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Evaluation of *in vitro* growth-inhibitory interactions between essential oils and their volatile compounds against *Staphylococcus aureus* in vapour phase

DOCTORAL THESIS

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Declaration

I, Marie Straková, hereby declare that this thesis entitled "Evaluation of growth-inhibitory interactions between essential oils and their volatile compounds against *Staphylococcus aureus* in vapour phase" was written 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, December 15, 2021

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Ing. Marie Straková

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Abstract

Due to the quick acquisition of drug resistance, *Staphylococcus aureus* is one of the most serious human pathogens. Nowadays, this bacterium is resistant to almost all antibiotics used. One of the strategies for successful treatment of infections caused by resistant microorganisms is the use of drug combinations. With the aim to develop a proof-of-concept approach that could be used in development of novel preparations for inhalation therapy that would be based on interactions between plant volatile agents [essential oils (EOs) and their constituents], we designed a new broth volatilization chequerboard method for *in vitro* determination of antimicrobial combinatory potential of plant volatiles simultaneously in liquid and vapour phases. The method is based on combination of standard chequerboard and new broth microdilution volatilization test, allowing calculation of fractional inhibitory concentration (FIC) values. The practical usability and accuracy of the novel technique was verified on three different combinations of plant volatile agents: 1) two plant-derived volatile compounds (carvacrol and thymol), 2) compound (8-hydroxyquinoline) and EO (*Cinnamomum cassia* EO), and 3) two EOs (*Origanum vulgare* and *Thymus vulgaris* EOs), which were tested against standard strains and clinical isolates of *S. aureus*. Results of all three tested interactions showed to produce additive antimicrobial effects against all tested strains in both phases. In several cases, they reached ΣFIC values lower than 0.6, which can be considered as a strong additive interaction. The most effective interactions in the vapour phase were observed against standard strain of *S. aureus* ATCC 25923 (ΣFIC = 0.51) when tested carvacrol and thymol, against a clinical isolate (ΣFIC = 0.56), when tested 8-hydroxyquinoline and *C. cassia* interactions, and against standard strain ATCC 29213 (ΣFIC = 0.59) when tested *O. vulgare* and *T. vulgaris* EOs. Using dual-column/dual-detector system for gas chromatography/mass spectrometry analysis of EOs, (E)-cinnamaldehyde was identified as the main constituent of *C. cassia* bark, while volatile oils from *O. vulgare* and *T. vulgaris* aerial parts consisted predominantly of carvacrol and thymol, respectively. The results show validity of our new broth volatilization chequerboard method, which allows cost and labour effective high-throughput antimicrobial screening of interactions between volatile agents with no need of special apparatus. These results can be potentially applied in development of various pharmaceutical applications that are based on volatile antimicrobials and can be used through inhalation therapy against respiratory infections

caused by *S. aureus*. However, further research focusing on *in vivo* evaluation will be necessary before its possible practical use.

Key words: antimicrobial interactions; broth volatilization chequerboard method; chemical composition; fractional inhibitory concentration; GC/MS analysis; respiratory infections; synergy

Abstrakt

Staphylococcus aureus je díky rychlému získávání rezistence k lékům jedním z nejzávažnějších lidských patogenů. V současnosti je tato bakterie rezistentní vůči téměř všem používaným antibiotikům. Jednou ze strategií jak úspěšně léčit infekce způsobené rezistentními mikroorganismy jako je *S. aureus* je užívání kombinací léků. S cílem vyvinout efektivní koncept, který by mohl být použit při vývoji nových přípravků pro inhalační terapii založených na interakcích mezi těkavými látkami rostlin (silicemi a jejich jednotlivými složkami), byla vyvinuta nová volatilizační šachovnicová metoda umožňující stanovení *in vitro* antimikrobiálního kombinačního potenciálu těkavých rostlinných látek současně v kapalné a plynné fázi. Tato metoda umožňující výpočet hodnot frakční inhibiční koncentrace (FIK), je založena na kombinaci standardní šachovnicové a nové bujónové mikrodiluční volatilizační metody. Praktická použitelnost a přesnost nové techniky byla ověřena na třech různých kombinacích silic a těkavých rostlinných látek: 1) na dvou těkavých rostlinných látkách (karvakrolu a thymolu), 2) na rostlinné látce (8-hydroxychinolinu) a silici (*Cinnamomum cassia*), a 3) na dvou silicích (*Origanum vulgare* a *Thymus vulgaris*), které byly testovány proti standardním kmenům a klinickým izolátům *S. aureus*. Výsledky všech tří testovaných interakcí prokázaly aditivní antimikrobiální účinky proti všem testovaným kmenům v obou fázích. V několika případech dosáhly hodnot ΣFIK nižších než 0,6, což lze považovat za silnou aditivní interakci. Nejúčinnější interakce v plynné fázi byly pozorovány proti standardnímu kmenu *S. aureus* ATCC 25923 (ΣFIK = 0,51) při testování karvakrolu a thymolu, dále proti klinickému izolátu (ΣFIK = 0,56), při testování interakcí 8-hydroxychinolinu se silicí z *C. cassia* a proti standardnímu kmenu ATCC 29213 (ΣFIK = 0,59) při testování kombinace silic *O. vulgare* a *T. vulgaris*. Chemickou analýzou za použití plynového chromatografu vybaveného dvěma kolonami a dvěma detektory byl v silici získané z kůry *C. cassia* identifikován (E)-cinnamaldehyd jako hlavní složka, zatímco silice z nadzemních částí *O. vulgare* obsahovala především karvakrol a silice z *T. vulgaris* thymol. Získané výsledky ověřily účinnost nové volatilizační šachovnicové metody, která umožňuje rychlý a cenově dostupný antimikrobiální screening interakcí mezi těkavými látkami (silicemi a jejich složkami) bez speciálního vybavení. Tyto výsledky mohou být potenciálně použity při vývoji různých farmaceutických léčiv založených na těkavých mikrobiálních látkách a dále

mohou být použity při inhalační terapii proti respiračním infekcím způsobeným *S. aureus*. Pro možné praktické využití však bude nezbytné provést další výzkum zaměřený na *in vivo* hodnocení farmakologických účinků.

Klíčová slova: antimikrobiální interakce; bujónová volatilizační šachovnicová metoda; frakční inhibiční koncentrace; GC/MS analýza; chemické složení; respirační infekce; synergie

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List of the abbreviations used in the thesis

8-HQ	8-hydroxyquinoline
ATCC	American type culture collection
CAS	Chemical Abstracts Service
CCEO	*Cinnamomum cassia* essential oil
CF	Cystic fibrosis
CFU	Colony forming unit
CLSI	Clinical and Laboratory Standards Institute
DMSO	Dimethylsulfoxide
ECHA	European Chemicals Agency
EFSA	European Food Safety Authority
EMA	European Medicines Agency
EO	Essential oil
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FDA	Food and Drug Administration
FIC	Fractional inhibitory concentration
FID	Flame ionization detector
FIK	Frakční inhibiční koncentrace
GC/FID	Gas chromatography/flame ionization detector
GC/MS	Gas chromatography/mass spectrometry
GRAS	Generally recognized as safe
LC_{50}	Median lethal concentration
LD_{50}	Median lethal dose
MH	Mueller-Hinton
MIC	Minimum inhibitory concentration
MRSA	Methicillin-resistant *S. aureus*
MTT	Thiazolyl blue tetrazolium bromide
ND	Not determined
NIST	National Institute of Standards and Technology
RI	Retention index
RT	Retention time

SA	*Staphylococcus aureus*
Std	Standard
VOC	Volatile organic compound
WHO	World Health Organisation
Σ	Sum

1 Introduction

1.1 Problem statement

Staphylococcus aureus, a leading cause of bacterial infections worldwide, is one of the most adaptable bacterial human pathogens. Although it is often found in human skin flora and mucous membranes (nasal area) as a benign commensal, it can also cause various serious illnesses such as bacteraemia, endocarditis, osteomyelitis, or pneumonia (Reddy et al. 2017). Furthermore, this pathogen is frequently present in the upper and lower respiratory tract during various illnesses such as cystic fibrosis (Das et al. 2013), chronic allergic diseases including chronic rhinosinusitis and asthma (Bachert & Zhang 2012; Kim et al. 2018), chronic obstructive pulmonary disease (Narewski et al. 2015), or secondary pneumonia occurring during seasonal influenza outbreaks (Rice et al. 2012). Due to its potential for a quick acquisition of drug resistance, this bacterium has become an alarming global problem (French 2010) and its treatment remains challenging to cope with due to the emergence of multi-drug resistant strains such as methicillin-resistant *S. aureus* (MRSA) (Taylor & Unakal 2020).

Essential oils (EOs) and other plant products have traditionally been used in medicine as natural remedies to treat respiratory tract infections. The delivery of therapeutic vapours and aerosols of EOs through inhalation to treat e.g. pharyngitis, bronchitis, and sinusitis has been used for thousands of years in various cultures (Fabio 2007; Stein & Thiel 2017; Yurdasiper et al. 2018). Due to their antimicrobial effects and high volatility, EOs and their constituents are of great potential for the development of novel antimicrobial drugs used in inhalation therapy as they have a broad spectrum of chemical diversity and can easily reach the upper and lower parts of the respiratory tract (Horvath & Acs 2015). Since it is more difficult for bacteria to develop resistance to the multi-component mixtures than to single-ingredient conventional antibiotics, the use of EOs as well as the combination therapy action of antimicrobial agents can be an effective strategy to overcome the problem with increasing antibiotic resistance of microorganisms, including *S. aureus*. Nevertheless, due to specific physico-chemical properties of EOs and their volatile components such as hydrophobicity and high

volatility, conventional antimicrobial susceptibility testing assays face specific issues in the drug research and development process.

The microdilution chequerboard assay is a commonly used method for the evaluation of antimicrobial interactions between two antimicrobial agents (including EOs and their constituents) in liquid media. Several methods to determine growth-inhibitory interactions between EOs and their volatile compounds in the gaseous phase have also been invented; however, in contrast to the well-established assays for the testing of antimicrobial combinatory effects in liquid media, they have not been standardized. Most of the methods used to evaluate combinatory activities of plant volatile vapours are based on modifications of the standard disc volatilization assay, which is not an appropriate method for the determination of minimum inhibitory concentrations (MICs) and therefore not even for fractional inhibitory concentrations (FICs). Moreover, these methods are not designed for a high-throughput screening and lack automation. In addition, they require high consumption of material and labour, and some assays need special equipment, which might not be commonly available.

1.2 Aims and focus of the study

Therefore, the main objective of this study was to develop an *in vitro* assay suitable for the determination of combinatory antibacterial effect of plant-derived volatile agents in the vapour phase and subsequently investigate the antistaphylococcal potential of their combinations.

The specific aims of this study were:

1. To develop a new broth volatilization chequerboard method to determine the growth-inhibitory effect of plant-derived volatile compounds against *S. aureus*.
2. To optimise this method for the evaluation of antistaphylococcal effect of a combination of a plant-derived compound with an EO and a combination of two EOs.
3. To analyse the chemical composition of EOs using a dual column/dual detector GC/MS system.

1.3 Significance of the study and link between the articles

With the aim to develop an effective proof-of-concept approach to generate novel preparations for the inhalation therapy of the respiratory illnesses that would be based on interactions between plant-derived volatile agents (EOs and their constituents), a new *in vitro* screening assay, named broth volatilization chequerboard method, was designed by our team for a simple and rapid determination of the growth-inhibitory effect of interactions between two plant-derived volatile agents (EOs and their constituents) simultaneously in the liquid and the vapour phase (Chapter 3). The new method is based on a combination of a standard chequerboard (Hsieh et al. 1993) and a new broth microdilution volatilization test (Houdkova et al. 2017). Compared to disc volatilization tests, our new screening assay allows to determine the combinatory effect of plant volatiles simultaneously in the liquid and the gaseous phase and it can easily compare MIC and FIC values in both liquid and solid media. This method is also suitable to test a broad range of concentrations in one 96-well microtiter plate, so it greatly reduces the consumption of material, it is suitable for high-throughput screening and the plate preparation can be automatized using a robotic pipetting system.

With the goal to verify the suitability and to confirm the accuracy of the new broth volatilization chequerboard method (designed in Chapter 3), the examination of the *in vitro* growth inhibitory potential of the three following combinations of plant volatile agents has been performed. First, the antibacterial activity of a combination of two plant-derived volatile compounds (namely carvacrol and thymol) has been evaluated against 12 antibiotic-resistant and sensitive forms of standard strains and clinical isolates of *S. aureus* strains (Chapter 3). Thereafter, the antistaphylococcal interactions between one compound (8-hydroxyquinoline) and one EO (hydrodistilled from *Cinnamomum cassia* bark) have been tested for their *in vitro* growth-inhibitory effect against the same bacterial strains (Chapter 4) and finally, the combination of two EOs (hydrodistilled from *Origanum vulgare* and *Thymus vulgaris)* has been tested against the same strains (Chapter 5). The combinations of EOs and volatile compounds tested in this research have been selected based on the results of our preliminary antimicrobial combinatory screenings performed as several combinations of different plant components and/or EOs against *S. aureus* ATCC 29213 (selected volatile agents produced the lowest FIC values).

Additionally, the chemical composition of the tested EOs was analysed by a GC/MS dual-column/dual-detector system with the aim to identify their constituents effective against *S. aureus* (Chapters 4-5). Two detectors and two capillary columns of different polarities were used in the GC/MS analysis to avoid the overlapping of signal peaks observed in the chromatogram and to achieve the best possible resolution of the compounds.

Apart from the invention of the new volatilization chequerboard method suitable for antimicrobial combinatory testing in vapour phase, this study brings, to the best of our knowledge, first reports on antistaphylococcal additive interactions of thymol with carvacrol and *O. vulgare* with *T. vulgaris* EOs in the vapour phase and on additive effects of the combinations of 8-hydroxyquinoline and *C. cassia* EO in both phases.

2 Literature review

2.1 *Staphylococcus aureus*

S. aureus is an eminent, ubiquitous, gram-positive, facultatively anaerobic sphere-shaped bacterium that is in human populations both a benign commensal and a common pathogen. It is responsible for a variety of infections, ranging from food poisoning and superficial skin and soft tissue infections to potentially life-threatening infections such serious bloodstream infections (bacteraemia and sepsis), endocarditis, meningitis, osteomyelitis, toxic shock syndrome, or pneumonia (Reddy et al. 2017). Although the anterior nares (or *vestibulum nasi*) appear to be the main reservoir and the most frequent site of this opportunistic pathogen's asymptotic colonization, it is also naturally present in axilla, rectum, vagina, gastrointestinal tract and skin (Sakr et al. 2018). According to Shukla et al. (2010), this pathogen is a component of the upper respiratory flora of up to 30% of normal individuals. Such extensive (yet harmless) colonization is widely considered to be a predisposition of an invasive infection (Prince 2013). Compared to skin and soft tissue infections caused by *S. aureus*, lower respiratory infections are less frequent, however associated with high mortality (Klevens et al. 2007). Moreover, *S. aureus* is also an important cause of pneumonia which is an acute respiratory illness secondary to infection and inflammation of the lung parenchyma when the alveoli of one or both lungs are filled with pus and fluids (exudate), which interferes with gas exchange, limits oxygen intake and makes breathing painful (Belleza 2019). Historically, staphylococcal pneumonia was observed in young infants and was initially implicated as a serious and often fatal respiratory complication of influenza during the 1918 ‘Spanish flu’ pandemic (Papanicolaou 2013; Morgene et al. 2018). Nowadays, *S. aureus* is an important cause of this respiratory disease in both community-acquired as well as hospital-acquired infections (Tong et al. 2015). Community-acquired pneumonia refers to pneumonia acquired outside of hospitals or long-term care facilities. By contrast, hospital-acquired pneumonia refers to pneumonia that develops at least 48 hours after hospital admission (Patterson & Loebinger 2012). *S. aureus*, though a common community pathogen, is found twice as frequently in pneumonia in hospitalized patients. The clinical course of pneumonia is characterized by high fever, chills, fatigue, cough with purulent bloody sputum production, fast and difficult breathing, and pleuritic chest

pain (Farver & Zander 2009). *S. aureus* is also frequently involved in secondary pneumonia occurring during seasonal influenza outbreaks and its co-infection with viruses is associated with significantly higher morbidity and mortality (Rice et al. 2012). Moreover, this bacterial pathogen is one of the first as well as the most common microbes that due to the poor clearance of viscous airway secretions, colonize and subsequently infect patients with cystic fibrosis (CF) (Das et al. 2013). Staphylococcal infections of CF patients are difficult to treat and the consequences of chronic lung inflammation as a response to the persistent microorganism may lead to considerably reduced lung function with malignant effects for the patients (Ulrich et al. 1998). Furthermore, this pathogen is known to be associated with chronic allergic diseases including chronic rhinosinusitis and asthma (Bachert & Zhang 2012; Kim et al. 2018). The presence of this microorganism can also complicate medical treatment of the chronic obstructive pulmonary disease (Narewski et al. 2015).

The success of *S. aureus* in general as a respiratory pathogen, may be attributed to various factors: its ability to adapt to the environment of the respiratory tract, substantial metabolic capabilities; genetic flexibility, both the ability to acquire and to mutate specific genetic elements, and the unique ability to exploit the immune responses that are evoked (Parker & Prince 2012).

2.1.1 Epidemiology of respiratory diseases

Respiratory diseases are among the leading causes of death and disability in the world representing 5 out of the 30 most common causes of death. According to the World Health Organization, more than 1 billion people suffer from either chronic or acute respiratory conditions and 4 million people die prematurely every year due to a chronic respiratory disease with infants and young children being the most susceptible. About 65 million people suffer from a chronic obstructive pulmonary disease and 3 million die from it every year. About 334 million people suffer from asthma, the most common chronic childhood disease. Pneumonia kills millions of people every year and is also a leading cause of death among children under 5 years old (WHO 2017).

S. aureus, as mentioned previously, is associated with all of these respiratory diseases, and its co-infection with other microorganisms often aggravates the course of the disease. However, its epidemiology is complicated due to different patterns of carriage. About 20 % of individuals are considered “persistent carriers” and almost

always carry one type of strain. Around 60% of the population are considered "intermittent carriers", they harbour *S. aureus* intermittently, and the strains change with varying frequency. A further 20 % are called "non-carriers" and almost never carry *S. aureus* (Kluytmans et al. 1997; Hurley 2018). The persistent carriage of *S. aureus* is more common in children than in adults, including the nasopharyngeal carriage which was found to be 48% among healthy children in the USA (Rosenfeld et al. 2012) and 36% in the Netherlands (Bogaert et al. 2004), however many people change their pattern of carriage between the age of 10 and 20 years (Kluytmans et al. 1997).

