588	1619	0.1	-	-	-	-	-	-	-	-	-	0.3	0.3	0.3	0.3	-	-	-	-	-	-	-	-	GC-MS, RI
Humulene-1,2-epoxide	1608	1625	0.8	0.7	1.9	1.9	-	0.2	-	-	-	-	-	-	-	-	0.6	0.6	1.0	0.9	2.5	0.4	2.8	1.5	GC-MS, RI
γ-Eudesmol	1632	[f]	-	-	-	-	-	-	-	-	-	-	-	-	-	0.2	-	-	-	-	-	-	-	-	GC-MS
α-epi-Cadinol	1640	1642	-	-	-	-	-	-	-	0.3	-	-	-	0.2	-	-	-	-	-	-	-	-	0.1	-	GC-MS, RI
Epicubenol	1613	1646	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.4	-	-	-	GC-MS, RI
Longifolenaldehyde	1631	1646	0.1	-	-	-	-	-	-	-	-	-	-	-	1.3	-	-	-	-	-	-	-	0.1	-	GC-MS, RI
Cedrelanol	[d]	1653	-	-	-	0.2	-	-	-	-	-	-	-	-	-	0.4	-	-	0.1	-	-	-	-	-	GC-MS, RI
10,10-Dimethyl-2,6-dimethylenebicyclo[7.2.0]undecan-5-ol	[d]	1651	-	-	-	-	-	-	-	-	-	-	-	-	-	-	tr	0.1	-	-	-	-	-	-	GC-MS, RI

Component	RI		Plant species/Column/Relative contents [%]																						Identif[c]
	Pub[a]	Obs[b]	Aol		Aop		Aor		Aos		Br		Ccb		Ccl		Cl		La		Rd		Ss		
			5	17	5	17	5	17	5	17	5	17	5	17	5	17	5	17	5	17	5	17	5	17	
11,11-Dimethyl-4,8-dimethylenebicyclo[7.2.0]undecan-3-ol	[d]	1654	-	-	-	0.5	-	-	-	-	-	-	-	-	-	-	0.2	0.2	-	-	1.5	0.3	0.2	-	GC-MS, RI
α-epi-Muurolol	1642	1655	1.2	1.1	0.6	0.4	0.1	-	1.0	0.6	-	-	0.5	0.2	-	-	-	-	-	-	-	-	-	-	GC-MS, RI
α-Muurolol	1646	1659	0.3	0.5	0.2	0.4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	GC-MS, RI
Cubenol	1646	[f]	-	-	-	-	-	-	-	0.5	-	-	0.9	0.1	-	-	-	-	-	-	-	-	-	-	GC-MS
β-Eudesmol	1650	[f]	-	-	-	-	-	-	-	-	-	-	-	0.8	-	-	-	-	-	-	-	-	-	-	GC-MS
Pogostole	1653	[f]	-	-	-	0.6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	GC-MS
Isospathulenol	1666	1659	-	-	-	-	-	-	-	-	-	-	0.3	1.5	2.1	2.1	-	-	-	-	0.4	-	-	-	GC-MS, RI
δ-Cadinol	1645	1661	-	-	-	-	-	-	0.5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	GC-MS, RI
α-Cadinol	1654	1668	1.9	1.8	0.9	1.1	0.2	0.1	1.1	1.5	-	-	0.5	0.6	-	-	-	-	-	-	-	-	-	-	GC-MS, RI
cis-10-Hydroxycalamenene	1661	1671	0.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	GC-MS, RI
Intermedeol	1666	1675	-	-	-	-	0.2	0.3	-	-	-	-	-	-	-	-	tr	-	-	-	-	-	-	-	GC-MS, RI
Neointermedeol	1656	1680	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.2	-	-	-	GC-MS, RI
trans-10-Hydroxycalamenene	1669	1681	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.2	-	-	-	GC-MS, RI
Cadalene	1676	1688	-	-	-	-	-	-	-	-	-	-	0.3	0.5	-	-	-	-	-	-	-	-	-	-	GC-MS, RI
Bulnesol	1671	[f]	-	-	-	-	-	-	-	0.4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	GC-MS
Mustakone	1677	1693	-	-	-	-	-	-	-	-	-	-	0.2	-	-	-	-	-	-	-	-	-	-	-	GC-MS, RI
α-Bisabolol	1685	1694	-	-	-	-	-	-	-	-	-	-	-	-	0.1	-	tr	-	-	-	-	-	-	-	GC-MS, RI, Std
Aristol-1(10)-en-9-ol	[d]	1699	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	tr	-	-	-	GC-MS, RI
(1R,7S)-Germacra-4(15),5,10(14)-trien-1β-ol	[d]	1700	0.1	-	-	1.0	-	-	-	-	-	-	-	-	0.7	0.2	-	-	-	-	1.9	-	0.8	1.0	GC-MS, RI
Shyobunol	1689	1724	-	-	-	-	-	-	21.7	4.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	GC-MS, RI
Eudesma-4,11-dien-2-ol	1690	[f]	-	-	-	-	-	-	-	-	-	-	-	-	-	0.1	-	-	-	-	-	-	-	-	GC-MS
Aristolone	1763	1776	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.3	-	-	-	GC-MS, RI
Isolongifolol	1723	1742	-	-	0.4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	GC-MS, RI
Oplopanone	1740	1753	0.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	GC-MS, RI
β-Costol	1767	[f]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.2	-	-	GC-MS
15-Hydroxy-α-muurolene	1780	1758	-	-	-	-	-	-	1.4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	GC-MS, RI
cis-Lanceol	1761	1785	-	-	0.6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	GC-MS, RI
Ambrial	[d]	1824	-	-	1.0	1.6	0.7	0.9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	GC-MS, RI
Perhydrofarnesyl acetone	[d]	1856	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.3	0.1	-	-	GC-MS, RI
Corymbolone	[d]	1898	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.1	-	GC-MS, RI
Farnesyl acetone	[d]	1939	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.1	-	-	-	GC-MS, RI
Kessanyl acetate	[d]	[f]	-	-	-	0.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	GC-MS
α-Vetivol	[d]	[f]	-	-	-	-	-	-	-	-	-	-	-	-	-	tr	-	-	-	-	-	-	-	-	GC-MS
Group sum [%]			**17.4**	**16.7**	**27.6**	**29.2**	**2.5**	**2.7**	**88.8**	**85.1**	**0.2**	**0.2**	**15.7**	**21.1**	**30.3**	**29.2**	**14.8**	**13.9**	**10.5**	**13.1**	**51.3**	**6.8**	**76.0**	**73.7**	

Component	RI		Plant species/Column/Relative contents [%]																						Identif[c]
	Pub[a]	Obs[b]	Aol		Aop		Aor		Aos		Br		Ccb		Ccl		Cl		La		Rd		Ss		
			5	17	5	17	5	17	5	17	5	17	5	17	5	17	5	17	5	17	5	17	5	17	
Higher isoprenoids																									
Geranyl linalool	2027	2039	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.1	-	-	-	-	-	GC-MS, RI
Phytol	2114	2119	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.1	-	-	-	-	-	-	-	GC-MS, RI
Group sum [%]			**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.1**	**0.0**	**0.1**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	
Others																									
1-Vinyl-5,5-dimethyl[2.1.1]bicyclohexane	[d]	922	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.3	0.3	GC-MS, RI
3-Hydroperoxyhexane	[d]	944	-	-	0.1	-	-	-	-	-	-	-	-	-	-	-	-	0.1	-	-	-	-	-	-	GC-MS, RI
1-(4-Methyl-3-cyclohexen-1-yl)ethanol	[d]	1152	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.3	0.2	-	-	-	-	GC-MS, RI
4-Isopropenylcyclohexanone	[d]	1161	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.0	0.8	-	-	-	-	GC-MS, RI
2-Methylene-6,6-dimethylbicyclo[3.2.0]heptan-3-ol	[d]	1161	0.2	0.1	0.4	0.1	-	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	GC-MS, RI
cis-3-Hexenyl-α-methylbutyrate	[d]	1234	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.1	-	-	-	GC-MS, RI
Cinerone	[d]	[f]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	GC-MS
5-Isopropenyl-2-methylenecyclohexyl hydroperoxide	[d]	1315	-	-	2.4	-	1.5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	GC-MS, RI
Ledane	[d]	1344	-	-	0.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	GC-MS, RI
6,6-dimethyl-bicyclo[3.1.1]hept-2-ene-2-carboxylic acid	[d]	[f]	-	-	-	0.4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	GC-MS
3a,9-Dimethyldodecahydro-3H-cyclohepta[d]inden-3-one	[d]	[f]	-	-	-	0.8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	GC-MS
Labda-8(17),12-diene-15,16-dial	[d]	2414	-	-	-	-	1.6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	GC-MS, RI
Group sum [%]			**0.2**	**0.1**	**3.1**	**1.3**	**3.1**	**0.1**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.1**	**1.3**	**1.0**	**0.1**	**0.0**	**0.3**	**0.3**	
Total identified			**93.9**	**92.8**	**88.5**	**91.2**	**91.6**	**91.2**	**95.2**	**93.6**	**98.8**	**98.4**	**96.5**	**92.0**	**90.8**	**93.0**	**98.3**	**96.6**	**97.4**	**96.1**	**90.0**	**92.2**	**91.8**	**90.3**	

[a)] Data taken from Adams (2007) [21] and from NIST (2017) [55]. [b)] Retention indices calculated from retention times on a HP-5MS column and based on C9–C29 alkanes. [c)] Identification method: GC-MS = Mass spectrum was identical to that of National Institute of Standards and Technology Library (ver. 2.0.f), RI = the retention index was consistent with that of the literature database, Std = constituent identity confirmed by co-injection of authentic standards. [d)] Literature data not available. [e)] Compound not detected in the sample. [f)] Retention indices were not calculated for compounds identified only by HP-17MS column. [g)] Retention indices were not calculated, as the retention times were outside the area of standard alkanes (C9–C29). [h)] tr = trace (<0.05). Aol – *Alpinia oxymitra* leaves, Aop – *Alpinia oxymitra* pericarp, Aor – *Alpinia oxymitra* rhizome, Aos – *Alpinia oxymitra* seeds, Br – *Boesenbergia rotunda*, Ccb – *Cinnamomum cambodianum* bark, Ccl – *Cinnamomum cambodianum* leaves, Cl – *Citrus lucida*, La – *Limnophila aromatica*, Rd – *Rhodamnia dumetorum*, Ss – *Sindora siamensis*, 5 – column HP-5MS, 17 – column DB-17MS

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7 *In vitro* growth-inhibitory effect of Philippine essential oils and their toxicity to lung fibroblasts

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Marketa Houdkova collected plant materials, distilled essential oils, tested antibacterial activity and analysed chemical composition. She prepared manuscript including its required revisions.