Out of the many staphylococcal infections and toxinoses, pneumonia is among the most prominent accounting for an estimated 50,000 staphylococcal infections per year in the United States only (Ragle et al. 2010). Moreover, hospital-acquired pneumonia mediated by *S. aureus* has been, despite the "appropriate" antimicrobial treatment, associated with significantly higher mortality (up to 37 %); much more than other nosocomial cases of pneumonia (Haque et al. 2012) and recent clinical observations have documented that mortality from pneumonia caused by methicillin-resistant *S. aureus* (MRSA) strains can exceed 50 % (Athanassa 2008). Also, the fulminant nature of the staphylococcal coinfection with influenza increases mortality rates that approach or even exceed 50 %, which also highlights an apparent synergy of these pathogens in the lung environment (Ragle et al. 2010). The prevalence and incidence of *S. aureus* infections in children with CF vary considerably over time as well as by country: In the USA, staphylococcal infections among CF patients have risen over time from 30 % in 1990 to 60 % in 2016 (CFFPR 2016; Hurley 2018) and over 25 % of CF individuals in the USA are nowadays found to have MRSA in respiratory culture specimens (Jennings et al. 2017). By contrast, data for the UK over the same time period appears to show the opposite, e.i. a decreasing trend (Hurley 2018). Moreover, according to The European Cystic Fibrosis Society Patient Registry (2017), the proportion of patients with a chronic *S. aureus* infection also varies by country, starting as low as 15 % in the UK and reaching up to 82 % in the Republic of Moldova (ECFSPR Annual Report 2017). In adults with CF, *S. aureus* infection rates also appear to be reduced with increasing age through adulthood (CFFPR, 2016).

2.1.2 Treatment

2.1.2.1 Systemic antibiotics and their combinations

The treatment of *S. aureus* has become increasingly problematic due to its potential to rapidly acquire drug resistance making it one of the most serious pathogens in the human population (Farver & Zander 2009; French 2010). Penicillin-resistant strains of *S. aureus* emerged shortly after the introduction of the antibiotic in the early 1940s (Rammelkamp & Maxon 1942; Lowy 2003) and nowadays 90-95 % of *S. aureus* strains are already penicillin-resistant (Sakoulas & Moellering 2008). The first semi-synthetic antistaphylococcal penicillins were developed around 1960 and methicillin-resistant *S. aureus* (MRSA) strains were observed within 1 year of their first clinical use (Turner et al. 2019). Currently, the global spread of MRSA is one of the most serious public health challenges worldwide, because apart from *β*-lactam antibiotics, MRSA strains have emerged with a concomitant resistance to other groups of antibiotics such as aminoglycosides, fluoroquinolones, glycopeptides, macrolides, and tetracyclines (Akpaka et al. 2017). Nevertheless, despite the emergence of resistant and multidrug-resistant *S. aureus* strains, there are several effective drugs in clinical use for which little resistance has been observed (Anstead et al. 2014). Vancomycin has been in clinical use for several decades and although its drawbacks have already been well described, it still remains the principal agent of choice in the treatment of MRSA (Davis et al. 2015; Bal et al. 2017). It is often combined with a second antibiotic, most often rifampin or gentamicin, for the treatment of serious MRSA infections (Deresinski 2009). Daptomycin and linezolid have also been used extensively during the last 10 years (Bal et al. 2017). New lipoglycopeptides (oritavancin, dalbavancin and telavancin), oxazolidinones (tedizolid) and third-generation cephalosporins (ceftaroline and ceftobiprole), have also shown good *in vitro* potency and *in vivo* efficacy in the treatment of MRSA, and have been approved by regulatory agencies since 2009 (Purrello et al. 2016, Bal et al. 2017). Many others such as the newer fluoroquinolones, oxazolidinones, and tetracyclines are in various stages of development. Furthermore, various combinations of antibiotic agents such as amoxicillin/clavulanic acid combination (=Co-amoxiclav, Augmentin) (EMA 2009; SUKL 2011), quinupristin/dalfopristin (=Synercid) (Manzella 2001), and trimethoprim/sulfamethoxazole (=TMP-SMX, Co-trimoxazole) (Cadena et al. 2011) have already been used as well to treat staphylococcal infections. Other drugs consisting

of combinations of two antibiotic agents, e.g. vancomycin and linezolid, vancomycin and daptomycin, daptomycin and linezolid, vancomycin and tetracyclines including tigecycline etc., have been tested against MRSA with the hope to obtain synergy which would address the shortcomings of vancomycin, however, with limited results so far. Surprisingly, although MRSA is by definition inherently resistant to nearly all β-lactam antibiotics, this class of drugs has consistently shown evidence of synergy with either daptomycin or vancomycin (Davis et al. 2015) and nowadays, an ongoing multicentre trial is investigating the role of the combination of vancomycin or daptomycin with β-lactam antibiotics (Bal et al. 2017). However, the use of some of these drugs (e.g. linezolid) to treat staphylococcal pneumonia and respiratory tract diseases in general remains debatable (Anstead et al. 2014) and e.g. daptomycin should not be used to treat pneumonia at all, as it is rendered inactive by pulmonary surfactant (Purrello et al. 2016).

In addition to the problems with staphylococcal resistance to antibiotics, lower respiratory tract infections are also difficult to treat due to the sequestration of microorganisms deep within the airways, where only limited portions of drugs gain access after a traditional systemic treatment (Wenzler et al. 2016). The only therapy available to treat *S. aureus* pneumonia are antibiotics which can, given an early recognition and prompt treatment of the patient, reduce the high mortality rate. Currently administered agents for the treatment of staphylococcal pneumonia (either community-acquired or healthcare-associated) are vancomycin or linezolid (Anstead et al. 2014; Purrello et al. 2016) and clindamycin (Kashyap et al. 2019), however, potential limitations of vancomycin for the treatment of *S. aureus* pneumonia have also been observed (Kollef & Micek 2005). Telavancin is indicated in Europe for nosocomial pneumonia only when the infection is known or believed to be caused by MRSA and when other treatments are not suitable; in particular it is attractive as an alternative to vancomycin in cases when MRSA pneumonia is difficult-to-treat (Purrello et al. 2016). Tigecycline is approved by the Food and Drug Administration agency for the treatment of community-acquired pneumonia. For CF, no current recommendations, or guidelines specific for MRSA in CF are found (Goss & Muhlebach 2011). Until the early 1990s, when MRSA was uncommon, attempts to treat and eradicate *S. aureus* were mostly based on a combination of two antibiotics: a semi-synthetic β-lactamase-resistant drug (dicloxacillin or flucloxacillin), rifampicin and fusidic acid (Esposito et al. 2019). Nowadays, antibiotics used to treat MRSA in CF patients are clindamycin, doxycycline, gentamycin, levofloxacin, linezolid,

rifampin, teicoplanin, tigecycline, TMP-SMX, tobramycin, vancomycin and others (Goss & Muhlebach 2011).

Nevertheless, it is well understood that effective antimicrobial therapy requires drug concentrations at the target site of infection. To reach the deep airways in sufficient concentrations, toxic doses of drugs would often need to be given systemically (Ambrose et al. 2010; Le et al. 2010). There might also be a risk of the degradation of active components in the gastrointestinal tract. In theory, a combination therapy may be an alternative which may overcome some of the old drug limitations (poor tissue penetration, slow bacterial killing and emerging resistance) and yield more time for new drugs to be routinely administered (Purrello et al. 2016).

2.1.2.2 Inhalation therapy

Although inhalation therapy has been used to treat respiratory diseases for over 4,000 years (Yurdasiper et al. 2018), the aerosol delivery of conventional antibiotics was first reported in the 1940s (Kuhn, 2001; Quon et al. 2014). Currently, pulmonary drug delivery is a focus of an extensive research affecting the treatment of various diseases including asthma, chronic obstructive pulmonary disease as well as CF (Yurdasiper et al. 2018).

Since *S. aureus* is commonly found on respiratory tract mucosa, antibiotic inhalation could be one of its possible treatments. The delivery of antibiotics via the pulmonary route has countless advantages over the more traditional routes as the lung is targeted directly. Direct access of antibiotics to the infection site in the lung parenchyma via inhalation could overcome problems the systemically administered (oral or intravenous) antibiotics encounter such as poor penetration into the lung parenchyma and narrow therapeutic windows between efficacy and toxicity (Wenzler et al. 2016). Moreover, inhalation lung delivery prevents the degradation of active components in the gastrointestinal tract and first pass metabolism in the liver (Kuzmov & Minko 2015; Bonaccorso et al. 2019). Since the inhaled antimicrobial agents have the capability of directly targeting the airways, higher drug concentrations can be achieved at the site of infection without the systemic adverse effects observed with the use of parenteral or oral antibiotic agents (Maselli et al. 2017). In fact, pulmonary drug delivery has very insignificant side effects given that the rest of the body is not exposed to antibiotics (Yurdasiper et al. 2018). Additional advantages of inhalation include rapid drug

absorption via highly vascularized mucosa (Bonaccorso et al. 2019) and rapid clinical response (Labiris & Dolovich 2003). Moreover, the high local concentration of the inhaled antibiotic agent could prevent biofilm formation, hence preventing the emergence of drug resistant bacteria (Lee et al. 2013). Despite these promising advantages, systemic inhalation delivery of therapeutics is not widely used yet (Kuzmov & Minko 2015).

In modern inhalation therapy, there are four main device types that are capable of delivering the drugs to the lungs of the patient. The first devices resembling nebulisers were developed in the 1860s (Levy et al. 2019) and currently, three basic types have been developed among them: air-jet (also known as pneumatic), ultrasonic, and vibrating mesh (Mansour 2018). Other types of devices are pressurized metered dose inhalers (pMDIs) that were introduced in the 1950s, dry powder inhalers (DPIs) from 1980s and soft mist inhalers (SMIs) that were invented after the year 2000 (Levy et al. 2019).

Currently, there are only two (or three) antibiotics that have been approved by the US Food and Drug Administration and are commercially available in the United States: aztreonam, tobramycin solution, and tobramycin powder. Moreover, one antibiotic, the colistimethate (colistin) dry powder for inhalation, has been approved by the European Medicines Agency. All above mentioned drugs have been approved for the treatment of *Pseudomonas aeruginosa* in people with CF (Daniels et al. 2016), therefore there is currently no commercially available antibiotic to treat *S. aureus* via the inhalation route, even though various studies show a significant potential of several antibiotics. For example, dry-powder vancomycin and combination of fosfomycin and tobramycin are currently in a late-stage development for supporting inhalation therapy and treatment of MRSA in CF patients (Quon et al. 2014; Curxpharmaceuticals 2020).

However, when delivering an antimicrobial agent to the lungs by an inhaler device, several issues with the pulmonary drug delivery system can occur. Numerous studies have shown that an incorrect inhalation device technique can compromise the delivery of the medication, increase the risk of exacerbations, result in higher health resource utilization, and even lead to premature mortality (Navaie et al. 2020). To reach the deep lung, particles are required to contain an optimal aerodynamic diameter (1–5 μm range), because if they are undersized, they will be exhaled and, on the other hand, if they are oversized, they affect the oropharynx and larynx (Yurdasiper et al. 2018). The deposition of aerosolized particles may also occur in other parts of the upper airways while the deposition of the medication in the lungs might be reduced due to patient-

specific respiratory tract physiology, especially in children and older people (Ibrahim et al. 2015). Moreover, the distribution of the antibiotic agent can be limited due to lung morphology and/or clearance mechanisms (alveolar and mucociliary macrophages) (Merchant et al. 2016). Furthermore, not all antibiotics are equally suited to inhalation (Nichols et al. 2019).

Due to the inappropriate use, overuse and misuse of antibiotics, increased antibiotic resistance, occurrence of side effects and falling eradication rates, there is an urgent need to search for new antimicrobial agents to combat bacteria such as *S. aureus*. Recently, the focus has been on medicinal plants, which are considered to be valuable sources of a broad spectrum of secondary metabolites possessing various biological activities that may be beneficial in therapeutic treatment and human health (Essawi and Srour 2000). Moreover, the use of more complex agents such as essential oils (EOs) and plant-derived volatile compounds as well as a combination therapy have proved to be generally effective strategies to overcome these problems.

2.2 Plant-derived volatiles

In addition to simple gases, such as oxygen, carbon dioxide and water vapour, plants synthesize and emit a high diversity of volatile chemicals, also known as essential oils (EOs), volatile oils, volatile organic compounds (VOCs), ethereal oils or essences, such as different terpenes, fatty acid derivatives, benzenoids, phenylpropanoids, and amino acid derived metabolites (Holopainen & Gershenzon 2010; Rosenkranz & Schnitzler 2016). These compounds, being crucial components of plants' phenotype and playing a dominant role in the ecology of plants, are the result of different plants responses, through the course of evolution, to their specific needs (Dicke & Loreto 2010). They have been involved in a broad number of ecological functions, as a consequence of the interactions between plants and biotic and abiotic factors. Plants use these volatiles to perform a variety of tasks such as attraction of pollinators, inter– and intra–organismic communication, defence against predators and insects, protection against certain environmental stressors, and for their thermo-tolerance. They are emitted by almost any kind of tissue and type of vegetation (grass, shrubs, trees, *etc*.) as aromatic compounds, green leaf volatiles and nitrogen-containing compounds (Vivaldo et al. 2017).

More than 100,000 chemical products are known to be produced by plants (Dicke & Loreto 2010) from which more than 1,700 floral volatile compounds from over 90 plant families have been isolated (Dudareva et al. 2006; Knudsen 2006; Fu et al. 2017). This remarkable accomplishment was made possible mainly by the rapid progress in gas chromatography mass spectrometry analyses (Baldwin 2010). The study of plant volatile compounds has long been restricted to floral volatiles, but studies on plant volatiles emitted from vegetative tissues have been emerging rapidly (Dicke & Loreto 2010). To date, more than 700 compounds have been reported as aroma or flavour in fruits and vegetables (Qualley & Dudareva 2009; Lubes & Goodarzi 2017) and according to Choudhary et al. (2017), there are more than 2,000 known volatiles in plants in total. The complex blend of these volatiles gives characteristic attributes to the whole plant, flower, or fruit (El Hadi et al. 2013; Lubes & Goodarzi 2017), because each volatile compound has a different smell, and the natural aroma or smell consists of hundreds of volatile compounds (Choudhary et al. 2017). Globally, plant volatiles constitute about 1 % of secondary plant metabolites (Dudareva et al. 2006; Dong et al. 2016).

Many of these plant volatiles have been used for centuries, perhaps since pre-Neolithic times, not only as flavouring agents and in manufacturing cosmetics and perfumes but also for their pharmaceutical properties (Figueiredo et al. 2008; Rosenkranz & Schnitzler 2016). Nowadays, EOs and plant-derived volatiles are commercially important especially for the pharmaceutical, agricultural, food, perfume, sanitary and cosmetic industries. EOs or some of their components are used in perfumes and make-up products, sanitary products, dentistry, agriculture, as food preservatives and additives, and as natural remedies. For example, carvone, geranyl acetate or limonene are used in perfumes, creams, soaps, make-up products, as fragrances and flavour additives, for oral and dental treatments, as household cleaning products and as industrial solvents (Ladan et al. 2011). Due to their antimicrobial and antioxidant properties, EOs and their individual volatile components (either extracted from plant material or synthetically manufactured) are widely used in food and food products, especially as food flavourings (Burt 2004; Figueiredo et al. 2008). They can also be used in the protection of crops, and against pests and plagues (Figueiredo et al. 2008). Moreover, EOs are used in massages and in aromatherapy (Ladan et al. 2011), including the inhalation and external application of the EOs (Bhavaniramya et al. 2019). EOs as total mixtures, or some of their components, can also be used for chemotaxonomic purposes (Figueiredo et al. 2008).

Currently, the greatest use of EOs in the European Union is in food (as flavourings), perfumes (fragrances and aftershaves) and pharmaceuticals (for their biological properties), whereas in aromatherapy, only a little more than 2% of the total market (Burt 2004).

2.2.1 Taxonomical distribution

Some plant volatile compounds are emitted from a wide range of plant species, whereas others are synthetized only by a specific plant taxon. For that reason, the composition of volatile emissions typically differs between plant species (Vivaldo et al. 2017; Conchou et al. 2019). For example, green leaf volatiles, C_6 molecules, playing an important role in plant defences, are very quickly produced and emitted by almost every green plant (Scala et al. 2013), whereas more specialized compounds, e.g. sulphur-containing glucosinolates are typically synthetized by Brassicales or furanocoumarins and their derivatives are produced by Apiales, Asterales, Fabales and Rosales (Agrawal 2011; Berenbaum & Zangerl 2008). There are about 3,000 EOs known in total, of which about 300 are commercially interesting and important especially for the flavour and fragrance markets (Van de Braak & Leijten 1999). These EOs are complex mixtures of concentrated aromatic volatile compounds derived from aromatic botanicals generally localized in temperate and warm regions like the Mediterranean and tropical countries. They belong to various genera of aromatic plants distributed over many various families such as Apiaceae, Asteraceae, Cupressaceae, Lamiaceae, Lauraceae, Myrtaceae, Poaceae, Pinaceae, Piperaceae, Rutaceae, and Zingiberaceae (Figueiredo et al. 2008; Raut & Karuppayil 2014). The production and emission of plant EOs and VOCs is not restricted to aboveground tissues (from which they are released into the atmosphere), but it also occurs belowground in their roots and rhizomes (from which they are released into the soil) (Dudareva et al. 2006; Dicke & Loreto 2010). Plant volatiles can be synthetized in all plant organs (bark, buds, flowers, fruits, leaves, roots, seeds, stems, twigs and wood) where they are stored in secretory cells, cavities, canals, epidermic cells or glandular trichomes (Solorzano-Santos & Miranda-Novales 2012). Plant volatiles, EOs as total mixtures, and secondary metabolites in general have been widely used for chemotaxonomic purposes and modern algorithms for data analyses confirm the narrow relationship between the volatile metabolome and plant taxonomy (Vivaldo et al. 2017; Conchou et al. 2019).