7.1 Introduction

Pneumonia, acute respiratory infection caused by bacterial pathogens such as *Haemophilus influenzae*, *Streptococcus pneumoniae*, and *Staphylococcus aureus*, is one of the leading causes of morbidity and mortality in developing countries, including the Philippines. The majority of severe episodes occurs in children under five years, the elderly and immuno-compromised individuals [1]. A timely antibiotic therapy can reduce its fatal cases; nevertheless, access to the health services is the most important health problem in the Philippines [2]. In this area, about 8,900 children die from pneumonia every year [1], whereas only 50% of them are treated with antibiotics [3].

The plant EOs are of great potential for development of novel antimicrobial preparations [4]. Since the presence of volatile compounds is characteristic for some plant taxa, chemotaxonomic research is frequent approach to their exploration. Due to the volatility of EOs, they are suitable for inhalation therapy, which is an effective way for the healing of respiratory ailments such as pneumonia. Their vapours can act directly on the site of infection in the respiratory system and simultaneously restricts systemic exposure, degradation of active components in the gastrointestinal tract, and associated toxicity [5]. In addition, it is more difficult for bacteria to develop resistance to these chemically diverse multicomponent mixtures than to single-ingredient conventional antibiotics [6]. During the last few years, several inhalation devices for EOs have been developed and patented [7].

In the Philippines, traditional medicine has been in practice for more than a thousand years and it still plays an important role in primary health care at present [8]. The Philippine archipelago is a centre of biodiversity where a large number of endemic plants has been reported to exhibit medicinal properties including plant species belonging to the essential oils-bearing families such as Zingiberaceae, Piperaceae, and Lauraceae [9]. Nevertheless, scientific validation and identification of many Philippine medicinal plants as well as determination of their anti-infective properties, chemical composition, and safety are needed. Recently, several *in vitro* studies have investigated antimicrobial potential of EOs derived from Philippine plant species that are easily available in traditional markets and in wild nature [10,11]. However, no experiments had determined antibacterial potential of their vapours against pathogens causing pneumonia until now.

In this article, we report examination of *in vitro* growth-inhibitory effect of EOs from six less known Philippine aromatic plants, namely *Alpinia brevilabris* C. Presl, *Alpinia cumingii* K. Schum., *Alpinia elegans* (C. Presl) K. Schum., *Callicarpa micrantha* Vidal, *Cinnamomum mercadoi* S. Vidal, and *Piper quinqueangulatum* Miq., against pneumonia causing bacteria in liquid and vapour phase by using novel broth microdilution volatilization method recently developed by our team [12]. Additionally, the cytotoxicity and chemical composition of tested EOs were analysed with the aim of assessing the relationship between their antimicrobial potential, chemistry, and safety for treatment of pneumonia.

7.2 Material and methods

7.2.1 Plant material

Based on chemotaxonomic criteria, six local plant species (*Alpinia brevilabris* C. Presl, *Alpinia cumingii* K. Schum., *Alpinia elegans* (C. Presl) K. Schum., *Callicarpa micrantha* Vidal, *Cinnamomum mercadoi* S. Vidal, and *Piper quinqueangulatum* Miq.) were selected as phytochemically less explored representatives of taxa containing EOs. The plant materials were collected in the foothills of Mt Makiling and Mt Pangasugan on the islands Luzon and Leyte, respectively in April–May 2017. All species were collected from wild populations, except *A. brevilabris* and *C. mercadoi*, which were obtained from plant nursery of the Visayas State University (VSU) and University of the Philippines Los Baños (UPLB), respectively. Ethnobotany expert Prof Ladislav Kokoska and botanist Dr Vaclav Zeleny authenticated 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. A detailed description of collected plant samples is summarized in Table 7.1.

Table 7.1 Philippine plant species selected for antibacterial and cytotoxicity testing

Scientific name	Family	Collection number	Area of collection	Weight of sample (g)	Essential oil yield % (v/w)	Essential oil colour
Alpinia brevilabris C. Presl	Zingiberaceae	02506KBFR4	Plant Nursery VSU	23.8	0.2	bright yellow
Alpinia cumingii K. Schum.	Zingiberaceae	02508KBFR6	Mt Pangasugan	25.4	0.4	pale yellow
Alpinia elegans (C. Presl) K. Schum.	Zingiberaceae	02509KBFR7	Mt Pangasugan	27.8	0.6	pale yellow
Callicarpa micrantha Vidal	Lamiaceae	02514KBFR3	Mt Pangasugan	23.4	0.5	pale yellow
Cinnamomum mercadoi S. Vidal	Lauraceae	02491KBFR7	Plant Nursery UPLB	17.8	1.3	pale yellow
Piper quinqueangulatum Miq.	Piperaceae	02531KBFR2	Mt Pangasugan	29.3	0.2	pale yellow

7.2.2 Essential oils isolation

EOs were obtained by hydrodistillation of dried leaves in 1 L of distilled water for 3 h using a Clevenger-type apparatus (Merci) according to the *European Pharmacopoeia* [13]. The EOs were stored in sealed glass vials at 4°C.

7.2.3 Bacterial strains and culture media

The following American Type Culture Collection (ATCC) strains were used: *Haemophilus influenzae* ATCC 49247, *Staphylococcus aureus* ATCC 29213, and *Streptococcus pneumoniae* ATCC 49619. 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. pneumoniae*). Broths pH were equilibrated to final value 7.6 using Trizma base (Sigma-Aldrich). All microbial strains and cultivation media were purchased from Oxoid. 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. pneumoniae* to ampicillin (≥84.5%), oxacillin (≥86.3%), and amoxicillin (≥90%), respectively (purchased from Sigma-Aldrich), were checked as positive antibiotic controls [14].

7.2.4 Antimicrobial assay

The antibacterial potential of plant EOs in liquid and vapour phase was determined using a broth microdilution volatilization method [12]. The experiments were performed in 96-well immune plates, covered by tight-fitting lids with flanges designed to reduce evaporation (SPL Life Sciences). Briefly, 30 μL of agar was pipetted into every flange on the lid, except the outermost flanges, and inoculated with bacterial suspension (5 μL). In the second part of this method, each sample of EOs was dissolved in DMSO (Sigma-Aldrich) 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. 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) were used for fastening the plate and lid together, with the handmade wooden pads for better fixing. 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) 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. 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 $\mu g/cm^3$, where 256; 128; 64; 32; 16; 8; 4; and 2 $\mu g/cm^3$ 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. All experiments were carried out in triplicate in three independent experiments and results were expressed as median/modal MICs values.

7.2.5 Cell culture

Lung fibroblast cells MRC-5 purchased directly from ATCC were propagated in Eagle's Minimum Essential Medium (EMEM) supplemented with 10% foetal bovine serum (FBS), 2 mM glutamine, 10 µL/mL non-essential amino acids, and 1% penicillin–streptomycin solution (10,000 units/mL of penicillin and 10 mg/mL of streptomycin), all these components purchased from Sigma-Aldrich. The cells were preincubated in 96-well microtiter plates at density of 2.5×10^3 cells per well for 24 h in a humidified incubator under atmosphere of 5% CO_2 at 37°C.

7.2.6 Cytotoxicity assay

The modified method for toxicity assessment of volatile agents [12] previously described by Mosmann [15] was used. Twelve two-fold serially diluted concentrations starting from 512 µg/mL of EOs dissolved in DMSO were prepared. The microtiter plates were covered by EVA capmats™ under humidified atmosphere of 5% CO_2 and cultivated for 72 h at 37°C. Thereafter, MTT (1 mg/mL) in EMEM solution was added to each well and plates were incubated for an additional 2 h under the same conditions. The media were removed and intracellular formazan product was dissolved in 100 µL of DMSO.

The solvent used did not affect the viability of the lung cells at the concentrations tested. The absorbance was measured at 555 nm and the percentage of viability was calculated when compared to an untreated control. The results of the cytotoxic effect were expressed as IC_{50} in µg/mL. The levels of cytotoxicity were classified as cytotoxic (IC_{50} < 2 µg/mL), moderately cytotoxic (IC_{50} 2–89 µg/mL), and non-toxic (IC_{50} > 90 µg/mL) [16]. Furthermore, 80% inhibitory concentration of proliferation (IC_{80}) was calculated as equivalent to MIC endpoint usually defined as 80% bacterial growth inhibition and therapeutic indices (TI) were defined as the ratio of IC_{50} or IC_{80} and MIC values.

7.2.7 GC-MS analysis

For determination of the main components of EOs tested, GC-MS analysis was carried out using dual-column/dual-detector gas chromatograph system Agilent GC-7890B equipped with autosampler Agilent 7693, fused-silica HP-5MS column (30 m × 0.25 mm, film thickness 0.25 µm, Agilent 19091s-433), DB-17MS column (30 m × 0.25 mm, film thickness 0.25 µm, Agilent 122-473), and flame ionization detector (FID) coupled with single quadrupole mass selective detector Agilent MSD-5977B (Agilent Technologies). The operational parameters: helium as carrier gas at 1 mL/min, injector temperature 250/200°C and oven temperature from 50 to 300°C/from 50 to 280°C for HP-5MS and DB-17MS, respectively. Samples of EOs diluted in *n*-hexane for GC-MS (Merck) at concentration 1 µg/mL and 1 µL of solution were injected in splitless mode. The mass detector was set to following conditions: ionization energy 70 eV, ion source temperature 200°C, scan time 1 s, mass range 30–600 m/z.

The identification of constituents was based on the comparison of their retention indices (RI) with the National Institute of Standards and Technology Library ver. 2.0.f, as well as authentic standards (Sigma-Aldrich) and literature [17]. The RIs were calculated for compounds separated by HP-5MS column using the retention times of *n*-alkanes series C9–C29 (Sigma-Aldrich). For each EO analysed, the final number of compounds was calculated as the sum of components simultaneously identified using both columns and the remaining constituents identified by individual columns only. The relative percentage contents of EO components were determined by FID and indicated for both columns.

7.3 Results

As a result of this study, all EOs exhibited some antibacterial efficacy, however, their effectiveness of EOs varied substantially in the range of 256–1,024 µg/mL in broth and 512–1,024 µg/mL on agar (Table 7.2). In liquid phase, the lowest MIC value was observed for *A. cumingii* and *A. elegans* (both 256 µg/mL) against *H. influenzae*. EOs of *A. brevilabris*, *C. micrantha,* and *P. quinqueangulatum* produced moderate antibacterial activity with MIC of 512 µg/mL. *C. mercadoi* possessed only weak inhibitory effect (1,024 µg/mL). No antibacterial activity in broth medium was determined against *S. pneumoniae*. The EO from *A. cumingii* was only one agent effective in gaseous phase with MIC value of 1,024 µg/mL against *H. influenzae*.

The values of IC_{50} and IC_{80} for human lung fibroblasts varied substantially in respective ranges of 27.7–215.3 µg/mL and 53.7–396.6 µg/mL (Table 7.3). Moderate toxicity was detected for all EOs except *C. mercadoi* and *A. brevilabris*, which were evaluated as non-toxic with respective IC_{50} of 215.3 and 91.6 µg/mL. In the case of IC_{80}, the most EOs were found as non-toxic. The lowest IC_{80} was observed for *C. mercadoi* then for *A. brevilabris*, *P. quinqueangulatum* and *C. micrantha* with values of 396.6, 168.8, 139.4 and 122.8 µg/mL, respectively. Similarly to IC_{50}, EOs of *A. cumingii* and *A. elegans* possessed moderate toxicity to lung cells. Nevertheless, therapeutic indices calculated from values for IC_{50} and IC_{80} (varying in range 0.03 – 0.39) indicated certain toxicological risk to the human lung cells.

Based on dual-column GC-MS analysis of six EOs from *A. brevilabris*, *A. cumingii*, *A. elegans*, *C. micrantha*, *C. mercadoi*, and *P. quinqueangulatum*, a total of 63, 54, 66, 65, 71, and 74 compounds were identified using both HP-5MS/DB-17MS columns, representing 95.4/93.7, 90.5/90.4, 91.2/90.0, 92.3/92.5, 93.9/90.4, and 92.8/90.0% of their total contents, respectively (Table 7.4). Monoterpenoids and sesquiterpenoids were observed as the leading chemical classes. In *A. brevilabris*, hedycaryol (26.8/27.6%) was the main constituent followed by *β*-eudesmol (11.3/11.0%), *α*-pinene (8.5/5.9%), and *γ*-eudesmol (7.8/4.6%). The most abundant component in *A. cumingii* EO was *β*-pinene (21.8/20.6%) followed by caryophyllene epoxide (14.8/14.7%), nonan-2-ol (7.0/6.8%), and myrtenol (6.8/2.4%).

Table 7.2 Antibacterial activity of Philippine essential oils and antibiotics in liquid and vapour phase against pneumonia causing bacteria grown in broth and agar media

	Bacteria/grown medium/MIC								
	Haemophilus influenzae			***Staphylococcus aureus***			***Streptococcus pneumoniae***		
	broth	**agar**		**broth**	**agar**		**broth**	**agar**	
Essential oils	**(µg/mL)**	**(µg/mL)**	**(µg/cm^3)**	**(µg/mL)**	**(µg/mL)**	**(µg/cm^3)**	**(µg/mL)**	**(µg/mL)**	**(µg/cm^3)**
Alpinia brevilabris	512.0	>1,024.0	>256.0	>1,024.0	>1,024.0	>256.0	>1,024.0	>1,024.0	>256.0
Alpinia cumingii	256.0	1,024.0	256.0	1,024.0	>1,024.0	>256.0	>1,024.0	>1,024.0	>256.0
Alpinia elegans	256.0	>1,024.0	>256.0	512.0	>1,024.0	>256.0	>1,024.0	>1,024.0	>256.0
Callicarpa micrantha	512.0	>1,024.0	>256.0	>1,024.0	>1,024.0	>256.0	>1,024.0	>1,024.0	>256.0
Cinnamomum mercadoi	1,024.0	>1,024.0	>256.0	>1,024.0	>1,024.0	>256.0	>1,024.0	>1,024.0	>256.0
Piper quinqueangulatum	512.0	>1,024.0	>256.0	512.0	>1,024.0	>256.0	>1,024.0	>1,024.0	>256.0
Positive antibiotic control									
Amoxicillin	-		-	-	-	-	0.3	ND	ND
Ampicillin	0.3	ND	ND	-	-	-	-	-	-
Oxacillin	-	-	-	0.25	ND	ND	-	-	-

MIC: minimum inhibitory concentration; ND: not determined; -: not tested

A. elegans was rich in content of caryophyllene epoxide (24.7/30.5%), followed by *α*-pinene (9.7/10.5%), isolongifolol methyl ether (4.2/3.8%), and linalool, which was detected by HP-5MS column only (4.1%). The analysis of *C. micrantha* identified aristolochene (17.6/10.2%), caryophyllene epoxide (14.6/17.8%), aciphyllene (9.2/14.1%), and *β*-caryophyllene (9.2/7.6%) as its major constituents. The EO of *C. mercadoi* was composed of high content of borneol (24.4/24.9%), *p*-cymene (16.8/13.5%), linalool (12.1/11.7%), and espatulenol (6.0/7.2%). Similarly, linalool was predominant compound (12.8/12.7%) of *P. quinqueangulatum*, followed by caryophyllene epoxide (8.7/9.1%), neointermedeol (8.4/5.2%), and eremophilene (7.5/7.2%).

Table 7.3 Cytotoxicity of Philippine essential oils in the lung fibroblast cells

Essential oils	IC_{50} ± SD (µg/ml)	IC_{80} ± SD (µg/ml)
Alpinia brevilabris	91.6 ± 0.3	168.8 ± 0.3
Alpinia cumingii	33.6 ± 1.2	67.7 ± 1.8
Alpinia elegans	27.7 ± 1.6	53.7 ± 0.9
Callicarpa micrantha	85.9 ± 8.2	122.8 ± 4.9
Cinnamomum mercadoi	215.3 ± 5.7	396.6 ± 8.6
Piper quinqueangulatum	38.8 ± 2.9	139.4 ± 20.0

IC_{50}: half maximal inhibitory concentration of proliferation in µg/ml; IC_{80}: 80 % inhibitory concentration of proliferation in µg/ml; SD: standard deviation

7.4 Discussion

In this study, the EOs were obtained by hydrodistillation from plant species belonging to typical essential oil-bearing plant families, namely *A. brevilabris* (Zingiberaceae), *A. cumingii* (Zingiberaceae), *A. elegans* (Zingiberaceae), *C. micrantha* (Lamiaceae), *C. mercadoi* (Lauraceae), and *P. quinqueangulatum* (Piperaceae). According to our best knowledge, the EOs from these species were isolated for the first time.

There is no previous literature reporting any antimicrobial effect for plant species tested here, except study on *C. mercadoi* bark and root crude extract, which showed week inhibitory activity against *S. aureus* [18]. However, the leaves EOs of related species such as *Alpinia rafflesiana*, *Cinnamomum longepaniculatum*, and *Piper caninum* [19-21] have been reported to possess certain degree of anti-*S. aureus* effect with respective MIC

values 7.81 µg/mL, 6.25 µL/mL, and 250 µg/mL. Although there is lack of data on antibacterial activity of above-mentioned EOs, several studies evaluating antibacterial potential of their main constituents have been published. Significant antibacterial activity of *β*-pinene, the main constituent of *A. cumingii* EO, has been detected against methicillin-resistant *S. aureus* with MIC value 6.25 µg/mL [22]. Growth-inhibitory effect was also proved for caryophyllene epoxide, dominant sesquiterpenoid of *A. elegans* and *C. micrantha* EOs [23] and linalool, present in *P. quinqueangulatum* EO [24].

Whereas data on inhalation toxicity of the EOs tested here are completely missing, several studies evaluated toxicity of taxonomically related species have previously been published. Contact toxicity was assessed for EO of *Alpinia purpurata* inflorescence since there was no mortality at any concentrations tested [25]. Another *in vivo* tests of acute oral toxicity in rodents showed that EOs from *Piper glabratum* leaves did not cause death or signs of toxicity up to dose of 1,000 mg/kg [26]. In contrast, *Cinnamomum zeylanicum* EO indicated significant cytotoxic effect in normal rat fibroblast F2408 over 15 µg/mL dose. However, treatment of the normal F2408 cells with the *C. zeylanicum* EO at varying periods did not cause any time-dependent cytotoxicity [27].

Because this is the first report on the EOs from *A. brevilabris*, *A. cumingii*, *A. elegans*, *C. micrantha*, *C. mercadoi*, and *P. quinqueangulatum*, the literature about their chemical composition is not available. However, analytical data from this study are consistent with previously published works on chemistry of the same genera. Similarly, like in *Alpinia* species analysed in our study, the abundant constituents of *Alpinia mutica* leaves were *α*- and *β*-pinene, linalool, bornyl acetate, and *β*-caryophyllene [28]. Borneol, the major constituent of *Cinnamomum camphora* var. *Borneol* [29], we detected in *C. mercadoi* at the highest content as well. The chemical profile of *Piper aduncum* EO [30] resembles composition of *P. quinqueangulatum* tested here with the high content of linalool, nerolidol, espatulenol, and caryophyllene epoxide. Similarly to studies describing chemical compositions of other *Alpinia* and *Piper* species [31-33], less volatile compounds such as fatty acids and phytol were detected in *A. brevilabris*, *A. cumingii*, and *P. quinquangulatum* EOs. With the aim to achieve a higher quality identification, the EOs were conducted on two columns with different polarities, non-polar HP-5MS and mid-polar DB-17MS, when ten, six, eleven, sixteen, fifteen, and nine additional

compounds were identified using DB-17MS column in *A. brevilabris*, *A. cumingii*, *A. elegans*, *C. micrantha*, *C. mercadoi*, and *P. quinqueangulatum* EOs, respectively.

7.5 Conclusion

In summary, this is the first report on antibacterial activity and chemical composition of EOs isolated from *A. brevilabris*, *A. cumingii*, *A. elegans*, *C. micrantha*, *C. mercadoi*, and *P. quinqueangulatum.* Although all EOs tested exhibited certain growth-inhibitory effect in broth against *H. influenzae* and *S. aureus*, only *A. cumingii* was active in vapour phase against *H. influenzae*. However, due to the moderate toxic effect to human lung cells, its usability as inhalation preparation seems to be limited. Nevertheless, further research focused on *in vivo* evaluation will be necessary to carry out in order to verify its potential for application in the therapy of respiratory infections.