2.2.2 Chemistry

Plants produce an amazing variety of volatiles comprising a great diversity of chemical structures which have been intensively investigated in the last few decades. According to their biosynthetic origin and chemical structure, plant volatiles can be grouped into two main groups: terpene hydrocarbons and oxygenated VOCs. In a few cases, sulphur compounds and furanocoumarins and their derivatives are also found (Vivaldo et al. 2017). Oxygenated compounds include esters, aldehydes, ketones, alcohols, phenols, and oxides, whereas terpene hydrocarbons are composed of two or more components with 5-C base units known as isoprene units. Isoprene (C_5H_8) is the smallest and most emitted terpene by plants (Rosenkranz & Schnitzler 2016). It is the focus of considerable research currently because of its influence on the lower troposphere where it contributes to the formation of ozone (Materic et al. 2015). Volatile terpenes are generally present in high amounts in many plant EOs and are responsible for several characteristic plant odours such as those associated with citruses, mints, and conifers (Rosenkranz & Schnitzler 2016). Terpenes are usually divided according to the number of isoprene units present in the molecule. Monoterpenes ($C_{10}H_{16}$) that are composed of two isoprene-like molecules and sesquiterpenes ($C_{15}H_{24}$) with three isoprene units are the major classes of terpenes (Materic et al. 2015), although the isoprene chains may also include diterpenes ($C_{20}H_{32}$) (Blowman et al. 2018). Both acyclic and cyclic monoterpenes and sesquiterpenes are present in plants and often possess various functional groups that are responsible for higher volatility (Rosenkranz & Schnitzler 2016). In the composition of EOs, monoterpenes are the prevailing compounds as they are responsible for 90 % of EOs overall (Blowman et al. 2018; Bhavaniramya et al. 2019). The most significant factors influencing the monoterpene emission are a combination of the ambient temperature, size of the monoterpene pool in plant tissue, and vapour pressure (Tingey et al. 1980; Materic et al. 2015). Sesquiterpenes are, on the other hand, due to their high reactivity and low vapour pressure which make them difficult to analyse, among the least-studied groups of VOCs (Duhl et al. 2008). In general, terpenoid substances are among the most valuable compounds produced by plants, side by side with alkaloids and phenolic substances, some of which are also volatiles, such as phenylpropanoids (Figueiredo et al. 2008).

A wide range of volatile aliphatic and aromatic compounds containing alcohol, aldehyde, ketone, acid or ester functional groups can also be found in plants, however usually at lower concentrations than terpenes. Despite that, they are of great aesthetic and commercial importance due to their characteristic odours associated with fruits and flowers (Rosenkranz & Schnitzler 2016). Moreover, several unsaturated hydrocarbons, although they are present only in extremely low concentrations, affect the odour of many fruits and vegetables. Among many plant VOCs, C_6 and some other compounds are typical for plant leaves. The characteristic 'green' or 'grassy' smell of newly cut grass emitted when leaf tissue is physical damaged, is caused by several six-carbon aldehydes and alcohols (green leaf volatiles) (Materic et al. 2015; Rosenkranz & Schnitzler 2016). Plant volatiles also include phytohormones, ethylene, and jasmonate and methyl salicylate that, together with green leaf aldehydes and jasmonic acid, they act to induce systemic acquired resistance to pests and diseases at distant sites both within and between neighbouring plants (Rowan 2011).

EOs, isolated from various aromatic plants by hydrodistillation and steam distillation (citrus oils by cold pressing) and the mixtures of volatiles (= volatile oils, frequently also termed „EOs"), which can be obtained by other methods, such as solvent extraction, maceration, enfleurage, supercritical fluid extraction and others, are volatile lipophilic aromatic liquids that are slightly soluble in water and highly soluble in organic solvents (Figueiredo et al. 2008, Baser & Buchbauer 2010). Chemical profile of obtained EOs (and therefore also the odor) is closely related to the extraction procedure employed and, hence, the choice of an appropriate extraction method becomes crucial (Baser & Buchbauer 2010). For example, sensory evaluation of the volatile extracts of *Citrus maxima* revealed that EO obtained by cold pressing was characterized by green, fruit, herbaceous, pummelo-like odours, whereas the EO obtained by hydrodistillation was represented by the strong fermented/overripe and alcohol/pungent notes. Meanwhile, the microwave-assisted extraction and ultrasonic-assisted extraction volatile extracts had similar odour, dominated by green and pummelo-like notes; and the supercritical CO_2 fluid extraction extract gave sweet and musk notes (Sun et al. 2014).

EOs have low molecular weight and boiling point and are usually colourless (Burt 2004). They are generally of lower density than water, with the exception of a few e.g. *Cinnamomum* sp., *Sassafras* sp., and *Chrysopogon* sp. (Dhifi et al. 2016). Due to different

growing conditions, each plant produces its own specific mixture of volatile constituents. These mixtures can be composed from 20 to 60 volatile components, at varying concentrations with two or three major compounds representing 20–70 % of all content that usually defines the biological properties of EOs (Bilia et al. 2014; Chouhan et al. 2017). However, various factors including physiological variations and environmental conditions during the plant's growth, geographic variations, genetic factors and evolution, amount of plant material/space, and manual labour needs determine the chemical variability and yield for each species (Figueiredo et al. 2008). Moreover, the composition of EOs is largely affected by extraction and isolation techniques. It has been found that traditional methods used for their extraction can cause the loss of some volatiles and degradation of unsaturated or ester compounds through thermal or hydrolytic effects, whereas the use of solvent extraction can cause the presence of toxic solvent residues. Furthermore, the obtained EOs are susceptible to degradation by other factors, such as light, heat and/or oxidation (Reyes-Jurado et al. 2015). It is well known that temperature influences compositions of EOs. EOs are complex mixtures of volatile organic compounds, they have a high vapor pressure and evaporate easily at room temperature. However, each component of EO has a different thermal stability. Until recently, no comparable study regarding thermal stability of EOs could be found in the literature (Turek et al. 2013). Only very recently, studies have been performed that pointed out the individual responses of EOs to varying storage temperatures. Terpenoids, especially mere terpenes and aldehydes, are commonly known to be thermolabile and susceptible to rearrangement processes at elevated temperatures. Terpenic conversion reactions upon heating have been reported both for isolated as well as for EOs (Turek & Stintzing 2013). Study of Benmoussa et al. (2016) clearly shows the chemical composition of *T. vulgaris* EO obtained by different methods; for example, chemical profile of this EO obtained by microwave-assisted hydrodistillation (MAHD), whose main advantage is its ability to rapidly heat the sample solvent mixture, resulting in its wide applicability for the rapid extraction of analytes, including thermally unstable substances (Kataoka 2019; Ghazanfari et al., 2020), can be compared with the chemical profile of *T. vulgaris* EO obtained by water distillation (HD). The results show that 11 components (94.69 % monoterpene hydrocarbons, 1.86 % monoterpene oxygenated compounds, 2.33% sesquiterpene hydrocarbons, and 0.37 % others) were detected in EO obtained by MAHD, whereas only 8 components were found in the EO obtained by HD (97.14 % monoterpene

hydrocarbons, 1.83 % sesquiterpene hydrocarbons, and 1.02 % others). Due to these obstacles, comparison of the EO chemical composition described in different studies is sometimes difficult, if not impossible (Figueiredo et al. 2008). However, thanks to their chemical composition, EOs (as well as individual volatile compounds) possess various biological activities such as antibacterial, anticancer, antifungal, antioxidant, anti-inflammatory, antiviral, *etc*…).

2.2.3 Analytical methods

Because many volatile compounds produced by plants, such as the constituents of EOs, are extensively used commercially as flavourings and fragrances, their analysis in the food and perfume industry has a long tradition (Bicchi 2004; Tholl et al. 2006). Moreover, the increasing scientific interest in the atmospheric chemistry, biochemistry, ecology, and physiology of plant volatiles has led to the development of a variety of systems for their analysis as well (Linskens & Jackson 1997; Millar & Sims 1998). Although the study of the volatile profiles of samples as well as the EOs composition and the identification of their individual volatile constituents is important for understanding the origin of their biological activities, chemical analysis may become a challenging, mainly since the majority of the compounds are present in minor quantities. Furthermore, a large group of monoterpenes includes numerous compounds with similar molecular formulas and a distinct structure type, as well as a great number of isomers. Therefore, their analysis requires techniques with low detection limits. However, due to the different physical and chemical properties of the volatile compounds, the efficiency of extracting volatile compounds from the sample also differ widely and thus, the obtained volatile profiles are highly method-dependent. Thus, no single analytical technique can give a complete profile of all volatiles. It appears that a combination of broad-spectrum profiling methods, and of targeted methods to analyse key volatiles that may occur at very low concentrations will continue to be used (Rowan 2011).

Even though a variety of techniques can be used to collect and concentrate volatile metabolites from the sample, there are two basic approaches: direct sampling of volatiles from the air (headspace) and solvent based volatile extraction methods (Rowan 2011). Headspace sampling is a non-destructive technique for collecting volatile compounds providing a more realistic volatile profile of living plants than traditional methods of solvent extraction of volatiles from plant tissues or steam distillation. Whether headspace

sampling is quantitative depends on the information obtained from the analysis. Rational arguments can be made stating that headspace is or is not quantitative. Thus, a simple answer does not exist, and one must have a deeper understanding of their analytical goals (Raynie 2019). It is usually classified into two types: static headspace (S-HS) and dynamic (D-HS) headspace. In the S-HS procedure, an equilibrium is established between the volatile substances contained in the sample and in the vapor phase above the sample in a gas-tight vial. After a certain time, necessary to establish equilibrium, a part of the gas phase is taken from the vial as it is free of non-volatile compounds, it can be directly analysed by GC. On the other hand, in the D-HS procedure, a flow of inert gas is used for continuous extraction of volatile compounds from a sample. The gas is bubbled through the sample and the displaced volatiles are trapped in an absorption adsorbent or cryogenic trap. The trap is then heated and the volatiles are released or desorbed and transferred into the chromatographic system for further analysis (Soria et al. 2015). The development of solid-phase microextraction (SPME) was an important advance in headspace analysis (Tholl et al. 2006). This technique is currently the preferred method to examine complex volatile mixtures in laboratories. Moreover, Hamilton Company developed the Hamilton SampleLock syringes with an easy-to-use twist valve and a positive rear stop that prevents loss of gaseous (as well as liquid) samples and plunger blowout (Hamilton 2020).

By far the most commonly employed instrumentation for profiling volatiles (i. e. to identify and quantify the volatile compounds of the sample or EOs) is gas chromatography coupled to the mass spectrometry detector (GC/MS). This technique is among the most suitable methods for volatile compounds analysis because it achieves the highest resolution of volatile profiles. To achieve an even higher quality identification of detected volatile constituents, the analysis can be conducted on two columns, which usually differ in polarity. In recent scientific studies it has been described that the use of a simultaneous dual-column/dual-detector system increases the resolution of the analysis leading to the improved identification and quantification of EO components (Marriott et al. 2001; Haggarty & Burgess 2017).

Although GC/MS is widely used, when it is not available, high-performance liquid chromatography (HPLC) is a useful alternative for an accurate quantitative analysis. Another method that can be used to detect and identify plant volatile compounds, is fractional distillation, which is commonly used to purify EOs or to concentrate the desirable parts

of EOs for specific applications. The main goal is to separate substances based on their different volatility (Nakatsu et al. 2000). Nuclear magnetic resonance (NMR) has not been widely used in volatile analysis except for the analysis of EOs. Although this is a relatively insensitive technique requiring larger sample amounts, it may be desirable when samples show high variability (Rowan 2011). Other methods, for example the mass spectrometry technique based on soft chemical ionization, selected ion flow tube-mass spectrometry (SIFT-MS) or proton transfer reaction mass spectrometry (PTR-MS) can be used as well (Tholl et al. 2006; Rowan 2011; Materic et al. 2015).

The quantitative aspects of EO analysis are not easy to deal with, not only because component identification is in general more important than quantitation, but also because the approach to it is often ambiguous (Rubiolo et al., 2009). Whether headspace sampling is quantitative depends on the information obtained from the analysis. Rational arguments can be made stating that headspace is or is not quantitative. Thus, a simple answer does not exist, and one must have a deeper understanding of their analytical goals (Raynie 2019). The quantitative composition of most EOs is very often reported in the literature in terms of relative percentage abundances, although this approach can unfortunately only give an approximate indication of the ratio between components in the sample under investigation. There can be no single absolute approach to quantitation, because of the complexity of EOs and the different methods to quantify their components (Rubiolo et al., 2009). Based on a literature survey carried out by Bicchi et al. (2008), different approaches to EO quantitative analysis are possible, depending on their use and destination. The most widely used approaches are: 1) relative percentage abundance, 2) internal standard normalized percentage abundance and quality characterization by statistical elaboration of the GC profile assumed as a parameter representative (fingerprint) of the sample investigated within a set, 3) true quantitation of one or more components (true quantitation) by a validated method) and and (4) quantitation by a validated method (Bicchi et al., 2008; Rubiolo et al., 2009). According to Raynie (2019), headspace sampling is very quantitative in terms of accuracy, precision, and other quantitative metrics when coupled with GC characterization and Antih et al. (2021) for example mentions, that the use of syringe headspace sampling technique could provide more accurate data than SPME method representing the true headspace distribution of the EO volatile agents, therefore prove a better technique when aiming for quantitative analysis.

2.2.4 Antimicrobial properties

Besides multiple biological properties, such as anticancer, antimutagenic, antioxidant, antiprotozoal, anti-inflammatory, antiviral, and immunomodulatory effects, plant-derived volatiles also possess a wide range of antimicrobial activities, including their antistaphylococcal effects. Within EOs, most of the antimicrobial activity has been found in the oxygenated terpenoids, e.g., phenolic terpenes and alcohols (Bassole & Juliani 2012). These volatile components have been known to possess antimicrobial properties against various microorganisms, such as both Gram-positive and Gram-negative bacteria (Cowan 1999). Moreover, volatile terpenes are also recognized for their ability to inhibit fungi (Cowan 1999; Hammer et al. 2003; Dambolena et al. 2008), viruses (Cowan 1999), nematodes (Gu et al. 2007) or even insects (Lee et al. 2003; Justicia et al. 2005). Furthermore, the interactions between the volatiles may produce synergistic effects. Several scientific reports have revealed that whole EOs usually show a greater antimicrobial activity than the mixtures of their main components, suggesting that the minor components are critical to the synergistic activity, even though additive and antagonistic effects have been observed as well (Bassole & Juliani 2012). Although the mode of their action is not fully understood, it might be linked to their lipophilic nature allowing them to destabilize the cell membrane integrity (Cowan 1999; Cox et al. 2000; Inouye et al. 2003).

The antimicrobial effects of many plant-derived volatiles have been extensively studied individually as well as in combinations against various pathogens. A number of scientific papers has been published in recent years on the antimicrobial activity of many EOs and their volatile constituents (Dormans & Deans 2000; Burt 2004; Fu et al. 2007; van Vuuren and Viljoen 2007; Gallucci et al. 2009; Goni et al. 2009; Puskarova et al. 2017; Reyes-Jurado et al. 2019). From these studies it is clear that these secondary plant metabolites have a potential in medical procedures and applications in the cosmetic, food and pharmaceutical industries. Due to their antimicrobial properties and high volatility as they have the ability to vaporize spontaneously in room temperature, EOs and their constituents have also a great potential to be used in inhalation therapy because they can easily reach the upper and lower parts of the respiratory tract (Horvath & Acs 2015). Inhalation of the volatile fraction from aromatic extracts or burning of plant material have been traditionally used to treat various respiratory diseases, such as asthma,

bronchitis and other infections, including the common cold (Pasdaran et al. 2016). Also, in the European Pharmacopoea, more than 25 EOs have an official status and the inhalation of volatile oils from e.g., *Eucalyptus globulus, E. polybractea* and *E. smithii, Foeniculum vulgare, Illicium verum, Melaleuca alternifolia, M. linariifolia, M. dissitiflora, Mentha* × *piperita, Pimpinella anisum, Thymus vulgaris* or *T. zygis* is frequently used for the treatment of respiratory tract infections (Horvath & Acs 2015).

Although the EOs and their constituents have been well-known for their volatile nature, compared with the abundance of evidence showing the effectiveness of EOs in their liquid phase, the potential of EO vapours is less researched, although gaining interest. Up to date, various reports have been published on the antimicrobial activity of EOs in their vapour phase as well, however primarily when testing them individually. The effectiveness of EOs and their components in their gaseous phase can be completely different from a direct contact in the liquid and solid phase. Some studies have reported that vapour generated by EOs of *Cymbopogon citratus* (Tyagi & Malik 2010), *E. globulus* (Tyagi & Malik 2011), *M. alternifolia* (Mondello et al. 2009), and several others including *Origanum syriacum, Thymbra spicata, F. vulgare* (Soylu et al. 2006) and *Lavandula vera* EOs (Tullio et al. 2007) have greater antimicrobial effect compared to EOs in liquid form applied by direct contact. it has been suggested that one of the reasons explaining this phenomenon is that the lipophilic molecules in the liquid phase associate to form micelles and therefore suppress that attachment of the EOs to the microorganism, whereas the vapour phase allows free attachment (Laird & Phillips 2012). Also due to the high number of monoterpenes in the vapour of EOs, it is easier for them to attack the bacteria compared to their liquid phase (Ghabraie et al. 2016).

As already mentioned previously, EOs and plant-derived volatiles may, if combined together, produce an antimicrobial synergy. Several experiments focused on the combinatorial antimicrobial action of EOs have previously been conducted against numerous bacteria including *S. aureus*. For example, menthol, when combined with geraniol showed to be synergistic against *S. aureus* ATCC 21212 (Gallucci et al. 2009). Synergy was also observed against *S. aureus* ATCC 12600 when 1,8-cineole was combined with limonene in ratios 9:1, 8:2, 7:3, 6:4 (van Vuuren & Viljoen 2007). The combination of 1,8-cineole and aromadendrene displayed synergism against different MRSA strains using time-kill assay, whereas the chequerboard assay demonstrated that the same combination of volatile compounds reduced the MIC in most cases in an additive

way (Mulyaningsih et al. 2010). Antistaphylococcal additive effects were also described for the combination of thymol/carvacrol (Lambert et al. 2001), cinnamaldehyde/eugenol (Moleyar & Narasimham 1992) or *Syzygium aromaticum/Rosmarinus officinalis* EOs (Fu *et al.* 2007).