Table 7.4 Chemical composition of Philippine essential oils tested

Component	RI		Plant species/Relative content [%]												Identification[c]
	Obs[a]	Pub[b]	*Ab*		*Ac*		*Ae*		*Cmi*		*Cme*		*Pq*		
			5	**17**	**5**	**17**	**5**	**17**	**5**	**17**	**5**	**17**	**5**	**17**	
Hydrocarbons															
4,8-Dimethyl-1,3,7-nonatriene	1119	1110	[f] -	-	-	-	-	-	-	-	-	-	0.4	0.4	GC-MS, RI
Group sum [%]			0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.4	0.4	
Carbonylic compounds															
Hexan-2-one	[d]	791	-	-	-	0.2	-	0.2	-	-	-	-	-	-	GC-MS, Std
Decan-2-one	[d]	1186	-	-	-	1.4	-	-	-	-	-	-	-	-	GC-MS
Decen-2-al	1281	1276	-	-	0.3	-	-	-	-	-	-	-	-	-	GC-MS, RI
Undecan-2-one	1296	1294	-	-	1.2	-	-	-	-	-	-	-	-	-	GC-MS, RI, Std
Pentadecan-2-one		1697	-	1.6	-	-	-	-	-	-	-	-	-	-	GC-MS
Group sum [%]			0.0	1.6	1.5	1.6	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	
Aliphatic alcohols															
Heptan-2-ol	901	902	-	-	3.4	3.0	-	-	-	-	-	-	-	-	GC-MS, RI
Octan-2-ol	1001	995	-	-	0.1	0.2	-	-	-	-	-	-	-	-	GC-MS, RI
Nonan-2-ol	1102	1098	-	-	7.0	6.8	-	-	-	-	-	-	-	-	GC-MS, RI
Decan-2-ol	[d]	1186	-	-	-	2.3	-	-	-	-	-	-	-	-	GC-MS, Std
Undecan-2-ol	1303	1301	-	-	2.0	-	-	-	-	-	-	-	-	-	GC-MS, RI
Octadecan-1-ol	2081	2077	-	-	-	-	-	-	-	-	-	-	1.2	1.0	
Group sum [%]			0.0	0.0	12.5	12.3	0.0	0.0	0.0	0.0	0.0	0.0	1.2	1.0	
Aromatic compounds															
2-Methyl-3-phenylpropanal	1248	1244	-	-	-	-	-	-	-	-	0.2	-	-	-	GC-MS, RI
3-Phenyl-2-butanone	1250	1244	-	-	0.2	-	-	-	-	-	-	-	-	-	GC-MS, RI
4-Phenyl-2-butanol	1264	1241	-	-	1.7	2.2	-	-	-	-	-	-	-	-	GC-MS, RI
4-Phenyl-2-butyl acetate	1401	1398	-	-	1.9	2.0	-	-	-	-	-	-	-	-	GC-MS, RI
Group sum [%]			0.0	0.0	3.8	4.2	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.0	

Component	RI		Plant species/Relative content [%]												Identification[c]
	Obs[a]	Pub[b]	*Ab*		*Ac*		*Ae*		*Cmi*		*Cme*		*Pq*		
			5	17	5	17	5	17	5	17	5	17	5	17	
Fatty acids															
Pentadecanoic acid	[d]	1878	-	-	-	1.0	-	-	-	-	-	-	-	-	GC-MS
Hexadecanoic acid	1970	1960	-	1.9	-	-	-	-	-	-	-	-	0.5	3.0	GC-MS, RI
Group sum [%]			0.0	1.9	0.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5	3.0	
Esters															
2-Heptyl acetate	1042	1043	-	-	0.4	0.4	-	-	-	-	-	-	-	-	GC-MS, RI
Formic acid, 3,7,11-trimethyl-1,6,10-dodecatrien-3-yl ester	1757	1752	-	-	-	-	0.8	-	-	-	-	-	-	-	GC-MS, RI
Group sum [%]			0.0	0.0	0.4	0.4	0.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	
Monoterpenoids															
Tricyclene	930	926	0.1	-	-	-	-	-	-	-	-	-	-	-	GC-MS, RI
α-Thujene	[d]	930	-	0.1	-	-	-	-	-	-	0.2	0.1	-	-	GC-MS, RI
α-Pinene	937	939	8.5	5.7	4.8	4.7	9.7	10.5	-	-	1,0	0.8	1.8	1.8	GC-MS, RI, Std
Camphene	954	953	1.0	0.7	0.3	0.1	0.4	0.3	-	-	0.5	0.3	0.1	-	GC-MS, RI, Std
Dehydrosabinene	958	956	0.1	-	0.3	0.3	0.7	0.5	-	-	-	-	-	-	GC-MS, RI
Sabinene	977	975	0.4	0.4	0.1	0.2	-	-	-	-	0.1	-	-	-	GC-MS, RI
β-Pinene	982	979	6.3	5.6	21.8	20.6	0.3	0.3	0.2	0.1	0.4	-	0.1	0.2	GC-MS, RI, Std
β-Myrcene	993	990	0.5	-	-	-	-	-	-	-	2.3	2.1	0.1	-	GC-MS, RI, Std
δ-2-Carene	1005	1002	-	-	-	-	-	-	-	-	0.1	tr	-	-	GC-MS, RI
α-Phellandrene	1009	1002	-	-	-	-	-	-	-	-	0.1	0.1	-	-	GC-MS, RI, Std
o-Cymene	1009	1026	-	-	-	-	0.3	-	-	-	-	-	-	-	GC-MS, RI
δ-3-carene	1014	1011	0.4	0.4	-	-	-	-	-	-	0.1	0.1	-	-	GC-MS, RI, Std
p-Cymene	1028	1024	1.5	1.1	0.3	0.6	0.5	-	-	-	16.8	13.5	0.1	-	GC-MS, RI, Std
Limonene	1032	1029	1.0	0.8	0.4	0.4	0.5	0.6	-	0.1	3.6	1.7	0.3	0.3	GC-MS, RI, Std
β-Phellandrene	[d]	1029	-	0.1	-	-	-	-	-	-	-	0.9	-	-	GC-MS
1,8-Cineole	1036	1031	-	-	0.2	-	3.0	3.7	-	-	-	-	2.2	2.1	GC-MS, RI, Std
cis-β-Ocimene	1039	1037	-	-	-	-	-	-	-	-	0.1	-	-	-	GC-MS, RI
trans-β-Ocimene	1050	1050	0.1	-	-	-	-	-	-	-	-	-	-	-	GC-MS, RI
cis-Linalool oxide (furanoid)	1076	1072	-	-	-	-	0.6	0.7	-	-	0.2	-	0.1	0.1	GC-MS, RI

Component	RI		Plant species/Relative content [%]												Identification[c]
	Obs[a]	Pub[b]	*Ab*		*Ac*		*Ae*		*Cmi*		*Cme*		*Pq*		
			5	17	5	17	5	17	5	17	5	17	5	17	
trans-Linalool oxide (furanoid)	1092	1086	-	-	-	-	0.8	-	-	-	0.3	0.2	0.2	0.1	GC-MS, RI
p-Cymenene	1094	1091	0.1	-	-	-	-	-	-	-	-	-	-	-	GC-MS, RI
Linalool	1102	1093	0.7	0.7	-	-	4.1	4.6	-	-	12.1	11.7	12.8	12.7	GC-MS, RI, Std
Perillene	[d]	1103	-	-	-	-	-	-	-	-	-	0.1	-	-	GC-MS
Rosefuran	[d]	1104	-	-	-	-	-	-	-	-	-	tr[g]	-	-	GC-MS
Umbellulol	1112	1107	-	-	-	-	0.8	-	-	-	-	-	-	-	GC-MS, RI
Camphen-6-ol	[d]	1113	-	-	-	-	-	0.1	-	-	-	-	-	-	GC-MS
p-Mentha-2,8-diene-1-ol	1113	1122	-	-	0.2	-	-	-	-	-	-	-	-	-	GC-MS, RI
β-Fenchol	1119	1121	0.1	0.1	tr	0.1	-	-	-	-	-	-	-	-	GC-MS, RI
Chrysanthenone	1122	1127	-	-	-	-	0.3	-	-	-	-	-	-	-	GC-MS, RI
cis-2-Menthenol	1127	1124	-	-	-	-	-	-	-	-	0.1	0.1	-	-	GC-MS, RI
α-Campholenal	1132	1126	0.1	0.1	0.5	0.5	0.5	0.4	-	-	-	-	-	-	GC-MS, RI
Pinocarveol	1146	1139	0.6	0.9	-	-	-	-	-	-	-	-	-	-	GC-MS, RI
Sabinol	1146	1142	-	-	5.1	4.2	0.5	0.8	-	-	-	0.7	-	-	GC-MS, RI
Verbenol	1150	1144	0.4	-	0.4	0.4	1.0	1.4	-	-	-	-	0.1	0.1	GC-MS, RI
Camphor	1152	1146	-	-	-	-	-	-	-	-	1.2	1.0	-	-	GC-MS, RI, Std
trans-2-Menthenol	[d]	1150	-	-	-	-	-	-	-	-	-	0.1	-	-	GC-MS
α-Pinocarvone	1170	1164	0.2	0.2	3.9	4.5	0.3	0.3	-	-	-	-	-	-	GC-MS, RI
Borneol	1173	1169	0.46	-	-	-	0.3	0.3	0.1	0.1	24.4	24.9	-	-	GC-MS, RI, Std
Ocimenol	[d]	1169	-	0.6	-	-	-	-	-	-	-	-	-	-	GC-MS
cis-Linalool oxide (pyranoid)	1178	1174	-	-	-	-	0.1	0.2	-	-	-	-	-	-	GC-MS, RI
Terpinen-4-ol	1184	1177	0.8	1.1	0.3	0.2	0.1	-	-	-	0.2	-	0.1	0.1	GC-MS, RI
p-Cymen-8-ol	1190	1182	0.2	-	-	-	-	0.1	-	-	-	0.2	-	-	GC-MS, RI
Myrtanal	1190	1198	-	-	0.2	0.1	-	-	-	-	-	-	-	-	GC-MS, RI
2,6-Dimethyl-3,7-octadien-2,6-diol	1192	1186	-	-	-	-	0.1	0.2	-	-	-	-	-	-	GC-MS, RI
Cryptone	1194	1185	-	-	-	-	-	-	-	-	1.2	1.0	-	-	GC-MS, RI
α-Terpineol	1197	1188	0.5	0.5	0.4	0.3	0.4	0.7	-	-	0.7	1.0	0.5	0.6	GC-MS, RI
Myrtenal	1203	1195	-	-	-	3.5	0.5	0.3	-	-	-	-	-	-	GC-MS, RI
Myrtenol	1204	1195	0.6	0.3	6.8	2.4	-	0.1	-	-	-	-	-	-	GC-MS, RI
trans-Carveol	1209	1216	-	-	-	-	-	-	-	-	1.0	-	-	-	GC-MS, RI
Carvotanacetol	1214	[e]	-	-	-	-	-	-	-	-	0.1	-	-	-	GC-MS, RI

Component	RI		Plant species/Relative content [%]												Identification[c]
	Obs[a]	Pub[b]	*Ab*		*Ac*		*Ae*		*Cmi*		*Cme*		*Pq*		
			5	**17**	**5**	**17**	**5**	**17**	**5**	**17**	**5**	**17**	**5**	**17**	
Verbenone	1217	1205	0.1	0.4	0.4	0.5	1.3	1.5	-	-	-	-	-	-	GC-MS, RI
cis-Carveol	1225	1229	-	-	0.2	-	0.2	0.3	-	-	-	-	-	-	GC-MS, RI
Fenchyl acetate	1226	1220	0.7	-	-	-	-	-	-	-	-	-	-	-	GC-MS, RI
Isobornyl formate	1236	1239	-	-	-	-	-	-	-	-	0.1	-	-	-	GC-MS, RI
Cuminaldehyde	[d]	1241	-	-	-	-	-	-	-	-	-	0.2	-	-	GC-MS
Carvone	1251	1243	-	-	-	-	0.1	0.1	-	-	-	-	-	-	GC-MS, RI, Std
Carvenone	[d]	1258	-	-	-	-	-	-	-	-	-	0.4	-	-	GC-MS
Bornyl acetate	1293	1288	7.0	5.8	-	-	-	-	-	-	-	-	-	-	GC-MS, RI, Std
Methyl myrtenate	1304	1294	-	-	-	-	-	-	-	-	-	-	0.5	-	GC-MS, RI
trans-Pinocarvyl acetate	1305	1298	-	-	-	-	0.9	0.7	-	-	-	-	-	-	GC-MS, RI
Carvacrol	1306	1299	-	-	-	-	-	-	-	-	0.3	0.3	-	-	GC-MS, RI, Std
Car-3-en-5-one	1321	1314	0.1	-	-	-	-	-	-	-	-	-	-	-	GC-MS, RI
2-Hydroxycineole acetate	1326	1342	-	-	-	-	-	-	-	-	2.8	3.7	-	-	GC-MS, RI
Methyl geranate	1327	1324	-	-	-	-	-	-	-	-	-	-	0.2	0.2	GC-MS, RI
p-Mentha-1,4-dien-7-ol	1330	1330	-	-	1.5	4.0	0.1	-	-	-	-	-	-	-	GC-MS, RI
Sobrerol	1386	1388	-	-	0.2	-	-	-	-	-	-	-	-	-	GC-MS, RI
Methyleugenol	1409	1403	-	-	-	-	-	-	-	-	0.3	0.5	-	-	GC-MS, RI
α-Ionone	1436	1430	-	-	-	-	-	-	-	-	-	-	0.06	-	GC-MS
Geranyl acetone	1458	1455	0.1	0.2	0.2	0.4	-	-	-	-	-	-	-	-	GC-MS, RI
β-Ionone	1495	1488	0.1	-	-	-	-	-	-	-	-	-	-	-	GC-MS, RI
Group sum [%]			32.8	26.0	48.5	48.0	28.4	28.7	0.3	0.3	70.3	65.7	19.3	18.3	
Sesquiterpenoids															
α-Cubebene	1358	1348	0.1	-	-	-	-	-	0.3	0.3	-	-	0.2	0.2	GC-MS, RI
Cyclosativene	[d]	1371	-	-	-	-	-	-	-	-	-	-	-	0.1	GC-MS, RI
Longicyclene	1377	1374	-	-	-	-	-	-	-	-	-	-	0.1	0.1	GC-MS, RI
α-Ylangene	1382	1375	-	-	-	-	-	-	0.2	0.1	-	-	-	-	GC-MS, RI
α-Copaene	1386	1376	0.2	0.2	-	-	-	-	1.1	1.1	0.1	-	0.6	0.5	GC-MS, RI
β-Bourbonene	1396	1388	-	-	-	-	-	-	0.1	tr	-	-	0.13	0.13	GC-MS, RI
β-Elemene	1401	1389	0.6	0.6	-	-	-	-	0.9	1.0	2.0	1.6	2.8	3.0	GC-MS, RI
Sesquithujene	1412	1405	-	-	-	-	-	-	0.1	-	-	-	-	-	GC-MS, RI

Component	RI		Plant species/Relative content [%]												Identification[c]
	Obs[a]	Pub[b]	*Ab*		*Ac*		*Ae*		*Cmi*		*Cme*		*Pq*		
			5	17	5	17	5	17	5	17	5	17	5	17	
Acora-3(7),14-diene	1421	1412	-	-	-	-	-	-	0.1	0.2	-	-	-	-	GC-MS, RI
β-Ylangene	[d]	1420	-	-	-	-	-	-	-	0.3	-	-	-	-	GC-MS
Cedrene	1430	1422	-	-	0.1	-	-	-	-	-	-	-	-	-	GC-MS, RI
β-Caryophyllene	1432	1419	1.1	1.0	-	-	1.0	1.0	9.2	7.6	2.5	2.0	2.1	1.8	GC-MS, RI, Std
γ-Elemene	1436	1436	-	-	0.4	-	-	-	-	-	-	-	-	-	GC-MS, RI
β-Copaene	1441	1432	-	-	-	-	-	-	0.4	0.3	-	-	0.3	0.2	GC-MS, RI
β-Humulene	1443	1438	0.10	-	-	-	-	0.1	-	-	-	-	-	-	GC-MS
α-Bergamotene	1444	1434	-	-	-	-	-	-	0.1	0.1	0.1	-	-	-	GC-MS, RI
γ-Patchoulene	1447	1441	-	-	-	-	-	-	tr	-	-	-	-	-	GC-MS, RI
Aromadendrene	1452	1441	-	-	-	-	-	-	-	0.1	-	-	tr	-	GC-MS, RI
β-Farnesene	[d]	1442	-	-	-	-	-	-	-	0.2	-	-	-	-	GC-MS, Std
Isogermacrene D	1452	1451	-	-	-	-	-	-	tr	-	-	-		-	GC-MS, RI
Selina-5,11-diene	1456	1446	-	-	-	-	-	-	tr	0.tr	-	-	0.1	-	GC-MS, RI
α-Himachalene	1462	1451	-	-	-	-	-	-	-	1.2	0.2	-	-	-	GC-MS, RI
α-Humulene	1467	1454	0.6	0.5	-	-	-	-	1.6	-	0.5	0.3	0.4	tr	GC-MS, RI, Std
Alloaromadendrene	1461	1460	0.1	0.1	-	-	0.1	-	0.6	0.6	-	-	-	-	GC-MS, RI
Cadina-3,5-diene	1462	1458	-	-	-	-	-	-	-	-	-	-	0.2	-	GC-MS, RI
β-Chamigrene	[d]	1477	-	-	-	-	-	-	-	0.7	-	-	-	-	GC-MS
γ-Muurolene	1487	1479	-	-	-	-	-	-	-	tr	0.1	0.2	-	-	GC-MS, RI
Amorpha-4,11-diene	1471	1482	-	-	-	-	-	-	1.3	2.9	-	-	-	-	GC-MS, RI
epi-β-Caryophyllene	1475	1474	-	-	-	-	-	-	-	-	2.5	2.5	-	-	GC-MS, RI
Eudesma-2,4,11-triene	1478	1479	-	-	-	-	0.2	-	0.3	-	-	-	-	-	GC-MS, RI
β-Selinene	1487	1490	0.1	0.3	-	-	2.4	2.7	6.5	5.6	-	-	-	-	GC-MS, RI
Germacrene D	1489	1485	-	-	-	-	-	-	3.8	3.9	-	-	0.4	0.5	GC-MS, RI
γ-Amorphene	[d]	1484	-	-	-	-	-	-	-	-	-	-	-	0.4	GC-MS
Aristolochene	1482	1488	-	-	0.6	0.5	-	0.4	17.6	10.2	-	-	-	-	GC-MS, RI
β-Guaiene	1492	1493	-	-	-	-	-	-	1.6	-	-	-	-	-	GC-MS, RI
Epicubebol	[d]	1494	-	-	-	-	-	-	-	-	-	tr	-	-	GC-MS
Viridiflorene	[d]	1496	-	-	-	-	-	-	-	-	-	-	-	tr	GC-MS
Eremophilene	1500	1501	0.4	-	0.6	-	1.6	1.3	-	3.4	tr	-	7.5	7.2	GC-MS, RI
β-Dihydroagarofuran	[d]	1503	-	-	-	-	-	0.26	-	-	-	-	-	-	GC-MS

Component	RI		Plant species/Relative content [%]												Identification[c]
	Obs[a]	Pub[b]	*Ab*		*Ac*		*Ae*		*Cmi*		*Cme*		*Pq*		
			5	17	5	17	5	17	5	17	5	17	5	17	
α-Selinene	1508	1498	0.3	0.1	-	-	-	-	-	-	-	-	-	-	GC-MS, RI
δ-Guaiene	1508	1506	-	-	-	-	-	-	-	-	0.2	-	-	-	GC-MS, RI
Aciphyllene	1509	1501	-	-	-	-	-	-	9.2	14.1	-	-	7.4	5.9	GC-MS, RI
α-Muurolene	1511	1500	-	-	-	-	-	-	-	-	0.3	-	-	-	GC-MS, RI
β-Curcumene	1516	1515	-	-	-	-	-	-	-	-	-	-	0.1	-	GC-MS, RI
β-Bisabolene	1517	1505	-	-	-	-	-	-	0.8	-	-	-	-	-	GC-MS, RI
γ-Cadinene	1526	1515	-	-	-	-	-	-	-	0.3	0.1	-	0.7	0.7	GC-MS, RI
Nootkatene	1527	1518	-	-	-	-	0.7	0.3	-	-	-	-	-	-	GC-MS, RI
Cubebol	1528	1515	0.1	0.1	-	-	-	-	-	-	-	-	-	-	GC-MS, RI
β-Cadinene	[d]	1515	-	-	-	-	-	-	-	-	-	-	-	0.47	GC-MS
α-Panasinsene	1532	1527	-	-	-	-	1.3	1.0	3.0	2.0	-	-	-	0.18	GC-MS, RI
δ-Cadinene	1527	1523	0.3	tr	-	-	-	-	1.3	1.2	0.2	-	-	-	GC-MS, RI
Calamenene	1535	1529	-	-	-	-	-	-	1.0	0.8	-	-	1.2	0.4	GC-MS, RI
Cubenene	1544	1542	-	-	-	-	-	-	-	-	-	-	0.1	-	GC-MS, RI
6-epi-shyobunol	[d]	[e]	-	-	-	1.0	-	-	-	-	-	-	-	-	GC-MS
Elemol	[d]	1549	-	0.1	-	-	-	-	-	-	-	-	-	-	GC-MS
α-Cadinene	1550	1538	-	-	-	-	-	-	0.2	-	-	-	0.1	0.3	GC-MS, RI
α-Calacorene	1556	1545	-	-	-	-	-	-	-	-	0.1	-	0.2	0.2	GC-MS, RI
Cadala-1(10),3,8-triene	1556	[e]	-	-	-	-	-	-	0.6	-	-	-	-	-	GC-MS, RI
Hedycaryol	1564	1559	26.8	27.6	-	-	-	-	-	-	-	-	-	-	GC-MS, RI
1,5-Epoxysalvial-4(14)-ene	1567	1573	-	-	-	-	2.8	-	-	-	-	-	-	-	GC-MS, RI
Isoshyobunone	1568	1535	-	-	-	-	-	-	1.4	-	-	-	-	-	GC-MS, RI
Nerolidol	1570	1563	0.7	0.8	-	-	1.2	1.5	0.4	0.5	-	-	5.5	5.8	GC-MS, RI
β-Spathulenol	1580	1582	-	-	-	-	0.2	-	0.2	0.2	-	-	-	-	GC-MS, RI
Caryophyllene epoxide	1583	1584	0.1	2.4	14.8	14.7	24.7	30.4	14.6	17.8	-	0.4	8.7	9.1	GC-MS, RI
Globulol	[d]	1590	-	-	-	-	-	-	-	-	-	0.3	-	-	GC-MS
Espatulenol	1593	1593	0.9	0.9	-	-	-	-	0.5	0.2	6.0	7.2	1.2	2.5	GC-MS, RI
Isoaromadendrene epoxide	1597	1594	-	-	-	-	3.1	3.0	0.4	0.7	-	-	-	-	GC-MS, RI
Viridiflorol	1600	1592	2.0	0.3	-	-	-	-	-	-	3.0	4.2	-	-	GC-MS, RI
Salvial-4(14)-en-1-one	1610	1603	-	-	-	-	-	-	-	-	-	-	0.3	0.4	GC-MS, RI
Cedrenol	1620	1642	-	-	-	-	-	-	-	-	-	-	0.2	-	GC-MS, RI