However, as in the case of testing antimicrobial activity of volatile agents individually, most of the experiments have only been performed in the liquid (/solid) phase and only a few authors have published studies on the interactions between EO vapours. Goni et al. (2009), for example, reported that when *Cinnamomum verum* and *S. aromaticum* EO vapours are combined, they exert an antagonistic effect against *Escherichia coli,* but a synergistic effect on a range of other bacteria such as *Bacillus cereus*, *Listeria monocytogenes* and *Yersinia enterolytica* (Goni et al. 2009). MRSA has been found to be reduced by an *in vitro* treatment consisting of a grapefruit extract called Citricidal™ combined with geranium oil or tea tree oil in vapour form or by a patchouli and tea tree EO vapour mixture (Edwards-Jones et al. 2004). Moreover, the antifungal effect of *C. verum* EO combined with mustard EO (containing > 95 % of allyl isothiocyanate) was evaluated against 10 different moulds by Clemente et al. (2019) with the results being mostly additive and synergistic. Doi et al. (2019) assessed the synergistic effects on antimicrobial activity of eleven different mixtures of *C. verum* and *Origanum vulgare* EOs in the vapour phase against *S. aureus*. Strong synergistic activities were found when ratios of cinnamon and oregano EOs were 8:1 and 9:1. *S. aureus* was more sensitive to EOs in the vapour phase than in the liquid phase (Doi et al. 2019). Furthermore, a combined effect of *C. citratus* and *Mentha arvensis* oil vapours with negative air ions (NAI) was investigated against *Pseudomonas fluorescens* by Tyagi and Malik (2010) and a significant enhancement in the bactericidal action of their combination was observed as compared to their individual action.

Currently, various phytomedicinal products based on a combination of medicinal plant EOs and/or their volatile constituents are available on the market. The herbal medicinal products are usually sold in the form of capsules, lozenges, tablets, tinctures, syrups, nasal drops, inhalers and sprays as non-prescription drugs, dietary supplements and confectionery. For the treatment of respiratory diseases, inhalers, sprays and nasal drops are particularly beneficial because their active components are delivered directly to

the site of infection in the airways (Houdkova 2018). Examples of these phytomedicinal products are mentioned below:

Biotussil is a traditional herbal medicinal product that is used in the form of oral drops for internal use as an auxiliary medicine for cold and cough. It is also recommended to treat both acute and chronic respiratory diseases, including rhinitis and sinusitis, aiding the formation and dissolving of mucus. It contains a combination of extracts from 8 medicinal herbs: extracts of *Gentiana lutea* root, *Primula veris* flower, *Plantago lanceolate* leaf, *T. vulgaris* herb, *Glycyrrhiza glabra* root, *Sambucus nigra* flower, and EOs of *F. vulgare* fruit and *P. anisum* fruit (SUKL 2015; Biomedica 2020).

GeloMyrtol is an herbal medical product that is recommended to treat several acute and chronic infections of the upper and lower airway system as acute and chronic rhinosinusitis, acute and chronic bronchitis, and chronic obstructive pulmonary disease. This medicament is composed of EOs from 4 plants: *E. 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. It is sold in the form of enteric-coated soft gelatin capsules (Paparoupa & Gillissen 2016).

Pinio-Nasal, previously called Pinosol, is a mixture of *Pinus sylvestris*, *M. piperita*, and *E. globulus* EOs, plant volatile compounds thymol and guaiazulene, and vitamin E (ratio 54:14:7:0.7:0.3:24). This medicine sold in form of nasal drops and nasal spray is indicated to treat rhinitis and other inflammatory diseases of the nose and nasopharyngeal mucosa. It is also suitable for inhalation devices and for aromatherapy, because the inhalation of its active substances can favourably affect infectious inflammatory airway diseases (inflammation of the larynx, trachea and bronchi) (RosenPharma a.s. 2014).

Vicks Inhaler is over the counter product containing a combination of several plant-derived volatile products, namely camphor, menthol, methyl salicylate and *Abies sibirica* EO. This inhalation remedy helps to relief stuffy nose and gives fast and temporary "mobile" relief from nasal clogging due to colds, hay fever or upper respiratory allergy (Vicks 2020).

2.3 Methods for evaluating interactions of antimicrobial agents

Several disc diffusion as well as dilution techniques such as microdilution chequerboard, e-test, and time-kill curve assays have been used for the evaluation of the antimicrobial combinatory interactions between two or even more antimicrobial agents in liquid and/or solid media. However, due to the specific physico-chemical properties of EOs and plant-derived volatile compounds such as high volatility and hydrophobicity, these conventional antimicrobial susceptibility testing methods face specific problems in the drug research and development processes. Low solubility of these compounds in water-based media (e.g. in broth) has to be overcome by adding emulsifiers or solvents (such as DMSO, ethanol, and Tween 80), which may alter the activity (Nedorostova et al. 2009), whereas the high volatility poses a risk of active substance losses due to evaporation (Laird & Phillips 2012). Furthermore, the transition of the vapours of EOs as well as the individual volatile compounds may affect the results of microplate assays. With templates with only one sample in each row of the plate, a simple change in the volatile antimicrobials layout may lead to significantly different results (Novy et al. 2014; Rondevaldova et al. 2015).

The interaction between EO components can produce four types of effects: additive, antagonistic, indifferent or synergistic. An additive interaction means that the effect of two antimicrobial agents is equal to the sum of the individual effects. Antagonism is observed when the effect of one or both compounds is less when they are applied together than when individually applied. Synergy is defined as a significantly greater activity provided by two agents combined than that provided by the sum of each agent alone (Burt, 2004) whereas the absence of interaction is defined as indifference. In most studies, when searching for antimicrobial synergy, the fractional inhibitory concentration indices (ΣFICs) were calculated for each antimicrobial combination (based on the obtained MICs) according to the following equation: $\Sigma FIC = FIC_A + FIC_B$, where $FIC_A = MIC_{A\ (in\ combination\ with\ B)} / MIC_{A\ (alone)}$, and $FIC_B = MIC_{B\ (in\ combination\ with\ A)} / MIC_{B\ (alone)}$. The interpretation of the FIC results may vary, but e.g. when evaluated according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST 2000), the ΣFIC index is interpreted as follows: synergistic interaction if $\Sigma FIC \leq 0.5$; additive effect if $\Sigma FIC > 0.5$ and ≤ 1; indifferent if $\Sigma FIC > 1$ and ≤ 2; and antagonism if $\Sigma FIC \geq 2$.

Assays for antimicrobial susceptibility testing (either of one single antimicrobial agent or of more agents in combination) can be performed either in direct contact with the pathogen or by the vapour phase, however in contrast to the well-established assays for testing of antimicrobial combinatory effects in liquid media, there are no standardized methods to identify interactions between volatile compounds in the gaseous phase, e.g. in accordance with the Clinical and Laboratory Standards Institute (CLSI) or the European Committee on Antimicrobial Susceptibility Testing (EUCAST). The disc volatilization assay, also called vapour diffusion test (Goni et al. 2009), vapour phase agar diffusion test (Wang et al. 20016), combinatorial vapour diffusion assay (Clemente et al. 2019) or vapour-contact assay (Doi et al. 2019) is a simple and probably the most frequently used method for the evaluation of combinatory activities of plant volatile vapours. Interactions between EOs or their volatile compounds have previously been measured in the gaseous phase using this method by several researchers (Sukatta et al. 2008; Goni et al. 2009; Wang et al. 2016; Clemente et al. 2019) who have also developed various modifications. The tests are carried out in inverted Petri dishes, where the solidified medium is exposed to vapours of combinations of EOs or their compounds by placing an impregnated disc on the lid of the dish. After incubation, zones of microorganism growth inhibition are measured on the agar surface. Subsequently, these zones are compared with the zones of inhibition of individual compounds, or the FICs are calculated. However, although methods based on a disc volatilization assay are a useful tool for a simple assessment of the antimicrobial potential of volatile agents in the gaseous phase, they also possess various disadvantages: they are not suitable to identify minimum inhibitory concentrations (MICs) (-therefore not even for FICs), suffer from the lack of automation and are not designed for a high-throughput screening. Other disadvantages include the relatively high consumption of material and labour because each concentration of each EO or the volatile compound must be tested on a separate Petri dish. The principles of some methods for antimicrobial combinatory testing in the gaseous phase are described below and shown in Figure 2.1.

Disc volatilization assay using sterile adhesive tapes

This is a modification of the standard disc volatilization method using Petri dishes, where after the agar inoculation, filter discs were loaded with one EO, spiked with another EO and subsequently the Petri dishes were sealed using either two parafilm tapes

(Clemente et al. 2019) or other sterile adhesive tapes (Goni et al. 2009) to prevent vapour outlet. In case of Goni et al. (2009), FIC values were calculated for each EO in the tested combination. Similarly, Doi et al. (2019) used this method as well.

In vitro dressing model

The *in vitro* dressing model is another alternative of the disc volatilization assay reducing the loss of active substances by evaporation, where the tested combinations of EOs were placed onto a small central area of either the Gamgee or the gaze layers. Subsequently, the agar plates were inoculated by a bacterial suspension and then covered with four layers of dressings containing Gamgee, gaze, Flamazine™ and Telfa Clear™ or Jelonet™. Modifications of the experiments were made to the primary layer by using/not using the reduced adherence dressings Flamazine™, Telfa Clear™ and Jelonet™ (Edwards-Jones et al. 2004).

Modified chequerboard assay in vials

Another method to identify antimicrobial combinatory effects of volatile compounds in the vapour phase is described in a paper by Ji et al. (2019), who used a modified chequerboard assay to measure the synergistic antifungal activities of two and three EO vapours. For the combination of 2 EOs, sixteen experimental vials were prepared. Agar was deposited in the 16 upper wells and inoculated by a fungus. Then, liquid EOs A and B were serially diluted twofold; EO A was deposited on each paper disc in four lower wells in each row of the experimental vials in twofold serial dilutions and EO B was similarly deposited in each column. This resulted in 16 combinations of EO A and B in 16 experimental vials. Immediately after depositing the diluted EO on paper discs, the lower and upper wells of the experimental vials were placed together, sealed with parafilm, and incubated. The agar was visually examined and when no colonies of the fungus were formed, the FICs were calculated. Similarly, the synergistic activity of a combination of three EO vapours was measured in 64 experimental vials (Ji et al. 2019).

Time kill assays

A disc volatilisation assay was performed to determine the time kill of EOs in the vapour phase in the study of Doi et al. (2019). Bacterium was exposed to EO combinations as well as to individual EOs and the diameter of bacterial inhibition was

measured after different time periods. The antimicrobial atmospheres were removed by changing the lids containing filter disc for sterile lids. After the incubation period, kill times were determined as the shortest time resulting in a visible bacteria growth inhibition. The determination of kill time was also used in the study of Tyagi and Malik (2010) who tested the effects of a combination of the EO vapours with negative air ions.

Airtight containers

Ji et al. (2019) examined the antifungal effects of combined EO vapours on inoculated beef jerky in airtight containers. A small Petri dish lid was placed inside the airtight container (= a round polystyrene dish fitted with a lid) and a piece of surface-sterilized and subsequently by a fungus inoculated beef jerky was placed on top of the lid. The upper well of the experimental vial used to determine the MIC of the fungus on the agar was placed upside down next to the small Petri dish lid. Subsequently, the combination of three EOs was deposited on paper discs (ratios 1:2:1) in the upper well of the vial inside the container. After the liquid EO was deposited, the lid of the container was applied and sealed with parafilm. After the incubation, the population of the fungus on beef jerky treated with a combination of the three EO vapours was compared to the population on jerky exposed to a single EO vapour.

Modified chequerboard assay performed in airtight containers

This method was applied by Aguilar-Gonzalez et al. (2015), who used hermetically sealed transparent plastic containers to test the antifungal activity of EO combinations in the vapour phase on inoculated strawberries using a chequerboard design. Different proportions of MICs of one EO (i.e. MIC, ½ MIC, ¼ MIC and ⅛ MIC) were combined with the same proportion of the other EO and poured on two filter papers, which were subsequently taped on the upper sides of the containers, one for each EO. Inoculated strawberries were placed on the containers' lids and then closed (upside down) and sealed with parafilm to avoid vapour leakage. After the acquisition of the EOs' MICs, the FICs were calculated.

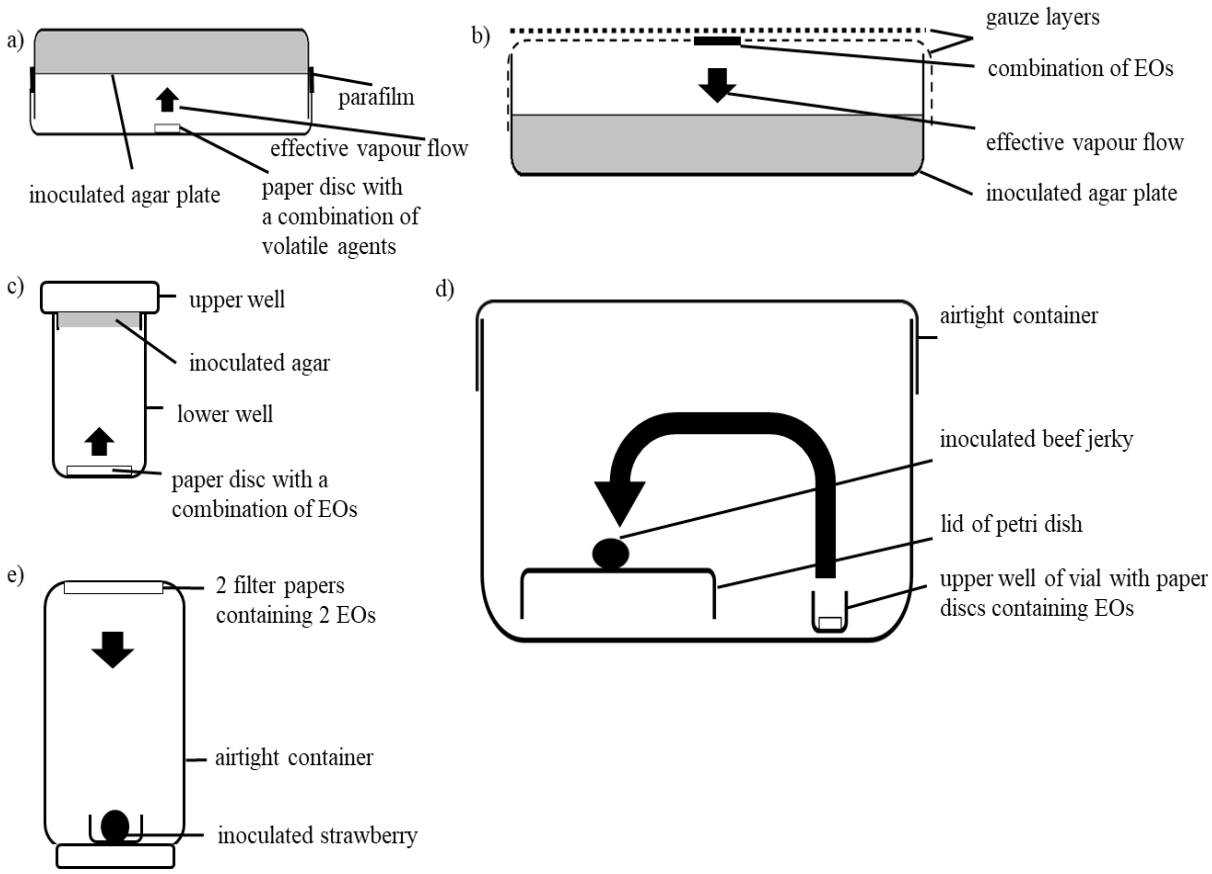


Figure 2.1 Schematic diagrams of methods for evaluating interactions of antimicrobial agents' vapours:

a) disc volatilization method using sterile adhesive tapes (parafilm) (Wang et al. 2016); b) *in vitro* dressing model volatilization test (Edwards-Jones et al. 2004); c) Experimental apparatus (vial) used for modified chequerboard assay in vials (Ji et al. 2019); d) airtight container (Ji et al. 2019), and e) airtight container used for modified chequerboard assay (Aguilar-Gonzalez et al. 2015). All diagrams are shown in a cross-sectional view.

EOs = essential oils.

3 The development of a new method for testing antibacterial interactions of volatile agents in the vapour phase and determination of the antistaphylococcal effect of interactions between two volatile compounds

Adapted from: **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 volatilization chequerboard method. Fitoterapia **129**:185–190.

Author contribution: Marie Strakova (born Netopilova) developed and optimized the broth volatilization chequerboard method and she performed experiments focused on the antistaphylococcal activity of plant volatile compound combinations tested in the liquid and vapour phase. She also processed and analysed obtained data and prepared the manuscript including its required revisions.

3.1 Introduction

Staphylococcus aureus is an important pathogen responsible for broad spectrum of diseases, ranging from food poisoning, mild skin and soft tissue infections to highly serious diseases such as endocarditis and osteomyelitis (Reddy et al. 2017). Currently, the global spread of methicillin-resistant *S. aureus* (MRSA) is one of the most serious public health challenges worldwide. It acquires resistance to all β-lactam agents as well as to other groups of antibiotics such as macrolides, fluoroquinolones, aminoglycosides, and glycopeptides (Reygaert et al. 2013; EARS-net 2018). Since *S. aureus* is commonly found on respiratory tract mucosa, the antibiotic inhalation could be one of its possible treatments. For example, dry-powder vancomycin and combination of fosfomycin and tobramycin are currently in late-stage development for supporting inhalation therapy and treatment of MRSA in cystic fibrosis patients (Quon et al. 2014).

The increase in bacterial resistance to antibiotics has also revived the interest in plant products as alternative antimicrobial agents to control pathogenic microorganisms (Hyldgaard et al. 2012). Plants produce secondary metabolites, which serve them as strong defence against predators and microbial pathogens due to their biocidal properties (Bassole & Juliani 2012). Their defence never rely on one particular class of compounds and secondary metabolites occur always as mixtures in plants. Thus, synergistic and antagonistic effects can either significantly enhance or reduce activities of single compounds (Hadacek 2002). Essential oils (EOs) are typical example of such complex mixtures, whereas many of them produce antimicrobial synergy (Bassole & Juliani 2012). Several experiments focused on combinatory action of EOs and their volatile constituents as well as on combinatory effects between phytochemicals and antibiotics have previously been conducted against numerous bacteria including *S. aureus*. Various methods such as chequerboard, time-kill curve, and e-test assays have been used for evaluation of their antimicrobial combinatory interactions (Doern 2014; Magi et al. 2015). However, due to the specific physico-chemical properties of EOs such as high volatility and hydrophobicity, these conventional methods face specific problems. Because of the low solubility of these compounds in water-based media (e.g. in broth), the surfactants are usually added, whereas high volatility causes a risk of active substances losses by evaporation (Kloucek et al. 2012). Furthermore, the transition of

vapours of EOs and their constituents may affect the results of microplate assays (Novy et al. 2014).

In contrast to well-established assays for testing of antimicrobial combinatory effects in liquid media, there are no standardized methods for determination of interactions between volatile compounds in the gaseous phase. Disc volatilization assay is probably the most frequently used method for evaluation of combinatory activities of plant volatile vapours. Interactions of EOs in the gaseous phase have previously been measured using this method by several researchers (Edwards-Jones et al. 2004; Goni et al. 2009; Aguilar-Gonzalez et al. 2015). The tests were carried out in Petri dishes, where solidified medium was exposed to the vapours of EO combinations by placing an impregnated disc on the lid of the dish. After incubation, zones of microorganism growth inhibition were measured on the agar surface. Subsequently, these zones were compared with the zones of inhibition of individual compounds (Edwards-Jones et al. 2004), or the fractional inhibitory concentrations (FICs) were calculated (Goni et al. 2009). However, this assay based on modification of standard agar disc diffusion test is not appropriate for minimum inhibitory concentration (MIC) determination (Jorgensen & Ferraro 2009) and suffers from the lack of automation (Jorgensen et al. 1999). On the other hand, the advantages of the microdilution procedure include the generation of MICs, reproducibility, economy of reagents and space that occurs due to the miniaturization of the test (Jorgensen et al. 1999; Jorgensen & Ferraro 2009). Since our new broth microdilution volatilization assay performed in 96-well microtiter plates, it allows determination of MIC values in both liquid and vapour phases (Houdkova et al. 2017), it has the potential to be modified for evaluation of combinatory effects of volatiles using chequerboard design and allowing determination of FIC indices.

Carvacrol and its isomer thymol are ones of the most extensively studied EO constituents. They are phenolic monoterpenoids, commonly present in EOs of Origanum and Thymus species (Hyldgaard et al. 2012), which are used as antiseptics in pharmacology, agriculture, cosmetics and food industry (Kumar et al. 2013). Beside the multiple biological properties (Fachini-Queiroz et al. 2012), they also possess wide spectrum of antibacterial activity, including their antistaphylococcal effects (Lambert et al. 2001; Hyldgaard et al. 2012). Several studies investigating the combinatory effects of carvacrol and thymol against various pathogenic microorganisms have previously been performed in liquid phase. Their interactions against *S. aureus* were also evaluated using

chequerboard assay and calculation of fractional areas (Lambert et al. 2001; Gallucci et al. 2009; Guarda et al. 2011). Nevertheless, the obtained results of studies mentioned above differ significantly, whereas synergistic (Guarda et al. 2011), antagonistic (Gallucci et al. 2009), and additive effects (Lambert et al. 2001) have been observed. In contrast to above mentioned papers showing their interactions in liquid media, there are no reports on combinatory effects of carvacrol and thymol in the vapour phase.

Therefore, the main aim of this study was to determine an *in vitro* inhibitory effect of carvacrol and thymol combination against twelve *S. aureus* strains simultaneously in vapour and liquid phase using broth volatilization chequerboard assay – a new method based on combination of standard microdilution chequerboard and new broth volatilization test (Houdkova et al. 2017) allowing calculation of FIC values.

3.2 Materials and methods

3.2.1 Chemicals

Carvacrol (97%, CAS: 499-75-2), thymol (99%, CAS: 89-83-8), oxacillin (86.3%, CAS: 7240-32-2), and thiazolyl blue tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (Prague, CZ). Dimethyl sulfoxide (DMSO) was obtained from Penta (Prague, CZ).

3.2.2 Bacterial strains and culture media

In this study, twelve *S. aureus* strains, including antibiotic-resistant and sensitive forms were used. American Type Culture Collection (ATCC) standard strains 25923, 29213, 33591, 33592, 43300, and BAA 976 were purchased from Oxoid (Basingstoke, UK) on ready-to-use bacteriological Culti-Loops, and clinical isolates (SA 1–6) were obtained from the Motol University Hospital (Prague, CZ). The identification of clinical isolates was performed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry as it is described in Rondevaldova et al. (2018). Cation-adjusted Mueller-Hinton (MH) broth (Oxoid, Basingstoke, UK) equilibrated to pH 7.6 with Trizma base (Sigma-Aldrich, Prague, CZ) and MH agar (Oxoid, Basingstoke, UK) were used as cultivation and assay media.

Stock cultures of bacterial strains were cultivated in broth medium at 37 °C for 24 h prior the testing. Turbidity of the bacterial suspension used for inoculation of both lid and plate, 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.

3.2.3 Broth volalatilization chequerboard method

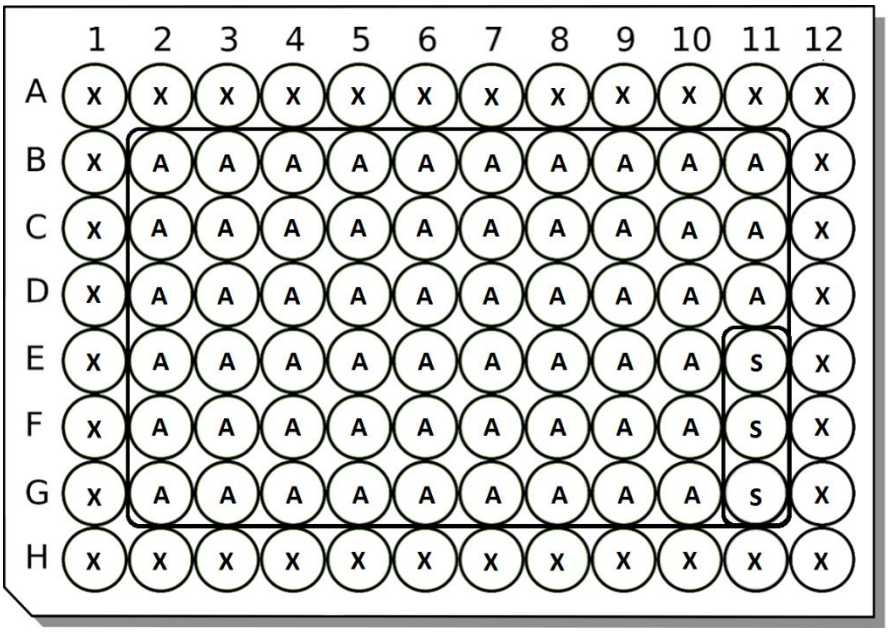
The broth microdilution volatilization method (Houdkova et al. 2017), modified according to the chequerboard assay design (Hsieh et al. 1993), was used for assessment of combinatory antimicrobial effect of carvacrol and thymol in the vapour and the liquid phase. The experiments were performed in white 96-well immunoplates (total well volume = 400 μL) covered by tight-fitting lids with flanges designed to reduce evaporation (SPL Life Sciences, Naechon-Myeon, Republic of Korea). Initially, 30 μL of agar was pipetted into every flange on the lid (with exception of outer most wells) and inoculated with 5 μL of bacterial suspension. The lid layout is shown in a. Subsequently, both carvacrol and thymol were dissolved in DMSO and diluted in the broth medium to initial concentrations of 2048 μg/mL (with maximum concentration of DMSO 1%). DMSO did not inhibit the growth of bacteria in broth and agar media. Assay plate preparation and serial dilutions were performed by the automated pipetting platform Freedom EVO 100 equipped with four-channel liquid handling arm (Tecan, Mannedorf, CH). In combinations, six two-fold serial dilutions of thymol from horizontal rows were subsequently cross-diluted vertically by six two-fold serial dilutions of carvacrol. The initial concentration used for both thymol and carvacrol was 2048 μg/mL. After that, plates were inoculated by bacterial suspensions. Each plate also contained sterility and growth control. Oxacillin was used as a positive control for verification of susceptibility of *S. aureus* strains in broth media. The outer most wells were not used to prevent edge effect. The plate layout is shown in Figure 3.1b. After the inoculation, plate and lid were fasten together by clamps (Lux Tool, Prague, CZ), with handmade wooden pads for better fixing (Figure 3.2) and incubated for 24 h at 37 °C.

MICs and combinatory effect in both liquid (in plate) and vapour (on lid) phase were evaluated by visual assessment of bacterial growth after colouring of metabolically active bacterial colony with 25 μL of MTT dye when the interface of colour change from yellow and purple (relative to that of colours in control wells) was recorded in agar and broth (a, 3.3b). MICs were defined as the lowest concentration that visually inhibited

growth of bacteria compared with the compound free growth control and expressed as in μg/mL. The final MIC value presented in this work is the average of MICs obtained from three independent experiments performed in triplicate. The MICs of independent experiments varied in maximum range of three dilutions.

Combinatory effect of volatile compounds was determined based on fractional inhibitory concentration indices (ΣFIC). For combination of compound A (thymol) and compound B (carvacrol), the ΣFIC is calculated according to the following equation: $\Sigma FIC = FIC_A + FIC_B$, where $FIC_A = MIC_{A\ (in\ combination\ with\ B)} / MIC_{A\ (alone)}$, and $FIC_B = MIC_{B\ (in\ combination\ with\ A)} / MIC_{B\ (alone)}$ and evaluated according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST 2000). The ΣFIC index was interpreted as follows: synergistic interaction if $\Sigma FIC \leq 0.5$; additive effect if $\Sigma FIC > 0.5$ and ≤ 1; indifferent if $\Sigma FIC > 1$ and < 2; and antagonism if $\Sigma FIC \geq 2$. The final ΣFIC value was calculated as average of ΣFICs obtained from three independent experiment performed in triplicate (data not shown).

a)



b)

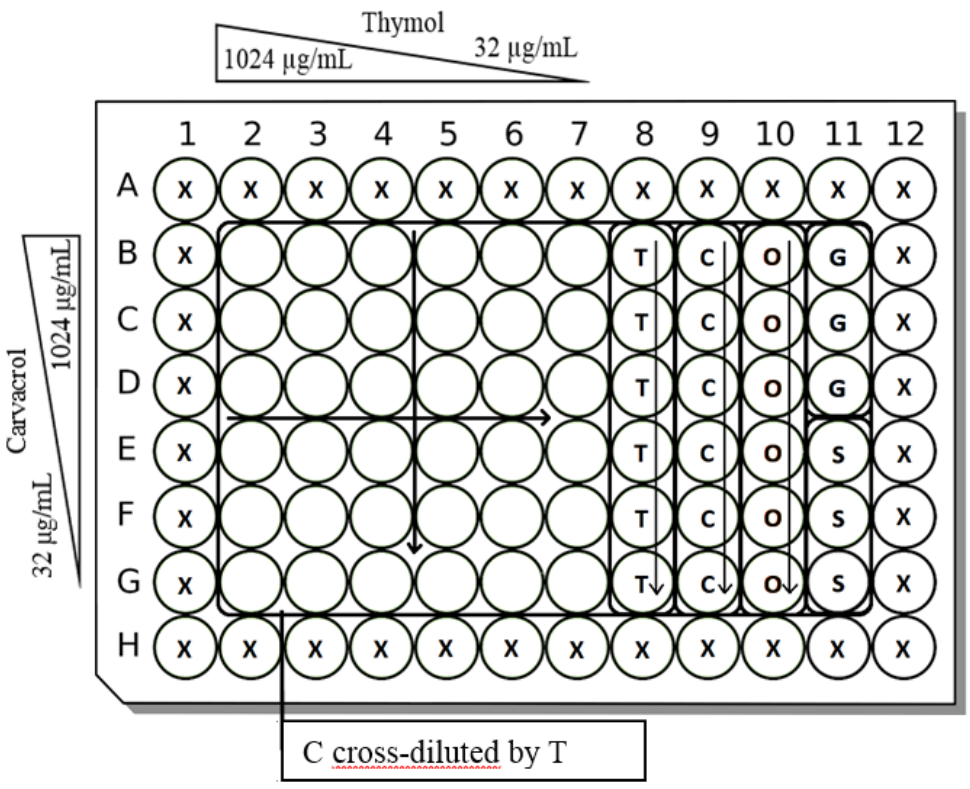
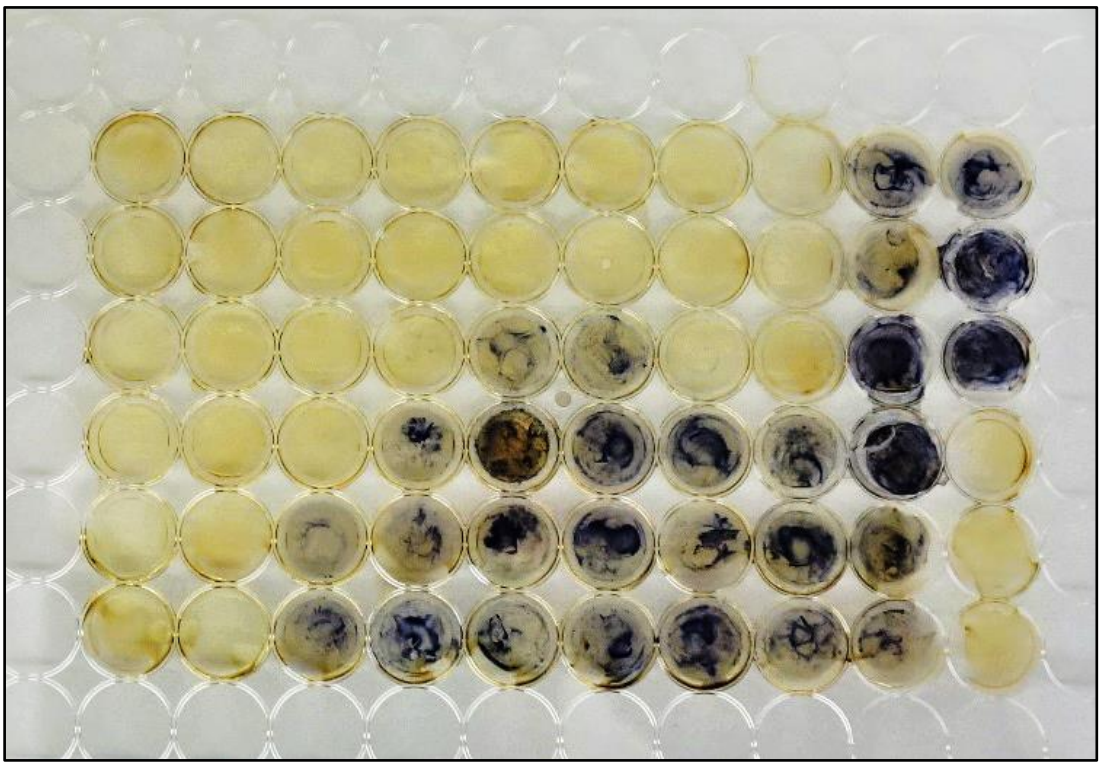


Figure 3.1 Lid (a) and plate (b) layouts of experiments demonstrating:

A: Inoculated agar; C: Carvacrol alone in two-fold dilutions (starting at concentration 2048 µg/mL); G: Growth control (infected medium control; 100% growth of bacteria); O: Oxacillin (positive antibiotic control) in two-fold dilutions; S: Sterility control (non-infected medium control; 0% growth of bacteria); T: Thymol alone in two-fold dilutions (starting at concentration 2048 µg/mL); X: flanges/wells not used.

Figure 3.2 Using clamps for fastening plate and lid

a)



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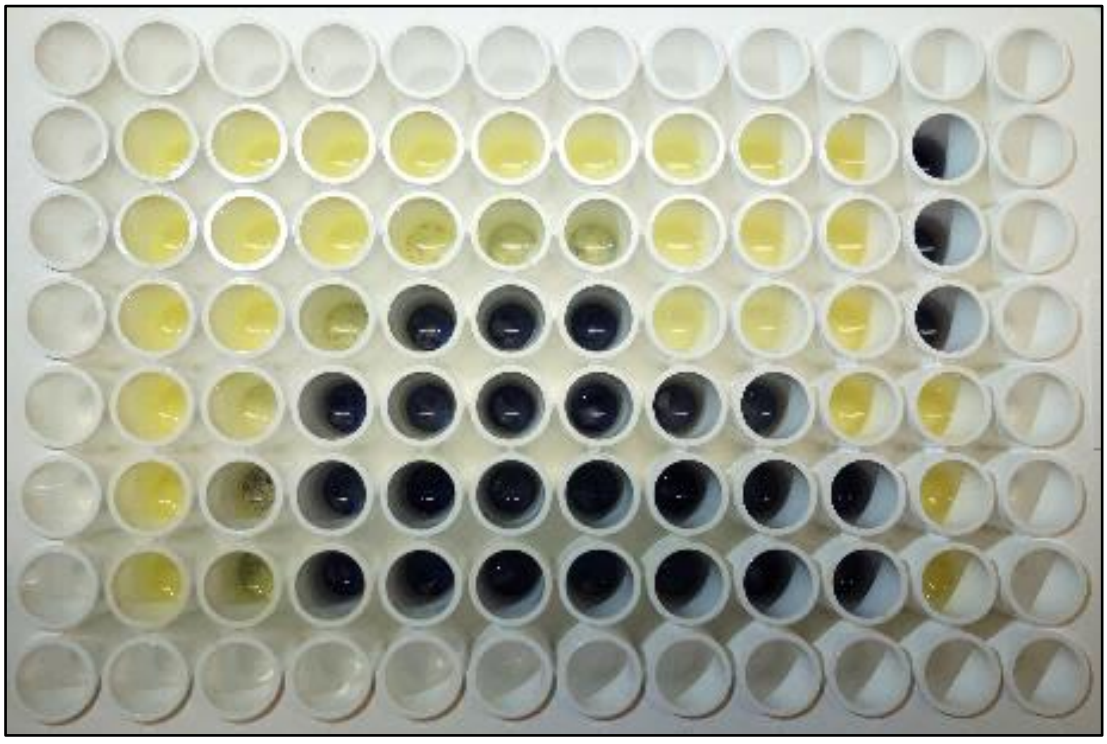

Figure 3.3 Evaluation of living bacterial colonies with MTT on lid (a) and in plate (b):

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

3.3 Results and discussion

Based on results of our preliminary experiments performed with combinations of eugenol (Sigma-Aldrich, Prague, CZ) with carvacrol, eugenol with thymol, and carvacrol with thymol against *S. aureus* ATCC 29213, the later combination were selected for more detailed evaluation because it produced the lowest FIC values when tested using new broth volatilization chequerboard method (unpublished data). In this study, we are describing combinatory *in vitro* growth-inhibitory effect of carvacrol and thymol on *S. aureus* in vapour and liquid phase using this newly developed assay.