Component	RI		Plant species/Relative content [%]												Identification[c]
	Obs[a]	Pub[b]	*Ab*		*Ac*		*Ae*		*Cmi*		*Cme*		*Pq*		
			5	**17**	**5**	**17**	**5**	**17**	**5**	**17**	**5**	**17**	**5**	**17**	
Longifolenaldehyde	1626	[e]	-	-	-	-	-	-	-	1.3	0.6	-	1.3	0.5	GC-MS, RI
Humulene-1,2-epoxide	1626	1608	-	-	1.5	1.7	3.8	4.2	1.3	1.7	-	0.5	-	0.7	GC-MS, RI
Selina-6-en-4-ol	1629	[e]	-	-	-	-	-	-	-	-	-	-	0.7	1.1	GC-MS, RI
γ-Eudesmol	1637	[e]	7.8	4.6	-	-	-	-	-	-	-	-	-	-	GC-MS, RI
epi-α-Muurolol	1656	1642	-	-	-	-	-	-	-	0.2	0.4	0.4	-	0.3	GC-MS, RI
Cubenol	1643	1646	-	-	-	-	-	-	0.4	tr	0.3	-	0.3	-	GC-MS, RI
Caryophylladienol	1649	1640	-	-	-	-	-	-	0.5	-	-	-	-	-	GC-MS, RI
Isospathulenol	1653	1653	-	-	-	-	-	-	-	-	1.0	1.0	-	-	GC-MS, RI
α-Eudesmol	1666	1653	6.7	7.4	-	-	1.0	-	-	-	-	-	-	-	GC-MS, RI
α-Cadinol	1670	1654	-	-	-	-	-	-	0.9	1.4	1.0	1.4	-	1.5	GC-MS, RI
β-Eudesmol	1671	1653	11.3	11.0	-	-	-	1.1	-	-	-	-	-	-	GC-MS, RI, Std
(-)-Isolongifolol methyl ether	1672	1672	-	-	-	-	4.2	3.8	-	-	-	-	-	-	GC-MS, RI
Valerianol	[d]	1658	-	2.3	-	-	-	-	-	-	-	-	-	-	GC-MS
Neointermedeol	1673	1662	-	0.29	-	-	-	0.1	2.5	0.6	-	-	8.4	5.2	GC-MS, RI
Intermedeol	1676	1666	-	-	-	-	0.6	0.6	1.5	-	-	-	-	-	GC-MS, RI
β-Costal	1683	1691	-	-	-	-	-	-	-	-	-	-	0.7	4.3	GC-MS, RI
Aromadendrene epoxide	1686	1641	-	-	-	-	-	-	1.3	1.1	-	-	-	-	GC-MS, RI
α-Santalol	1686	1675	0.3	-	-	-	-	-	-	-	-	-	-	-	GC-MS, RI
Cadalene	1690	1676	-	-	-	-	-	-	0.4	-	-	-	-	-	GC-MS, RI
Cedr-8-en-13-ol	1692	1688	-	-	tr	-	-	-	0.5	-	-	-	-	-	GC-MS, RI
β-Nootkatol	1704	1715	-	-	-	-	0.3	0.1	-	-	-	-	-	-	GC-MS, RI
Muurol-5-en-4-one	1706	1689	-	-	-	-	-	-	0.6	1.1	-	-	-	-	GC-MS, RI
Aristol-1(10)-en-9-ol	[d]	1704	-	-	-	-	-	-	-	1.0	-	-	-	-	GC-MS
Juniper camphor	[d]	1709	-	-	-	-	-	-	-	1.3	-	-	-	-	GC-MS
Widdrenal	1724	1724	-	-	-	-	-	-	-	-	0.4	0.3	-	-	GC-MS, RI
Guaiol acetate	[d]	1726	-	-	-	-	-	-	-	-	-	0.2	-	-	GC-MS
Valerenal	[d]	1727	-	-	-	-	-	-	-	0.2	-	-	-	-	GC-MS
Vetiselinenol	1735	1731	-	-	-	-	-	-	-	-	0.3	0.3	-	-	GC-MS, RI
γ-Costol	1735	1746	-	-	-	-	-	-	-	-	-	-	0.4	1.4	GC-MS, RI
Valerenol	1738	1736	-	-	-	-	-	-	-	-	0.4	-	2.4	-	GC-MS, RI

Component	RI		Plant species/Relative content [%]												Identification[c]
	Obs[a]	Pub[b]	*Ab*		*Ac*		*Ae*		*Cmi*		*Cme*		*Pq*		
			5	**17**	**5**	**17**	**5**	**17**	**5**	**17**	**5**	**17**	**5**	**17**	
Oplopanone	1754	1745	-	-	-	-	-	-	-	-	0.1	-	-	-	GC-MS, RI
α-Vetivol	1762	1756	-	-	-	-	-	-	-	-	-	-	1.8	-	GC-MS, RI
Aristolone	1767	1763	-	-	-	-	0.7	-	-	-	-	-	0.3	-	GC-MS, RI
β-Costol	1784	1767	-	-	-	-	-	-	-	-	-	-	2.4	0.7	GC-MS, RI
β-Cyperone	1791	1759	-	-	-	-	1.3	1.5	-	1.8	-	-	-	0.5	GC-MS, RI
α-Costol	[d]	1774	-	-	-	-	-	-	-	-	-	0.7	2.3	-	GC-MS, RI
Methyl isocostate	1797	1792	-	-	-	-	-	-	-	-	-	-	0.1	0.7	GC-MS, RI
Saussurea lactone	1798	1806	-	-	-	-	0.8	-	-	-	-	-	-	-	GC-MS, RI
Cryptomeridiol	[d]	1813	-	0.8	-	-	-	-	-	-	-	-	-	-	GC-MS
Nootkatone	1829	1820	-	-	-	-	1.8	2.3	-	0.8	-	-	-	-	GC-MS, RI
Neophytadiene	1848	1840	0.4	-	-	-	-	-	-	-	-	-	0.2	-	GC-MS, RI
Hexahydrofarnesyl aceton	1855	1850	1.3	-	0.8	0.9	0.2	-	-	-	-	-	0.4	-	GC-MS, RI
Kessanyl acetate	[d]	1878	-	-	-	-	-	0.7	-	-	-	-	-	-	GC-MS
Platambin	1881	1867	-	-	-	-	-	-	0.6	0.5	-	-	-	-	GC-MS, RI
(8S,14)-Cedrandiol	1909	1889	-	-	-	-	-	-	-	-	-	-	0.2	0.3	GC-MS, RI
Farnesyl acetone	1929	1924	-	-	0.5	0.5	-	-	-	-	-	-	-	-	GC-MS, RI
Carissone	1951	1936	-	-	-	-	0.3	-	-	-	-	-	-	-	GC-MS, RI
Columellarin	1982	1967	-	-	-	-	-	-	-	-	-	-	0.5	-	GC-MS, RI
Group sum [%]			62.3	62.3	20.2	20.5	58.8	58.39	91.2	90.7	22.1	23.5	65.1	58.6	
Higher isoprenoids															
Phytol	2110	2111	0.3	1.4	-	-	-	-	-	-	-	-	0.6	0.8	GC-MS, RI
Group sum [%]			0.3	1.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.6	0.8	
Others															
6,6-Dimethyl-2-methylenebicyclo[3.2.0]heptan-3-ol	-	1157	-	-	0.6	0.4	-	-	-	-	-	-	-	-	GC-MS, RI
4-Hydroxy-6-isopropyl-3-methyl-2-cyclohexen-1-one	1425	1432	-	-	-	-	-	-	-	-	0.5	-	-	-	GC-MS, RI
Pinonic acid	[d]	1442	-	-	-	-	-	0.2	-	-	-	-	-	-	GC-MS

Component	RI		Plant species/Relative content [%]												Identification[c]
	Obs[a]	Pub[b]	*Ab*		*Ac*		*Ae*		*Cmi*		*Cme*		*Pq*		
			5	17	5	17	5	17	5	17	5	17	5	17	
2-Isopropyl-4α,8-dimethyl-1,2,3,4,4α,5,6,7-octahydronaphthalene	1496	1492	-	-	-	-	0.9	-	-	-	-	-	1.9	1.5	GC-MS, RI
7-Isopropyl-4,10-dimethylenecyclodec-5-enol	1685	[e]	-	-	1.1	-	-	-	-	-	-	-	-	-	GC-MS, RI
6-Isopropenyl-4,8a-dimethyl-1,2,3,5,6,7,8,8a-octahydro-naphthalen-2-ol	1686	[e]	-	-	-	-	-	-	-	-	0.5	0.1	1.6	1.9	GC-MS, RI
(1R,7S,E)-7-Isopropyl-4,10-dimethylenecyclodec-5-enol	1701	1695	-	0.5	0.9	2.0	2.3	2.3	0.8	1.5	0.3	0.9	2.2	4.5	GC-MS, RI
1-Isopropyl-4,8-dimethylspiro[4.5]dec-8-en-7-one	1707	1700	-	-	1.0	-	-	-	-	-	-	-	-	-	GC-MS, RI
Cyclododecanol	[d]	[e]	-	-	-	-	-	-	-	-	-	0.2	-	-	GC-MS
Group sum [%]			0.0	0.5	3.6	2.4	3.2	2.5	0.8	1.5	1.3	1.2	5.7	7.9	
Total identified			**95.4**	**93.7**	**90.5**	**90.4**	**91.2**	**90.0**	**92.3**	**92.5**	**93.9**	**90.4**	**92.8**	**90.0**	

a) Retention indices calculated from retention times on a HP-5MS column and based on C9–C29 alkanes. **b)** Data taken from Adams [17] and NIST [34]. **c)** Identification method: GC-MS = Mass spectrum was identical to that of National Institute of Standards and Technology Library (ver. 2.0.f), RI = the retention index was consistent with that of the literature database, Std = constituent identity confirmed by co-injection of authentic standards. **d)** Retention indices were not calculated for compounds identified only by HP-17MS column. **e)** Literature data not available. **f)** Compound not detected in the sample. **g)** tr = trace (<0.05). Ab – *Alpinia brevilabris*, Ac – *Alpinia cumingii*, Ae – *Alpinia elegans*, Cmi – *Callicarpa micrantha*, Cme – *Cinnamomum mercadoi*, Pq – *Piper quinqueangulatum*, 5 – column HP-5MS, 17 – column DB-17MS

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8 Conclusions

The current study reports development of an effective proof-of-concept approach applicable in the development of new preparations for the inhalation therapy of respiratory diseases, which overcomes some specific disadvantages of conventional standardised methods commonly used for evaluation of antimicrobial potential and cytotoxic effect of plant volatiles. This concept is based on *in vitro* testing of growth-inhibitory effect of plant volatile compounds and EOs against pneumonia causing bacteria and evaluation their toxicity to human lung cells using combination of novel broth microdilution volatilization method and modified MTT cytotoxicity assay, followed by analysis of chemical composition of antibacterially effective EOs using dual column/dual detector GC-MS system. In the first experiment (Chapter 5), the assessment of plant-derived volatile compounds verified the appropriateness of both methods. Two following experiments (Chapters 6 and 7) confirmed suitability of newly developed approach for antibacterial susceptibility testing, safety evaluation and chemical analysis of EOs.

Newly developed broth microdilution volatilization method is the key achievement of the study. According to our results, this assay is suitable for simple and rapid simultaneous determination of *in vitro* growth-inhibitory effect of plant volatile compounds and EOs in the liquid and the vapour phase at different concentrations. Cost and labour effectiveness are the main benefits of the method that allows high-throughput screening of volatile agents without the need of special apparatus. However, the MIC values of antibacterial potential of plant volatiles in vapour phase are only indicative, because exact concentrations of evaporated antibacterial agents that influence bacterial growth on agar medium are not detected in this assay. If the distribution of volatiles is uniform in liquid and gaseous phase, the concentrations can be expressed as weight of volatile agent per volume unit of a well, whereas their real values will be 256, 128, 64, 32, 16, 8, 4 and 2 $\mu g/cm^3$ for 1,024; 512; 256; 128; 64; 32; 16 and 8 µg/mL, respectively. Limited condition for bacterial growth in small volume of agar applied on the lid and necessary use of clamps for fastening plate and lid together are other weakness of this method. Since our new broth microdilution volatilization method is performed using serially produced microplates that are not designed for this purpose, development of microplate lid-based device for antimicrobial testing in vapour phase could overcome these difficulties. In addition, the broth microdilution method was accompanied by

modified MTT assay using EVA capmat^{TM} as vapour barrier cover with aim to preliminary assess the inhalation safety of tested volatiles to the human lung cells on microtiter plates and to evaluate their suitability for application in the inhalation treatment.

According to the results of growth-inhibitory effects of six plant derived volatile compounds (carvacrol, cinnamaldehyde, eugenol, 8- hydroxyquinoline, thymol, and thymoquinone), each volatile produced certain level of antibacterial activity against standard strains of *H. influenzae, S. aureus*, and *S. pneumoniae* (Chapter 5). 8-hydroxyquinoline and thymoquinone were found as the most active antibacterial agents, nevertheless they were evaluated as toxic towards human lung cells lines. As mentioned in chapters 6 and 7, EOs isolated from various parts of seven Cambodian (*Alpinia oxymitra*, *Boesenbergia rotunda*, *Cinnamomum cambodianum*, *Citrus lucida*, *Limnophila aromatica*, *Rhodamnia dumetorum*, and *Sindora siamensis*) and six Philippine (*Alpinia brevilabris*, *Alpinia cumingii*, *Alpinia elegans*, *Callicarpa micrantha*, *Cinnamomum mercadoi*, and *Piper quinqueangulatum*) plant species were tested for their antibacterial and cytotoxic effect. All samples exhibited a certain degree of antibacterial activity against at least one bacteria associated with respiratory infections (*H. influenzae, S. aureus*, and *S. pneumoniae*). Nevertheless, only *A. oxymitra* rhizomes EO inhibited growth of *S. pneumoniae*. Since this bacterium is one of the main pneumonia causing pathogens, a systemic high-throughput screening of herbal volatiles and their constituents is necessary to find effective sources of antibacterial agents, as well as assessment of combinatory antibacterial effect of volatile compounds could be beneficial approach [100,101].

With aim to compare amount of effective antibacterial agent to the concentration causing toxicity therapeutic indices were calculated. Considering the cytotoxic level of volatile compounds and EOs tested, only *A. oxymitra* pericarp EO was classified as non-toxic to the human lung cell lines and at the same time, effective against *H. influenzae* in liquid and solid medium. However in case of other EOs and volatile compounds, their specific modifications such as nanoencapsulation or preparation of synthetic derivatives of plant volatiles can solve problem with toxicity of antibacterial agents during their delivery to the site of infection in the respiratory system [102]. Moreover, further research

focused on *in vivo* testing is needed as different results of cytotoxicity may be obtained in such conditions.

The chemical composition of EOs isolated from Cambodian and Philippine plant species was determined using GC-MS equipped by dual column/dual detector system that provides complementary information and higher quality identification of detected components. Flame ionization detector is effective especially for quantification, while mass spectrometer produces data useful for chemical-structure elucidation and compound qualification [103]. The identification and quantification of plant EOs can be difficult when they are composed of substances showing similar chromatographic retention behaviour [104]. All samples were separated on two capillary columns of different polarity (non-polar HP-5MS and more polar DB-17MS) to overcome these limitations because this approach increase the resolution of the analysis leading to the more complex volatile sample separation and higher identification probability. Nevertheless, selection of more different stationary phases in the terms of their polarity can be more effective approach for separation and detection of broader spectrum of compounds [105]. Moreover, application of solid-phase microextraction has a great efficiency in EOs' composition analysis whose quantification principle is based on timed accumulation of evaporated analytes in the coating; therefore, this technique allows to determine components responsible for antibacterial effect in vapour phase.

In our opinion, the future practical applications of proof-of concept approach consisting of broth microdilution volatilization and modified MTT methods accompanied by dual column/dual detector GC analysis, lies not only in development of pharmaceutical preparations, but it could also be applicable for development of disinfection and sterilization agents in healthcare facilities, in agriculture for exploring possibilities of protection of stored agricultural product (e.g. controlled-atmosphere storage), and in food industry for preservation and shelf-life extension of food products. However further optimization and modification of this assay is necessary for other possibilities of its use.

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10 Appendices

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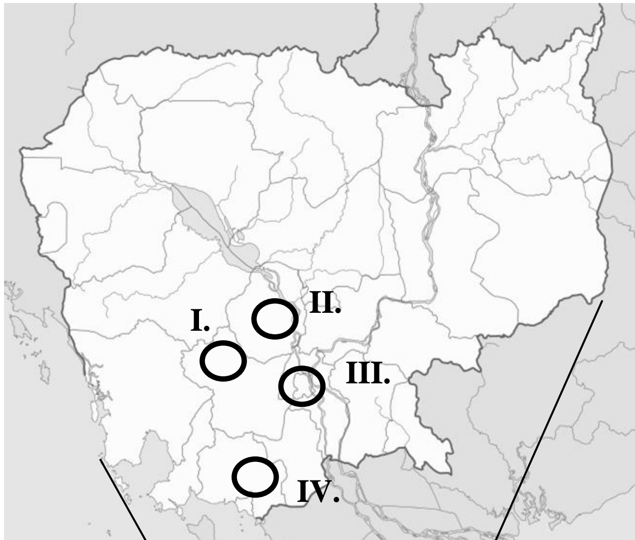
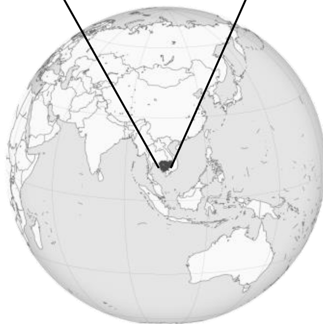
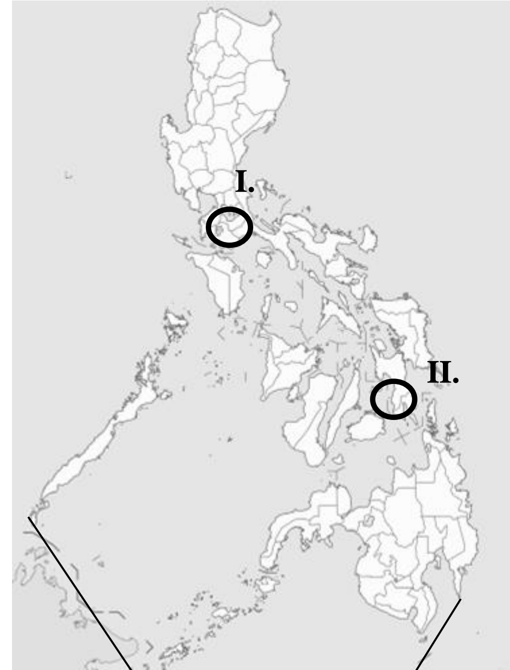
Appendix 1: Collection sites of tested plant material

(A) Cambodia





(B) Philippines



I. Mt Aoral

II. Oudong

III. Phnom Penh - Psar Thmei, Chbar Ampov, Cham Kar Dong

IV. Angkor Chey

I. Mt Makiling, Plant nursery UPLB

II. Mt Pangasugan, Plant nursery VSU

Wikipedia Commons, 2015

Appendix 2: Plant species photographic illustrations

Cambodian plant species

Alpinia oxymitra - a) leaves with fruits, b) fruit, c) pericarp and seeds (Kokoska, 2016)

Boesenbergia rotunda - rhizomes (Houdkova, 2016)