The detailed results of individual MICs of both compounds against twelve staphylococcal strains as well as the MICs of their combinations with corresponding ΣFIC values are shown in Tables 3.1 and 3.2. The results showed that carvacrol exhibited a weak antistaphylococcal effect with MICs ranging from 370 to 1593 μg/mL and from 484 to 1024 μg/mL in agar and broth media, respectively. Similarly, the respective ranges of thymol MICs were 341–1707 μg/mL and 355–1024 μg/mL in vapour and liquid phases. These results correspond well with the antistaphylococcal effect previously determined by various authors using broth microdilution method, whereas their respective MIC values were ranging from 200 to 3810 μg/mL for carvacrol and from 300 to 7530 μg/mL for thymol (Gallucci et al. 2009; Rua et al. 2011; Houdkova et al. 2017). Regarding previous reports on antistaphylococcal effect of carvacrol and thymol vapours, Wang et al. (2016) found a weak activity of both compounds against MRSA ATCC 33591 using disc volatilization test up to concentration 1000 μg/disc. In our experiments, the same strain was susceptible to both agents at the MICs 626 μg/mL. These results may vary due to differences in the methods used. In comparison with Houdkova et al. (2017), who reported MIC values of carvacrol and thymol against *S. aureus* ATCC 29213 in vapour phase equal to 256 and 128 μg/mL, respectively, MIC values of this study were a little bit higher since we obtained the MIC values of 370 and 355 μg/mL, respectively. In our opinion, the differences in time-dependent evaporation losses of both compounds might be responsible for these slight MICs variations (Novy et al. 2014), which are probably caused by longer time necessary for preparation of chequerboard plate designed experiment than for simple MIC plate layout.

Considering their combinatory activity, thymol and carvacrol produced the additive antimicrobial effect against all strains tested. In several cases (10 combinations

of these volatile compounds in vapour and 15 combinations in broth), they reached ΣFIC values lower than 0.6, which can be considered as a strong additive interaction. The best result was found in vapour phase against standard strain of *S. aureus* ATCC 25923 at combination of 128 μg/mL of carvacrol and 16–256 μg/ mL of thymol (ΣFIC = 0.51) and in liquid phase against clinical isolate SA 4 at combination of 256 μg/mL of carvacrol and 256 μg/mL of thymol (ΣFIC = 0.53). In both cases, the addition of carvacrol resulted in almost 4-fold reduction in the MIC of thymol alone. Our results are in accordance with Lambert et al. (2001), who have reported that carvacrol and thymol in combination show additive effects against *S. aureus*. However, other authors have found synergistic and antagonistic effects (Gallucci et al. 2009; Guarda et al. 2011). These discrepancies can be caused by different methodologies for testing of antibacterial effect and *S. aureus* strains used. According to our best knowledge, this is the first report on additive interaction of carvacrol and thymol in vapour phase.

Both carvacrol and thymol are agents that have frequently been used in agricultural, pharmaceutical, food, and cosmetic products, whereas they take a prominent place in oral health care products such as mouthwashes and toothpastes (Kumar et al. 2013; Wang 2016). Due to their significant antimicrobial activities, thymol containing EOs are active ingredients of various commercial products used for treatment and alleviation of respiratory infections. These facts are confirming the safety status of both compounds to human health. Their harmlessness can also be supported by Food and Drug Administration Generally Recognised as a Safe (GRAS) List, which includes thymol and carvacrol containing plant materials such as *Thymus vulgaris* L., *T. zygis* var. *gracilis* Boiss, and *Oregano* spp. Regarding the inhalation toxicity, which is a crucial aspect of inhalation administration, the LD_{50} values for both compounds are not determined. However, the material safety data sheets for their technical grades note that it is irritating to humans when exposed by inhalation (M and U International 2008; EPA 1993). Although there are no data on safety of thymol and carvacrol vapours combination, it can be supposed that their combinatory effect can lower efficient doses and related possible toxicity of these compounds. As a result of this study, new method for screening of combinatory effect of volatile compounds simultaneously in vapour and liquid phase was developed and successfully tested on two volatile compounds, carvacrol and thymol.

The above-mentioned results demonstrate the validity of our novel broth volatilization chequerboard assay, which combines principles of classical microdilution

chequerboard test and broth microdilution volatilization method (Houdkova et al. 2017). Previously developed techniques based on disc volatilization method provide qualitative results only, while quantitative data (MIC endpoints) indicating the degree of susceptibility are necessary for proper determination of combinatory effects expressed as FICs. Although the MIC and FIC values can be recorded for vapours of EOs by disc volatilization method (Goni et al. 2009), it is well known that the diameter of the zone of inhibition is influenced by the rate of diffusion of the antimicrobial agent throughout the agar, which is the main limiting factor of the results quantification (Jorgensen & Ferraro 2009). In comparison with disc diffusion and volatilization tests, our new screening assay allows determination of combinatory effect of plant volatile compounds simultaneously in liquid and gaseous phase as well as it can easily compare MIC and FIC values in both liquid and solid media. Moreover, this method is also suitable for testing of a broad range of concentrations in one 96-well microtiter plate, so it greatly saves consumption of material and it is suitable for high-throughput screening. It has been roughly estimated that our new method is almost 9x cheaper than disc diffusion method (only agar and plastic consumption were used for calculation). Another advantage of our method is a possibility of automation of assay plate preparation by the automated pipetting machine. Although the new assay's advantages are obvious, the method does not solve specific limitations of previously developed techniques caused by physico-chemical properties of tested volatiles. Depending on their vapour pressure and evaporation temperature, the final concentrations of antimicrobials and their combinations may be affected by loses caused by the evaporation of volatile compounds during test preparation as well as by transitions between liquid and gaseous systems. For this reason, observed MICs and FICs of phenolic EO compounds 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 one-fourth, that means 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 in the well evenly. Therefore, in case of concentrations used in our experiment, they can be ranging from traces up to 341.3 μg/mL of air (for 1024 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/head-space techniques and gas chromatography/mass

spectrometry analysis. Despite the fact that interpretation of FIC data slightly above or below the critical theoretical cut-off of 1.0 as additive interaction seem to put a positive spin on findings (Odds 2003), we recommend the EUCAST scale that includes additive effect for evaluation our results of broth volatilization chequerboard method (EUCAST 2000). The reason is that final concentrations of antimicrobial agents used for calculation of MIC values are in fact lower due to their spontaneous transitions between liquid and gaseous systems. Nevertheless, the indicative FIC values obtained by broth volatilization chequerboard assay are suitable for interpretation of screening experiments focused on identification of combinatory interactions of volatile antimicrobial agents in vapour phase.

Table 3.1 *In vitro* inhibitory activity of thymol in combination with carvacrol against *S. aureus* in liquid phase

S. aureus strain	MICs alone (μg/mL)			Thymol in combination with listed carvacrol concentrations (μg/mL)									
	C	T	O	+ C 512		+ C 256		+ C 128		+ C 64		+ C 32	
				MIC	ΣFIC	MIC	ΣFIC	MIC	ΣFIC	MIC	ΣFIC	MIC	ΣFIC
SA ATCC 25923	512	455	0.25	16	1.04	39	0.58	171	0.62	313	0.80	341	0.80
SA ATCC 29213	512	512	0.5	32	1.06	171	0.83	313	0.86	853	1.79	512	1.06
SA ATCC 33591	967	1024	341	32	0.57	284	0.54	512	0.63	512	0.57	569	0.59
SA ATCC 33592	683	683	64	55	0.90	300	0.84	512	0.99	654	1.05	654	1.00
SA ATCC 43300	484	484	43	16	1.24	18	0.57	142	0.56	284	0.72	341	0.78
SA BAA 976	967	910	16	32	0.57	256	0.56	512	0.72	512	0.65	569	0.67
SA 1	740	740	3	36	0.80	178	0.62	370	0.69	512	0.84	512	0.80
SA 2	1024	1024	256	313	0.80	853	1.08	1024	1.13	1024	1.06	1024	1.03
SA 3	512	569	32	32	1.06	156	0.77	313	0.79	484	0.99	569	1.06
SA 4	967	967	128	32	0.57	256	0.53	512	0.67	512	0.60	625	0.70
SA 5	853	796	1	57	0.68	298	0.69	484	0.80	626	0.89	683	0.91
SA 6	1024	1024	1	46	0.55	313	0.56	512	0.63	512	0.56	569	0.59

ATCC: American type culture collection; C: Carvacrol; MIC: minimum inhibitory concentration – the values are expressed as an average from three independent experiments, each performed in triplicate (rounded to integers); O: Oxacillin; SA: *Staphylococcus aureus*; T: Thymol; ΣFIC: sum of fractional inhibitory concentrations; the combinatory effect is evaluated as follows: synergy ΣFIC ≤ 0.5; additive ΣFIC >0.5 and ≤1; indifferent ΣFIC >1 and ≤ 2 (rounded to 2 decimal places).

Table 3.2 *In vitro* inhibitory activity of thymol in combination with carvacrol against *S. aureus* in vapour phase

S. aureus strain	MICs alone (μg/mL)		Thymol in combination with listed carvacrol concentrations (μg/mL)									
	C	T	+ C 512		+ C 256		+ C 128		+ C 64		+ C 32	
			MIC	ΣFIC	MIC	ΣFIC	MIC	ΣFIC	MIC	ΣFIC	MIC	ΣFIC
SA ATCC 25923	484	398	16	1.11	18	0.58	105	0.51	199	0.63	228	0.66
SA ATCC 29213	370	355	32	1.60	71	0.95	185	0.91	427	1.39	398	1.30
SA ATCC 33591	626	626	32	0.89	117	0.60	313	0.71	370	0.69	427	0.75
SA ATCC 33592	484	484	27	1.12	89	0.71	356	0.99	455	1.07	484	1.07
SA ATCC 43300	427	427	16	1.37	16	0.71	89	0.55	199	0.65	256	0.70
SA BAA 976	796	796	39	0.72	281	0.66	398	0.67	512	0.75	427	0.58
SA 1	626	569	32	0.89	60	0.52	228	0.61	370	0.77	341	0.66
SA 2	1593	1707	512	0.63	967	0.74	1024	0.70	1024	0.66	1024	0.64
SA 3	569	512	32	0.98	128	0.71	284	0.78	427	0.95	370	0.78
SA 4	683	540	32	0.84	142	0.66	327	0.82	398	0.86	341	0.69
SA 5	967	967	57	0.59	274	0.54	427	0.57	540	0.62	540	0.59
SA 6	1138	1024	92	0.55	427	0.65	512	0.61	740	0.78	796	0.80

ATCC: American type culture collection; C: Carvacrol; MIC: minimum inhibitory concentration – the values are expressed as an average from three independent experiments, each performed in triplicate (rounded to integers); O: Oxacillin; SA: *Staphylococcus aureus*; T: Thymol; ΣFIC: sum of fractional inhibitory concentrations; the combinatory effect is evaluated as follows: synergy ΣFIC ≤ 0.5; additive ΣFIC > 0.5 and ≤ 1; indifferent ΣFIC > 1 and ≤ 2 (rounded to 2 decimal places)..

3.4 Conclusions

New screening method for determination of combinatory antimicrobial effect in liquid and gaseous phase has been developed in this study. Two volatile compounds, carvacrol and thymol, were successfully tested in both phases by the new broth volatilization chequerboard assay against twelve strains of *S. aureus* and their MIC and FIC values were obtained. To the best of our knowledge, this is the first report on additive interaction of thymol and carvacrol in the vapour phase. Moreover, according to our results, this new effective high-through-put screening method is suitable for simple and rapid determination of combinatory antibacterial potential of plant volatiles at different concentrations and it enables evaluation and comparison of combinatory effect of two volatile compounds simultaneously in liquid and gaseous phase.

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4 Antistaphylococcal effect of interactions between a volatile compound and an essential oil

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Author contribution: Marie Strakova (born Netopilova) prepared the concept and design of the study as well as she performed experiments focused on antistaphylococcal effect of essential oil and plant compound combination. She also analysed chemical composition of the essential oil, processed the obtained data, and prepared manuscript including its required revisions.

4.1 Introduction

Antibiotic resistance is nowadays occurring in nearly all bacteria that infect people, including *Staphylococcus aureus* (Li & Webster 2018). This pathogen is a leading cause of bacterial infections worldwide and has been responsible for broad spectrum of diseases, ranging from superficial skin and soft tissue infections to life-threatening infections such as bacteraemia, endocarditis, osteomyelitis or pneumonia (Reddy et al. 2017). Due to its potential for rapid acquisition of drug resistance, this bacterium is considered to be one of the most serious pathogens in humans (French 2010). Currently, the global spread of methicillin-resistant *S. aureus* (MRSA) is one of the most serious public health challenges, because besides β-lactam antibiotics, MRSA strains have emerged with concomitant resistance to other groups of antibiotics such as aminoglycosides, fluoroquinolones, glycopeptides, macrolides, and tetracyclines (Akpaka et al. 2017). Since *S. aureus* is a pathogen associated with a wide range of infections affecting the respiratory tract, taking up antibiotics through inhalation could be one way of administering the drug. At another level, and in view of the ever-increasing resistance against antibiotics, a possible strategy for increasing efficiency in fighting *S. aureus*-related diseases, an antimicrobial combinatory effect may be used. An example of such treatment is the combination of fosfomycin and tobramycin, a novel treatment that is in the late-stage development of an inhalation therapy of cystic fibrosis, and that produced activity against a broad spectrum of bacteria, including MRSA (Quon et al. 2014).

Increase in bacterial resistance to antibiotics has renewed the interest in plant products as sources of alternative/adjunct antimicrobial agents to control pathogenic microorganisms (Hyldgaard et al. 2012) and caused resurgence in the use of herbal medicines worldwide. Plants serve as significant sources of volatile compounds (e.g. terpenes, phenylpropanoids, alkaloids and fatty and amino acid derivatives) (Dudareva et al. 2013) that are present in different plant parts in the form of essential oils (EOs) exhibiting various biological properties that include antibacterial, antifungal, and antiviral effects (Chouhan et al. 2017). Currently, a broad spectrum of phytochemicals and their mixtures, including EOs, are used as antibacterial and antifungal agents to treat or reduce the risk of various infectious diseases. An example of such herbal medical product is GeloMyrtol (G. Pohl-Boskamp, Hohenlockstedt,

Germany) that is used for the treatment of respiratory diseases. This product, recommended for reducing the risk of acute exacerbations from chronic bronchitis is obtained from various EOs produced by plants such as *Citrus limon*, *C. sinensis*, *Eucalyptus globulus*, and *Myrtus communis* (Kokoska et al. 2019). In general, antibacterial activity of any EO may depend on one major compound only. However, new findings show that interactions with other compounds in the oils are also important (Chouhan et al. 2017) whereas possible synergistic or antagonistic effects between EO constituents can either significantly enhance or reduce activities of single compounds (Hadacek 2002). Although numerous studies have focused on the interactions between EOs and volatile constituents in liquid phase (Bassole & Juliani 2012), there is only a limited number of studies dealing with their combinatory effects in vapour phase (Aguilar-Gonzalez et al. 2015).

One of the species with a great tradition in herbal medicine is *Cinnamomum cassia*, an evergreen tree native to southern China (Firmino et al. 2018) that was primarily used for the treatment of diarrhoea, upset stomach, bad breath, and other digestive problems, as well as for relief of poor appetite, nausea, cramps, and intestinal gas (Hoehn & Stockert 2012). Today, the German Commission E recognizes the use of two cinnamon species (*Cinnamomum verum* and *C. cassia*) to treat loss of appetite, dyspeptic complaints, bloating and flatulence (Costello et al. 2016). It is also thought that *C. c*can be used effectively to lower blood glucose levels in type 2 diabetes mellitus (Hoehn & Stockert 2012), and numerous food supplements which are based on *C. cassia* can be currently found on the market. Furthermore, *C. cassia* EO (CCEO) possesses antibacterial effects whereas its antistaphylococcal activity was previously described by several authors (Ooi et al. 2006; Melo et al. 2015; Firmino et al. 2018). It has also been tested in combination with classic antibiotics against multidrug-resistant bacteria (Atki et al. 2019). 8-hydroxyquinoline (8-HQ, synonyms: 8-oxychinolin, 8-quinolinol, oxine), a quinoline alkaloid previously found in the roots of *Centaurea diffusa* and *Sebastiania corniculata* (Kim et al. 2006; Yang et al. 2012), is a plant-derived volatile agent that is used as a preservative in cosmetics (Andersen 2006), as fungicide and insecticide in agriculture, as well as in development of new drugs based on its derivatives (Fernandez-Bachiller et al. 2010; Prachayasittikul et al. 2013). It has also been reported to exhibit antibacterial activities, including antistaphylococcal effects (Prachayasittikul et al. 2013; Houdkova et al. 2017). Moreover, synergistic inhibitory activity of 8-HQ with its metal

chelates has been reported for various fungi (Gershon et al. 1989) and yeasts (Nicoletti et al. 1999). Furthermore, Houdkova et al. (2017) also documented its antibacterial effect against *S. aureus* (and other bacteria) in gaseous phase.

Based on the results of our preliminary experiments performed as combinations of different EOs (*Armoracia rusticana*, *C. cassia*, *C. verum*, *Cymbopogon flexuosus*, *Elettaria cardamomum*, *Syzygium aromaticum*, and *Vetiveria zizanioides*) with various plant-derived volatiles (8-HQ, carvacrol, cineole, terpinen-4-ol, thymol, thymoquinone, *α*-pinene) against *S. aureus* ATCC 29213, the combination of CCEO with 8-HQ was selected for more detailed evaluation due to the lowest fractional inhibitory concentration (FIC) values that it had produced when tested (unpublished data). Moreover, to the best of our knowledge, the combinatory antistaphylococcal activity of CCEO and 8-HQ have not previously been studied. Therefore, we decided to test the interactions between these two agents in both liquid and vapour phases against standard strains and clinical isolates of *S. aureus*.

4.2 Materials and methods

4.2.1 Chemicals

Oxacillin (86.3%), thiazolyl blue tetrazolium bromide (MTT) and 8-HQ (99%) were purchased from Sigma-Aldrich (Prague, CZ). Dimethylsulfoxide (DMSO) and n-hexane were obtained from Penta (Prague, CZ) and Merck KGaA (Darmstadt, DE), respectively. Methyl octanoate and other standards (*α*-pinene, borneol, bornyl acetate, camphene and caryophyllene) were purchased from Sigma-Aldrich, Prague, CZ.

4.2.2 Plant material and preparation of essential oil

Dried bark of *C. cassia* was purchased from a commercial supplier (U Salvatora, Prague, CZ). After grounding and homogenization by a Grindomix apparatus (GM100 Retsch, Haan, DE), the residual moisture (15.82 %) was determined gravimetrically at 130 °C for 1 h by Scaltec SMO 01 analyser (Scaltec Instruments, Gottingen, DE) according to the Official Methods of Analysis of AOAC INTERNATIONAL (2012). CCEO was obtained by hydrodistillation of dried plant material in 1 L of distilled water using a Clevenger-type apparatus (Merci, Brno, CZ) as described in the European

pharmacopoeia (2013). EO thus obtained was subsequently stored in sealed glass vials at 4 °C and yield (based on dry plant weight) of CCEO was calculated, with a result of 0.88 % (v/w).

4.2.3 Bacterial strains and culture media

Twelve antibiotic-resistant and sensitive forms of *S. aureus* strains were used in this study. Standard strains of the American Type Culture Collection (ATCC) 25923, 29213, 33591, 33592, 43300, and BAA 976 were purchased from Oxoid (Basingstoke, UK) on ready-to-use bacteriological Culti-Loops. Clinical isolates (SA 1-6) obtained from Motol University Hospital (Prague, CZ) were selected based on the previous antimicrobial susceptibility testing (data not shown) as representatives of methicillin-sensitive *S. aureus* (SA 1, SA 5, SA 6) and MRSA (SA 2, SA 3, SA 4) strains and were identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry as described in Rondevaldova et al. (2018).

Mueller-Hinton (MH) broth (Oxoid, Basingstoke, UK) was used as a cultivation medium and both MH agar (Oxoid, Basingstoke, UK) and MH broth were used as assay media. The pH of cation-adjusted MH broth was equilibrated to a final value of 7.6 with Trizma base (Sigma-Aldrich, Prague, CZ). Stock cultures of bacterial strains were cultivated in broth medium at 37 °C for 24 h prior to testing. The bacterial suspension's turbidity used for inoculation of both plate and lid, was adjusted to 0.5 McFarland standard by Densi-La-Meter II (Lachema, Brno, CZ) to reach the final concentration of 10^7 CFU/mL.

4.2.4 Antimicrobial assay

The antibacterial potential of CCEO in combination with 8-HQ in liquid and vapour phase was determined using broth volatilization chequerboard method previously developed by our team (Netopilova et al. 2018). The technique is based on the combination of classical microdilution chequerboard test and broth microdilution volatilization method (Houdkova et al. 2017) allowing determination of combinatory effect of plant volatile agents simultaneously in liquid and vapour phase as well as comparison of MIC and calculation of FIC values in both liquid and solid media. Experiments were performed in white, 96-well immunoplates (total well volume = 400 µL) covered by tight-fitting lids with flanges designed to reduce evaporation (SPL Life

Sciences, Naechon-Myeon, Republic of Korea). In the first part of the procedure, 30 µL of agar was pipetted into every flange on the lid (with exception of the outermost wells) and inoculated with 5 µL of bacterial suspension. Subsequently, both CCEO and 8-HQ were dissolved in DMSO and diluted in the broth medium to get the initial concentrations of 2048 µg/mL and 32 µg/mL respectively, with maximum DMSO content of 1%.

The preparation of plate assay and serial dilutions were performed by an automated pipetting platform Freedom EVO 100 equipped with a four-channel liquid handling arm (Tecan, Mannedorf, CH). In combinations, six two-fold serial dilutions of CCEO from horizontal rows were subsequently cross-diluted vertically by six two-fold serial dilutions of 8 HQ. The final volume in each well was 100 µL, with the exception of the outermost wells which were left empty to prevent edge leakage effect. After that, plates were inoculated by bacterial suspensions. Each plate also contained inoculated and non-inoculated broth which served as growth and sterility controls, respectively. Oxacillin was used as a positive control for verification of susceptibility of *S. aureus* strains in broth medium. After the inoculation, plate and lid were fasten together by clamps (Lux Tool, Prague, CZ), with handmade wooden pads for better fixing and incubated for 24 h at 37 °C. The DMSO assayed as the negative control at concentration of 1% did not inhibit any of *S. aureus* strains tested either in broth or agar media.

Minimum inhibitory concentrations (MICs) and combinatory effects in both liquid and vapour phases (i.e. in plates and on lids) were evaluated by visual assessment of bacterial growth after colouring of metabolically active bacterial colonies with 25 µL of MTT dye in a concentration of 600 µg/mL when the interface of colour changed from yellow and purple (relative to that of colours in control wells). An MIC is defined as the lowest concentration that visually inhibits bacterial growth compared to the compound free growth control and is expressed in µg/mL. The final MIC value presented in this work is the average of MICs obtained from three independent experiments that were performed in triplicate.

Combinatory effect of volatile compounds was determined based on results of calculated fractional inhibitory concentration indices (ΣFIC). For combination of compound A (CCEO) and compound B (8-HQ), the ΣFIC is calculated according to the following equation: $\Sigma FIC = FIC_A + FIC_B$, where $FIC_A = MIC_{A\ (in\ combination\ with\ B)} / MIC_{A\ (alone)}$, and $FIC_B = MIC_{B\ (in\ combination\ with\ A)} / MIC_{B\ (alone)}$ and evaluated according to the

European Committee on Antimicrobial Susceptibility Testing (EUCAST 2000). The ΣFIC index was interpreted as follows: synergistic interaction if ΣFIC ≤ 0.5; additive effect if ΣFIC > 0.5 and ≤ 1; indifferent if ΣFIC > 1 and ≤ 2; and antagonistic if ΣFIC ≥ 2.

4.2.5 Gas chromatography/mass spectrometry analysis (GC/MS analysis)

For determination of the main components of CCEO, GC/MS analysis was carried out using the dual-column/dual-detector gas chromatograph Agilent GC-7890B system equipped with an Agilent 7693 autosampler, two columns, i.e. a fused-silica HP-5MS column (30 m × 0.25 mm, film thickness 0.25 μm) and a DB-HeawyWAX column (30 m × 0.25 mm, film thickness 0.25 μm), and a flame ionisation detector (FID) coupled with a single quadrupole mass selective Agilent MSD-5977B detector (Agilent Technologies, Santa Clara, CA, USA). The operational parameters were helium as carrier gas at 1 mL/min injector temperature 250 °C for both columns. The oven temperature of both columns was raised after an isothermic period of 3 minutes from the temperature of 50 °C to 280 °C with heating rates of 3 °C/min until the temperature reached 120 °C, then 5 °C/min until 250 °C; after 5 minutes of holding time on 250 °C the heating rate was 15 °C/min until it reached 280 °C. The programme ended with an isothermic period of 20 min. CCEO was diluted in *n*-hexane for GC/MS at a concentration of 20 μL/mL and 1 μL of methyl octanoate was added as internal standard. One μL of the CCEO solution was injected in split mode (split ratio 1:50). The mass detector was set to the following conditions: ionisation energy 70 eV, ion source temperature 230 °C, scan time 1 s, mass range 40–600 m/z.

Identification of constituents was based on comparison of their retention indices (RI) and retention times (RT) and spectra with the National Institute of Standards and Technology Library ver. 2.0.f (NIST, USA), as well as with authentic standards and literature (Adams 2007). The RI was calculated for compounds separated by the HP5-5MS column using the retention times of *n*-alkanes series ranging from C_8 to C_{40} (Sigma-Aldrich, Prague, CZ). For the analysed EO, the final number of compounds was computed 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.

4.3 Results

Results of our *in vitro* antimicrobial interactions between CCEO and 8-HQ showed the additive effects against all 12 *S. aureus* strains in both liquid and vapour phases. Based on the GC/MS analysis, we identified 26 compounds in total in the EO of *C. cassia* bark, where the majority of compounds present in the tested EO belonged to the monoterpenoid and sesquiterpenoid groups.

4.3.1 GC/MS analysis

In total, we evidenced 26 compounds in the CCEO using both HP-5MS/DB-HeawyWAX columns, representing 99.27/98.93 % of their total contents. The complete chemical composition of CCEO is provided in Table 4.1. Although the analysis showed that the most numerous constituents of *C. cassia* bark EO were monoterpenoids and sesquiterpenoids, (E)-cinnamaldehyde was the predominant compound representing 86.48/86.64 %. Other compounds detected in significantly lower amounts were cinnamyl acetate (3.53/6.66 %), *α*-copaene (1.57/0.95 %), bornyl acetate (0.90/0.65 %), and caryophyllene (1.03/0.65 %). Two compounds, δ-cadinene and sabinene, were only found by HP-5MS column (0.79 and 0.21 %, respectively), whereas seven compounds (caryophyllene oxide, coumarin, eugenol, humulene, linalool, *β*-pinene, *γ*-muurolene) were only detected present by DB-HeawyWAX column.

4.3.2 Antimicrobial activity

The detailed results of the *in vitro* growth-inhibitory effect of individual MICs of CCEO and 8-HQ against 12 staphylococcal strains, including clinical isolates, as well as the MICs of their combinations with corresponding ΣFIC values are summarized in Tables 4.2 and 4.3 for the liquid and vapour phase, respectively. Results show that 8-HQ exhibited a strong antistaphylococcal effect with MICs ranging from 7 to 20 µg/mL and from 2 to 8 µg/mL in agar and broth media, respectively, while respective CCEO MICs were ranging from 512 to 853 µg/mL in vapour phase and from 512 to 1136 µg/mL in liquid phase. Considering their combinatory activity, CCEO in combination with 8-HQ produced an additive antimicrobial effect against all strains tested. In several cases (i.e. for 1 combination of these volatile agents in the vapour phase and 5 combinations in broth), they reached ΣFIC values lower than 0.6, which can be considered as a strong

additive interaction. The most effective combination inhibiting *in vitro S. aureus* growth was found in the liquid phase against strain ATCC 29213 at 71 µg/mL of CCEO and 1 µg/mL of 8-HQ (ΣFIC=0.503), and in the vapour phase against a clinical isolate of SA 6 at 455 µg/mL of CCEO and 0.5 µg/mL of 8-HQ (ΣFIC=0.564).

Table 4.1 Chemical composition of *C. cassia* bark essential oil.

	[1] RI		Component	[2] C	[3] Column		[4] Identification	
					HP-5MS	DB-HeawyWAX	HP-5MS	DB-HeawyWAX
	Obs.	Lit.			[%]	[%]		
1	930	939	*α*-Pinene	MH	0.47	0.21	GC/MS, RI, Std	GC/MS
2	945	954	Camphene	MH	0.23	0.11	GC/MS, RI, Std	GC/MS
3	960	952	Benzaldehyde	A	0.01	0.18	GC/MS, RI	GC/MS
4	973	975	Sabinene	MH	0.21	-	GC/MS, RI	-
5	1027	1029	Limonene	MH	0.27	0.16	GC/MS, RI	GC/MS
6	1030	1031	Eucalyptol	MO	0.84	0.48	GC/MS, RI	GC/MS
7	1164	1162	Benzenepropanal	A	0.34	0.65	GC/MS, RI	GC/MS
8	1167	1169	Borneol	MO	0.03	0.27	GC/MS, RI, Std	GC/MS
9	1178	1177	Terpinen-4-ol	MO	0.50	0.40	GC/MS, RI	GC/MS
10	1191	1188	*α*-Terpineol	MO	0.91	0.70	GC/MS, RI	GC/MS
11	1222	1219	Z-Cinnamaldehyde	A	0.27	0.59	GC/MS, RI	GC/MS
12	1275	1270	E-Cinnamaldehyde	A	86.48	84.64	GC/MS, RI	GC/MS
13	1288	1288	Bornyl acetate	MO	0.90	0.65	GC/MS, RI, Std	GC/MS
14	1380	1374	*α*-Copaene	SH	1.57	0.95	GC/MS, RI	GC/MS
15	1419	1412	*α*-Bergamotene	SH	0.48	0.29	GC/MS, RI	GC/MS
16	1425	1419	Caryophyllene	SH	1.03	0.65	GC/MS, RI, Std	GC/MS
17	1449	1446	Cinnamyl acetate	O	3.53	6.66	GC/MS, RI	GC/MS
18	1505	1499	*α*-Muurolene	SH	0.40	0.25	GC/MS, RI	GC/MS
19	1530	1523	*δ*-Cadinene	SH	0.79	-	GC/MS, RI	-
20	-	-	*β*-Pinene	MH	-	0.11	-	GC/MS
21	-	-	Linalool	MO	-	0.11	-	GC/MS
22	-	-	Humulene	SH	-	0.09	-	GC/MS
23	-	-	*γ*-Muurolene	SH	-	0.09	-	GC/MS

24	-	-	Caryophyllene oxide	SO	-	0.11	-	GC/MS
25	-	-	Eugenol	SO	-	0.46	-	GC/MS
26	-	-	Coumarin	O	-	0.14	-	GC/MS
			Chemical classes					
			Aldehydes		87.11	86.05		
			Monoterpene hydrocarbons		1.19	0.59		
			Oxygenated monoterpenes		3.17	3.06		
			Sesquiterpene hydrocarbons		4.27	2.32		
			Oxygenated hydrocarbons		-	0.11		
			Others		3.53	6.80		
			Total identified [%]		**99.27**	**98.93**		

[1)] RI = retention indices; Exp = retention indices determined relative to a homologous series of n-alkanes (C_8-C_{40}) on a HP-5MS column, Lit = literature RI values (Adams, 2007; NIST, 2019); [2)] C = Class; A - Aldehydes, MH - Monoterpene hydrocarbons, MO - Oxygenated monoterpenes, O - Others, SH - Sesquiterpene hydrocarbons, SO - Oxygenated sesquiterpenes; [3)] Column = composition of essential oil detected on HP-5MS and DB-HeawyWAX columns; [%] = relative percentage content; - = not detected; [4)] 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 matching literature database; Std = constituent identity confirmed by co-injection of authentic standards

Table 4.2 *In vitro* inhibitory activity of *C. cassia* EO in combination with 8-hydroxyquinoline against *S. aureus* in liquid phase

Staphylococcus aureus strains	MICs alone (µg/mL)			CCEO in combination with listed 8-HQ concentrations (µg/mL)			
				+ HQ 1		+ HQ 0.5	
	CCEO	8-HQ	O	MIC	ΣFIC	MIC	ΣFIC
SA ATCC 25923	1136	8	0.5	469	0.521	768	0.708
SA ATCC 29213	1024	2	0.5	71	0.503	683	0.883
SA ATCC 33591	512	2	256	50	0.597	256	0.750
SA ATCC 33592	683	3	128	213	0.746	910	1.619
SA ATCC 43300	910	8	85	188	0.659	1024	1.708
SA ATCC BAA 976	853	3	64	178	0.563	1024	1.500
SA 1	626	4	2	302	0.679	740	1.356
SA 2	512	4	256	156	0.589	512	1.142
SA 3	967	4	171	427	0.728	740	0.897
SA 4	1024	2	256	160	0.637	512	0.764
SA 5	626	3	1	270	0.833	910	1.698
SA 6	512	2	1	57	0.611	284	0.806

8-HQ: 8-hydroxyquinoline; ATCC: American type culture collection; CCEO: *Cinnamomum cassia* essential oil; MIC: minimum inhibitory concentration – the values are expressed as an average from three independent experiments, each performed in triplicate (rounded to integers); O: Oxacillin; SA: *Staphylococcus aureus*; ΣFIC: sum of fractional inhibitory concentrations; the combinatory effect is evaluated as follows: synergy ΣFIC ≤0.5; additive ΣFIC>0.5 and≤1; indifferent ΣFIC>1 and≤2 (rounded to 3 decimal places).

Table 4.3 *In vitro* inhibitory activity of *C. cassia* EO in combination with 8-hydroxyquinoline against *S. aureus* in vapour phase

Staphylococcus aureus strains	MICs alone (µg/mL)			CCEO in combination with listed 8-HQ concentrations (µg/mL)			
				+ HQ 1		+ HQ 0.5	
	CCEO	8-HQ	O	MIC	ΣFIC	MIC	ΣFIC
SA ATCC 25923	512	7	-	256	0.667	370	0.806
SA ATCC 29213	853	15	-	512	0.683	569	0.717
SA ATCC 33591	512	9	-	313	0.726	341	0.724
SA ATCC 33592	683	10	-	626	0.993	683	1.052
SA ATCC 43300	683	11	-	597	0.990	512	0.832
SA ATCC BAA 976	683	18	-	512	0.807	512	0.779
SA 1	683	20	-	512	0.805	569	0.876
SA 2	569	9	-	313	0.670	341	0.668
SA 3	740	16	-	569	0.863	569	0.831
SA 4	740	10	-	512	0.792	512	0.738
SA 5	796	11	-	683	0.993	683	0.941
SA 6	853	12	-	455	0.605	455	0.564

8-HQ: 8-hydroxyquinoline; ATCC: American type culture collection; CCEO: *Cinnamomum cassia* essential oil; MIC: minimum inhibitory concentration – the values are expressed as an average from three independent experiments, each performed in triplicate (rounded to integers); O: Oxacillin; SA: *Staphylococcus aureus*; ΣFIC: sum of fractional inhibitory concentrations; the combinatory effect is evaluated as follows: synergy ΣFIC ≤0.5; additive ΣFIC>0.5 and≤1; indifferent ΣFIC>1 and≤2 (rounded to 3 decimal places).

4.4 Discussion

As far as the chemical composition of CCEO is considered, our results obtained from GC/MS analysis correspond well with those in previously published papers (e.g. Ooi et al. 2006; Firmino et al. 2018) confirming that (E)-cinnamaldehyde is occurring in CCEO in significantly higher quantities (85.06 %, and 90.22%, respectively) than other compounds. Similarly, the results of antistaphylococcal activity of 8-HQ obtained in this study correspond well with our previous findings (Houdkova et al. 2017) as well as with those of Yang et al. (2013) who reported a MIC of 10 µg/mL against *S. aureus* KCCM 11335 in liquid phase. The obtained CCEO MICs are in accordance with results of Ooi et al. (2006), who also reported an inhibitory effect of CCEO against *S. aureus* with a liquid phase MIC of 600 µg/mL. On the other hand, Firmino et al. (2018) reported a MIC value of 250 µg/mL against *S. aureus* ATCC 6538 but this difference can be probably explained by the different *S. aureus* strain used. Similarly, Atki et al. (2019) evidenced CCEO MIC value of 4.88 µg/mL against *S. aureus* ATCC 25923 which is many times lower than in our own study; however, this might be caused by a different chemical composition of the EO and by the lower density of bacterial suspension used (10^6 CFU/ml). Although components present in the highest concentrations in the EOs are not necessarily responsible for explaining the greatest part of activity (Chouhan et al. 2017), it can be assumed that the antistaphylococcal activity caused by our CCEO may be caused by action of (E)-cinnamaldehyde, which is its major compound and according to Ooi et al. (2006) shows potent and comparable antibacterial activity with CCEO itself. However, the other compounds present in the EO (e.g. α-copaene, caryophyllene, cinnamyl acetate etc.) might contribute to the total antimicrobial effect of the CCEO as well.