Cinnamomum cambodianum – trunk with young twigs

(Novy, 2016)

Limnophila aromatica – a) aerial part, b) leaves (Houdkova, 2016)

Citrus lucida - fruit (Houdkova, 2016)

Rhodamnia dumetorum – inflorescence and fruits (Le, 2012)

Sindora siamensis – a) fruits, b) dry pericarp (Novy, 2016)

Philippine plant species

Alpinia brevilabris – a) inflorescence, b) leaves (Houdkova, 2017)

Alpinia cumingii – a) inflorescence, b) infrutescence, c) leaves (Houdkova, 2017)

Alpinia elegans – a) infrutescence, b) fruits and flower (Houdkova, 2017)

Callicarpa micrantha – a) leaves and infrutescence, b) infrutescence, c) inflorescence

(Houdkova, 2017)

Cinnamomum mercadoi – a) young branches, b) leaf (Houdkova, 2017)

Piper quinqueangulatum – a) vine, b) leaf (Houdkova, 2017)

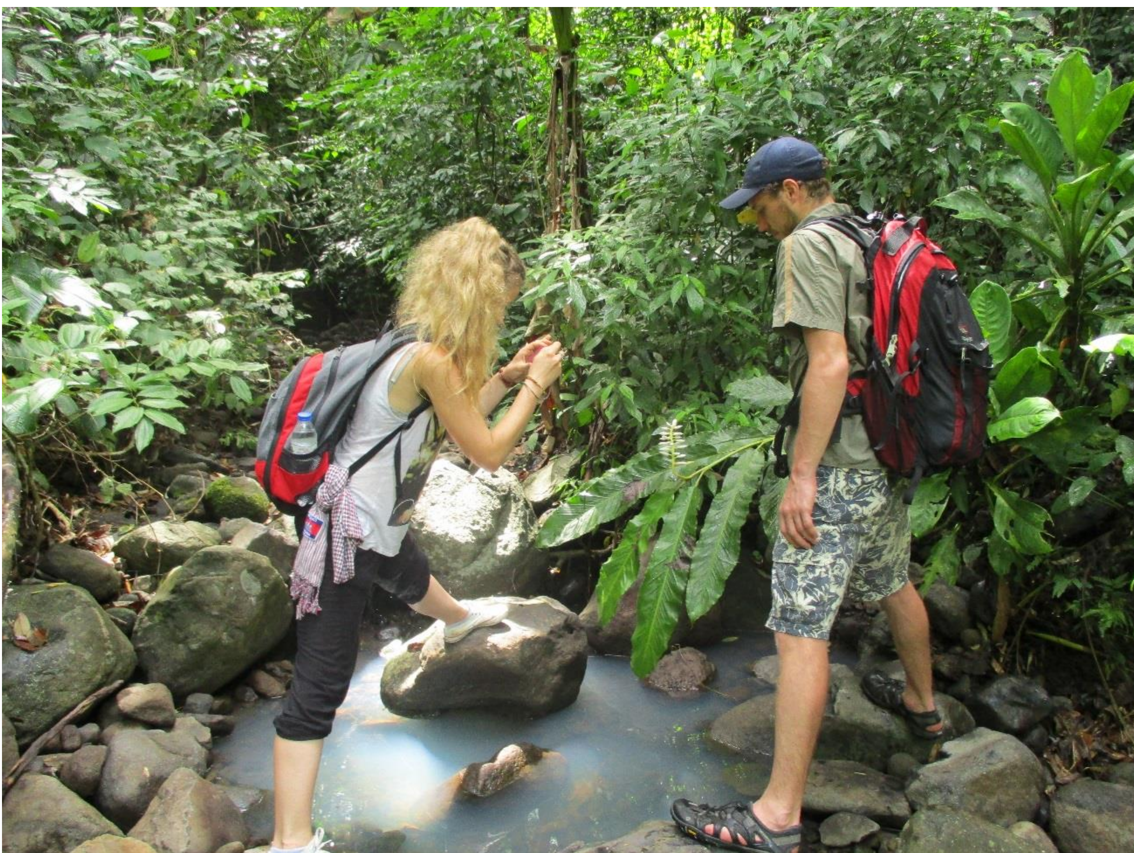
Appendix 3: Collecting plant material

Cardamom Mountains, Cambodia (Novy, 2016)

Mount Aoral, Cambodia (Novy, 2016)

(Houdkova, 2016)

Local herbal market, Cambodia (Novy, 2016) (Houdkova, 2016)



Mount Pangasugan, Philippines (Rondevaldova, 2017)

Appendix 4: *Curriculum vitae*

PERSONALIA

Name	**Ing. Markéta Houdková**
Address	Sruby 34, 565 44
Mobile phone number	+420 721 564 763
Office phone number	+420 22438 3413
E-mail 1	houdkovam@ftz.czu.cz
Date of birth	30 July 1990
Nationality	Czech
Marital status	Single

EDUCATION

2015 – 2018 **Doctoral Study**

Czech University of Life Sciences Prague
Faculty of Tropical AgriSciences
Study Programme: Tropical and Subtropical Agriculture
Thesis: Vapours of essential oils and their constituents as *in vitro* inhibitors of pneumonia causing microorganisms

2013 – 2015 **Master's degree**

Czech University of Life Sciences Prague
Faculty of Agrobiology, Food and Natural Resources
Study Programme: Nutrition and Foodstuffs
Thesis: Possibilities of goat's milk utilization in food processing (graduated with distinction)

2009 – 2013 **Bachelor's degree**

Czech University of Life Sciences Prague
Faculty of Agrobiology, Food and Natural Resources
Study Programme: Nutrition and Foodstuffs
Thesis: Goat milk utilization in food processing

WORK EXPERIENCE

02/2018 – Present **Laboratory technician**

Laboratory of Ethnobotany and Ethnopharmacology, Faculty of Tropical AgriSciences, Czech University of Life Sciences Prague

	- antibacterial activity testing, GC-MS analysis
04/2012 – 12/2012	**Article processing in the editorial system “Agronavigator”**
	Institute of Agricultural Economics and Information, Prague
	- writing abstracts of scientific articles focused on foodstuffs, food processing, and food safety on web portal “Agronavigator.cz”

ABROAD EXPERIENCES

07/2018	**PhD Summer Science Camp in Indonesia**
	USU Medan; HKBP Medan, MATANA University in Jakarta, Atma Jaya Catholic University in Jakarta, SWCU Salatiga
	- research presentation “Vapours of essential oils and their constituents as *in vitro* inhibitors of pneumonia causing bacteria”
04/2017 – 06/2017	**Student mobility in Philippines**
	Visayas State University, Baybay
	- plant sample collecting, essential oils distillation
07/2016 – 08/2016	**Student mobility in Cambodia**
	Royal University of Agriculture, Phnom Penh
	- plant sample collecting, essential oils distillation, lectures focused on essential oils

PROJECT PARTICIPATION

2018	Influence of different technological processing methods on important antioxidants in colour genotypes of wheat (CIGA 20182004)
2018	Chemical composition and biological activity of medicinal and edible tropical plants (IGA 20185019)
2017	Advanced methods for *in vitro* evaluation of antimicrobial activity of plant compounds, extracts and essential oils (CIGA 20175001)
2017	Biologically active compounds in medicinal and edible tropical plants (IGA 20175020)
2016	Tropical plants as source of biologically active and nutritionally important compounds (IGA 20165009)

LANGUAGE SKILLS

Czech:	mother tongue
English:	proficient user
French:	Basic user

Appendix 5: List of author's publications

Publications in scientific journals:

Houdkova M, Rondevaldova J, Doskocil I, Kokoska L (2017) Evaluation of antibacterial potential and toxicity of plant volatile compounds using new broth microdilution volatilization method and modified MTT assay. *Fitoterapia* 118: 56-62. (IF 2.642)

Houdkova M, Urbanova K, Doskocil I, Rondevaldova J, Novy P, Nguon S, Chrun R, Kokoska L (2018). *In vitro* growth-inhibitory effect of Cambodian essential oils against pneumonia causing bacteria in liquid and vapour phase and their toxicity to lung fibroblasts. *South African Journal of Botany* 118: 85-97. (IF 1.442)

Houdkova M, Doskocil I, Urbanova K, Tulin EKCB, Rondevaldova J, Tulin AB, Kudera T, Tulin EE, Zeleny V, Kokoska L (2018) Evaluation of antipneumonic effect of Philippine essential oils using broth microdilution volatilization method and their lung fibroblasts toxicity. *Natural Product Communications* 13: 1-8. (IF 0.809)

Netopilova M, **Houdkova M**, Rondevaldova J, Kmet V, Kokoska L (2018) Evaluation of *in vitro* growth-inhibitory effect of carvacrol and thymol combination against *Staphylococcus aureus* in liquid and vapour phase using new broth microdilution volatilization chequerboard method. *Fitoterapia* 129: 185-190. (IF 2.642)

Scientific conference contributions:

Houdkova M, Krkonoskova A, Rondevaldova J, Kokoska L (2016) *In vitro* method for simultaneous determination of antibacterial potential of plant volatile compounds in liquid and vapour phase using microtiter plates. *Trends in Natural Product Research: a young scientists meeting of PSE and IUNG-PIB*, Pulawy, Poland, 30.5.-2.6. 2016. Book of Abstracts, poster P06, p. 83.

Houdkova M, Krkonoskova A, Rondevaldova J, Kokoska L (2016) *In vitro* method for simultaneous determination of antibacterial potential of plant volatile compounds in liquid and vapour phase using microtiter plates. *Regional Workshop: Promotion of neglected and underutilized indigenous crop species for food security and nutrition in Southeast Asia and the EU*, Battambang, Cambodia, 20.-22.7. 2016. Book of Abstracts, short lecture P11, p. 11.

Rondevaldova J, **Houdkova M**, Kokoska L (2017) Antimicrobial and Antioxidant activity of Cambodian neglected edible and medicinal plants. *1st International Conference on Food*, Environment and Culture, Baguio, Philippines, 15.-18.5. 2017. Book of Abstracts, poster P-22, p. 90.

Houdkova M, Rondevaldova J, Kokoska L (2017) New broth microdilution volatilization method for evaluation of antibacterial potential of plant volatile compounds. *48th International Symposium on Essential Oils*, Pecs, Hungary, 10.-13.9. 2017. Book of Abstracts, short lecture OP-04, p. 45.

Houdkova M, Rondevaldova J, Kokoska L (2018) Antibacterial effect of plant volatiles against *Pseudomonas aeruginosa* assessed by using broth microdilution volatilization method. *2nd Nommensen International Conference on Technology and Engineering*, Medan, Indonesia, 19.-20.7. 2018. Book of Abstracts, short lecture p. 42.