Several authors have demonstrated synergistic actions as well as additive effects of CCEO in combination with conventional antibiotics against different bacterial strains. For example, Atki et al. (2019) reported synergistic effects of CCEO against *S. aureus* when combined with ampicillin or chloramphenicol, and additive effects when combined with streptomycin. Furthermore, interactions between different EOs have recently been studied in view of augmenting their antibacterial effect without increasing their concentration. The effect of a combination of *Cinnamomum* sp. EO with other EOs or a volatile compound have been reported as well. Clemente et al. (2016) evidenced that the

combination of *C. verum* bark EO fortified with cinnamaldehyde and allyl isothiocyanate showed an additive effect against *Escherichia coli*, *Pseudomonas aeruginosa*, and some other bacteria species. However, only a few scientists searching for antimicrobial synergy of EOs and/or volatile phytochemicals also addressed how these compounds would behave in gaseous phase. Although there are only a few studies reporting the combinatory effects of certain *Cinnamomum* species with other EOs or their volatile compounds in the vapour phase (e.g. Clemente et al. 2016), there is no report on the combinatory effect of CCEO vapours with other EOs or volatile compounds. In contrast to the well-researched CCEO, the interactions of 8-HQ with other antimicrobial agents have not previously been studied. Moreover, to the best of our knowledge, this is the first report on an antimicrobial combinatory effect of CCEO with 8-HQ in vapour phase as well as the first investigation of antibacterial activity of these individual compounds in combination with other agents in the gaseous phase.

According to the United States Food and Drug Administration (USFDA), *C. cassia* is generally recognized to be safe (GRAS) in amounts commonly found in food (USFDA 2019). In addition, cinnamon oil as well as cinnamaldehyde demonstrate very significant and effective antimicrobial activities against a broad range of bacterial pathogens, whereas results reported from animal studies further confirm that cinnamaldehyde is non-toxic and non-carcinogenic to mammals (Ooi et al. 2006). The European Chemicals Agency (ECHA) reported that the acute oral median lethal dose (LD_{50}) of *C. cassia* oil in rats was estimated to be greater than 2000 mg/kg of body weight (bw). Similarly, the LD_{50} of cinnamaldehyde was found to be 2220 mg/kg of bw, which indicates that cinnamaldehyde does not exhibit acute toxicity to rats when taken orally (ECHA 2014). Cinnamaldehyde added to the feed of rats at 1000 and 2500 mg/kg of feed for 16 weeks caused no adverse effects (Ooi et al. 2006). On the other hand, based on a mice acute oral study of Dickhaus and Heisler (1981) as cited in European Chemicals Agency (ECHA 2014), who set 8-HQ LD_{50} values at 177 mg/kg bw, and supported by a document of the European Medicines Agency that reported oral LD_{50} values ranging from 220 to 280 mg/kg bw in mice, it was proposed to classify 8-HQ as acutely toxic (as H301, category 3). However, the MICs obtained in our study for the liquid phase were ranging from 2 to 8 µg/mL, which most likely responds to the values many times lower. Regarding inhalation toxicity, which is a crucial aspect of inhalation administration, median lethal concentration (LC_{50}) values were not determined neither for 8-HQ, nor for *C. cassia* for

the inhalation route. However, the data on low acute dermal toxicity of rats (LD_{50} > 10000 mg/kg bw) of 8-HQ suggest its possible inhalation safety (EFSA 2011). It can similarly be assumed that LC_{50} values of *C. cassia* might be close to those of cinnamaldehyde which were estimated to be 68.88 mg/L for rats (ECHA 2014). Based on the latter value it can be predicted that neither cinnamaldehyde nor CCEO are toxic through inhalation. The most common adverse effects of *C. cassia* include the occurrence of allergic reactions to cinnamaldehyde, which is believed to be the allergen compound for some sensitive people in cosmetics, food and perfumes as well as in toothpastes (Ooi et al. 2006). Although MICs of CCEO and 8-HQ are higher than those of conventional antibiotics (such as amphotericin B, oxacillin, streptomycin, etc.), the natural antibiotic combinatory effects of these agents may be an alternative solution to circumvent the antibiotic resistance of a number of pathogenic microbes, which is a global medical problem nowadays. Due to the safety of CCEO and cinnamaldehyde and the very low 8-HQ MIC observed in our study, as well as on the basis of the research of Rajamanickam et al. (2019) who confirmed that cinnamaldehyde is an effective phytochemical against most bovine respiratory diseases, it can be assumed that the results of CCEO and 8-HQ combinations could be potentially applied in development of various pharmaceutical applications that are based on volatile antimicrobials. These combinations could decrease the minimum effective dose of the agents, thus reducing their possible adverse effects and treatment costs. However, further research to achieve a better understanding of the action mechanisms, further *in vivo* experiments and clinical trials on CCEO or its active compounds in combination with 8-HQ are still necessary to determine their pharmacodynamics and pharmacokinetics. The possible future antimicrobial combinatory testing of CCEO together with 8-HQ derivatives (e.g. 5-chloro-7-iodo-8HQ, 5-chloro-8HQ), which have previously been reported to exhibit a significant antimicrobial activity against different strains of *S. aureus* (Cherdtrakulkiat et al. 2016; Cherdtrakulkiat et al. 2019), could also bring a better understanding of interactions between these agents.

4.5 Conclusions

In summary, the present study reports the results of antistaphylococcal interactions between two volatile agents, CCEO and 8-HQ, that were tested by a broth

volatilization chequerboard assay. This combination exhibited additive effects against all 12 *S. aureus* strains in both liquid and vapour phases. To the best of our knowledge, this is the first report on additive effects of 8-HQ and CCEO combinations in both phases. Moreover, the chemical composition of CCEO was analysed by GC/MS using two capillary columns of different polarity. Although (E)-cinnamaldehyde was the prevailing phytochemical found in the tested EO, compounds belonging to the classes of monoterpenoids and sesquiterpenoids were the most numerous identified. These results can be potentially applied in development of various pharmaceutical applications that are based on volatile antimicrobials and can be used through inhalation therapy against respiratory infections caused by *S. aureus.* However, further research focusing on *in vivo* evaluation will have to be carried out in order to verify its potential practical use.

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5 Antistaphylococcal effect of interactions between two essential oils

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Author contribution: Marie Strakova (born Netopilova) prepared concept and design of the study as well as she performed experiments focused on antistaphylococcal combinatory effect of essential oils' interactions. She also analysed chemical composition of essential oils, processed the obtained data, and prepared manuscript including its required revisions.

5.1 Introduction

Staphylococcus aureus is a gram-positive bacterium that has been responsible for a broad spectrum of diseases, ranging from food poisoning and superficial skin and soft tissue infections to life-threatening infections such as bacteraemia, endocarditis, osteomyelitis, pneumonia, or toxic shock syndrome (Reddy et al. 2017). It is notorious for its ability to quickly become resistant to any antibiotic, which makes this bacterium one of the most serious pathogens in humans, and its treatment is often difficult (French 2010). In humans, *S. aureus* can occur as both a benign commensal and a harmful pathogen. Besides being a common colonizer of the skin, it also asymptomatically and permanently colonizes the anterior nostrils of up to 30% of the normal human population (Kluytmans & Wertheim 2005; Sakr et al. 2018), which is widely considered to be a predisposition of invasive infection (Prince 2013). Since *S. aureus* is a microorganism that is associated with a broad spectrum of infections affecting the respiratory tract, taking up antibiotics through inhalation could be one of its possible treatments. Moreover, the combination of two or even more antibiotic agents may be used as a possible strategy for increasing efficiency in fighting *S. aureus*-related diseases, including respiratory infections. An example of such treatment is a combination of fosfomycin and tobramycin that is currently in the late-stage development of an inhalation therapy of cystic fibrosis (MacLeod et al. 2012; Curxpharmaceuticals 2020). It was demonstrated that fosfomycin synergistically enhances the activity of tobramycin against a wide range of bacteria, including *S. aureus* (MacLeod et al. 2012; McCaughey et al. 2012). However, inhalation of solid antimicrobial agents, as well as the use of inhaler devices, may often be problematic, especially in children and the elderly (Ibrahim et al. 2015). Therefore, there is a need to search for new antimicrobial agents to combat bacteria affecting the respiratory tract and for easier ways how to deliver antimicrobials into the lower respiratory tract.

Recently, as concerns about the increasing bacterial resistance to conventional antibiotics are growing, the use of medicinal plants, their unique properties, and possibilities of applications are more frequently proposed as an option for treating these problems. The use of agents of complex chemical composition, such as essential oils (EOs), as well as the therapy based on a combination of drugs, have already shown to be generally effective strategies to overcome issues with microbial resistance. In general, the

antibacterial activity of any EO may depend on one major compound only; however, new findings show that interactions with other compounds in the oils are also important (Chouhan et al. 2017), whereas possible synergistic or antagonistic effects between EO constituents can either enhance or reduce activities of single compounds (Hadacek, 2002). Similarly, EOs, when used in combination, can initiate a synergistic antimicrobial effect. Various experiments focused on interactions between EOs and their volatile constituents have previously been conducted against numerous microorganisms. In the extensive review of Leigh-de Rapper and van Vuuren (2020) that was focused on EOs against pathogens of the respiratory tract, synergy was determined for 34% of the EOs combinations. However, only a limited number of studies dealing with the combinatory effects have been performed in the vapour phase, as recently reviewed in Houdkova and Kokoska (2020). One of the reasons is that there is only a limited number of assays suitable for qualitative evaluation of antimicrobial interactions of volatile agents in the gaseous phase. Recently, we have developed a new broth volatilization chequerboard method allowing evaluation of the antimicrobial activity of volatile agents in the vapour phase, of which the accuracy was verified on a combination of two plant-derived compounds (Netopilova et al. 2018) as well as on the combination of a compound with an EO (Netopilova et al. 2020). However, the usability of this method for the determination of the combinatory antimicrobial effect of two EOs has not been proven yet.

Origanum vulgare L. (oregano) and *Thymus vulgaris* L. (thyme) are aromatic spice herbs belonging to the Lamiaceae family. They are native to the Mediterranean region and neighboring countries (Modnicki & Balcerek 2009; Murillo-Amador et al. 2013), and in folk medicine, they have been used as remedies to treat respiratory disorders (e.g., coughs and bronchitis) as expectorants, dyspepsia as well as urinary tracts disorders (Modnicki & Balcerek 2009; Javed et al. 2013; Murillo-Amador et al. 2013; Teixeira et al. 2013). Although the principal components of oregano and thyme EOs are carvacrol and thymol, respectively, their chemical compositions vary depending on geographical region and season of collection (Faleiro et al. 2003). Both plants are also used in pharmaceutical industries, including the products for the treatment of respiratory infections. For example, the extract of *T. vulgaris* is used in two oral over-the-counter products Bronchipret Saft and Bronchipret TP (Bionorica, Neumarkt, Germany), which are used for the treatment of respiratory tract illnesses, cough, and bronchitis (Kokoska

et al. 2019). Furthermore, EOs derived from these plants have been shown to exhibit a broad range of considerable biological activities, including their antimicrobial effect, which has been mostly attributable to the presence of phenolic compounds, such as carvacrol and thymol (Santoro et al. 2007; Kacaniova et al. 2012; Fournomiti et al. 2015); however, other minor constituents such as monoterpene hydrocarbons *γ*-terpinene and *p*-cymene contribute to the antibacterial activity of the oils as well (Santoro et al. 2007; Fournomiti et al. 2015). To date, numerous studies concerned with the chemical composition and antimicrobial activity of *O. vulgare* and *T. vulgaris* EOs have been published. Differences between the antimicrobial activities of various chemotypes of these oils have been described (de Martino et al. 2009; Schmidt et al. 2012). Furthermore, it was also proved that the antimicrobial effect of these EOs might be comparable to their main component alone (Jafri & Ahmad 2020). Due to their antimicrobial properties, EOs (including *O. vulgare* and *T. vulgaris* EOs) could be used as alternatives to conventional antimicrobial agents, especially against antibiotic-resistant pathogens (Fournomiti et al. 2015). So far, numerous studies regarding the antibacterial activity of *O. vulgare* and *T. vulgaris* EOs alone against a wide range of microorganisms, including *S. aureus*, have been published (Ozkalp et al. 2010; Boskovic et al. 2015; de Carvalho et al. 2015). Both EOs have also previously been tested against *S. aureus* in combination with other EOs (Al-Bayati, 2008; Honorio et al. 2015) as well as with classic/conventional antibiotics (van Vuuren et al. 2009). Moreover, their synergistic and additive inhibitory activity with each other has previously been reported against *S. aureus* as well (Stojkovic et al. 2013; Gavaric et al. 2015). However, although there are numerous articles on the antistaphylococcal activity of *O. vulgare* and *T. vulgaris* EOs tested in the broth and agar, substantially fewer articles dealing with their antibacterial effects against *S. aureus* have been published using the vapour phase (Lopez et al. 2007; Nedorostova et al. 2009). Moreover, to the best of our knowledge, the combinatory antistaphylococcal activity of *O. vulgare* EO and *T. vulgaris* EO have not previously been studied in the gaseous phase.

Based on the results of our preliminary screenings performed as several combinations of different EOs (*Cinnamomum cassia*, *C. verum*, *Cymbopogon flexuosus*, *O. vulgare*, *Syzygium aromaticum*, and *T. vulgaris*) against *S. aureus* ATCC 29213 (the lowest fractional inhibitory concentration (FIC) values in the vapour phase ranged from 0.59 to 1.25), the combination of *O. vulgare* EO with *T. vulgaris* EO was selected for more detailed evaluation due to its lowest FIC values that it had produced (unpublished data).

Therefore, the aim of the present study was to determine the antibacterial combinatory potential of EOs hydrodistilled from *O. vulgare* and *T. vulgaris* against standard strains and clinical isolates of *S. aureus* in both the vapour and liquid phases. Since the methods currently available for the determination of antimicrobial interactions of EOs in the vapour phase are based on disc diffusion assay, which yields only qualitative information about the antimicrobial agent combination, the accuracy of these techniques is limited because it is difficult to distinguish indifferent from synergistic interaction. For this reason, the validation of the qualitative broth volatilization chequerboard method for testing of combinatory antimicrobial effect of two different EOs was an additional objective of this study.

5.2 Materials and methods

5.2.1 Chemicals

Oxacillin (86.3%, CAS: 7240-38-2) and thiazolyl blue tetrazolium bromide (MTT, 98%, CAS: 298-93-1) were purchased from Sigma-Aldrich (Prague, CZ), whereas dimethylsulfoxide (DMSO, CAS: 67-68-5) and *n*-hexane (CAS: 110-54-3) were obtained from Penta (Prague, CZ). Methyl octanoate (≥99.8%, CAS: 111-11-5) and other standards (3-carene (99%, CAS: 498-15-7), borneol (97%, CAS: 464-45-9), bornyl acetate (95%, CAS: 5655-61-8), camphene (97.5%, CAS: 79-92-5), camphor (98%, CAS: 464-49-3), carvacrol (97%, CAS: 499-75-2), caryophyllene oxide (99%, CAS: 1139-30-6), linalool (97%, CAS: 78-70-6), *p*-cymene (99%, CAS: 99-87-6), thymol (99%, CAS: 89-83-8), α-pinene (≥99%, CAS: 7785-70-8), α-terpinene (85%, CAS: 99-86-5), β-caryophyllene (98.5%, CAS: 87-44-5), β-pinene (≥99.0%, CAS: 18172-67-3), and γ-terpinene (97%, CAS: 99-85-4)) were purchased from Sigma-Aldrich, Prague, CZ.

5.2.2 Plant material and preparation of essential oils

The dried aerial parts of *O. vulgare* and *T. vulgaris* were purchased from a commercial supplier (U Salvatora, Prague, CZ). Initially, they were homogenized by a Grindomix apparatus (GM100 Retsch, Haan, DE). Subsequently, the residual moisture contents of both samples were determined gravimetrically at 130 °C for 1 h by Scaltec SMO 01 analyser (Scaltec Instruments, Gottingen, DE) in triplicate, and results were

expressed as arithmetic averages according to the Official Methods of Analysis of the Association of Official Agricultural Chemists (2012). Both EOs were obtained by hydrodistillation of dried plant material in 1 L of distilled water using a Clevenger-type apparatus (Merci, Brno, CZ) according to the procedure described in the European Pharmacopeia (2013) and stored in sealed glass vials at 4 °C.

5.2.3 Bacterial strains and culture media

In this study, 12 strains of *S. aureus* were used, including antibiotic-resistant and sensitive forms. Standard strains of the American Type Culture Collection (ATCC) 25923, 29213, 33591, 33592, 43300, and BAA 976 were purchased from Oxoid (Basingstoke, UK) on ready-to-use bacteriological Culti-Loops. Clinical isolates (SA 1-6) obtained from Motol University Hospital (Prague, CZ) were selected based on the previous antimicrobial susceptibility testing (data not shown) as representatives of methicillin-sensitive *S. aureus* (SA 1, SA 5, and SA 6) and methicillin-resistant *S. aureus* (SA 2, SA 3, and SA 4) strains and were identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry as described in Rondevaldova et al. (2018).

Mueller–Hinton (MH) broth was used as a cultivation medium, and both MH agar and MH broth purchased from Oxoid (Basingstoke, Hampshire, UK) were used as assay media. The pH of cation-adjusted MH broth was equilibrated to a final value of 7.6 with Trizma base (Sigma-Aldrich, Prague, CZ). Stock cultures of bacterial strains were cultivated in broth medium at 37 °C for 24 h prior to the testing. The bacterial suspension's turbidity used for the inoculation of both plate and lid, was adjusted to 0.5 McFarland standard by Densi-La-Meter II (Lachema, Brno, CZ) to reach the final concentration of 10^7 CFU/mL.

5.2.4 Antimicrobial assay

The *in vitro* antibacterial combinatory potential of *O. vulgare* EO in combination with *T. vulgaris* EO in the liquid and vapour phase was determined using a broth volatilization chequerboard assay previously developed in our laboratory (Netopilova et al. 2018). The method is based on the combination of classical microdilution chequerboard test and broth microdilution volatilization technique (Houdkova et al. 2017), allowing for the determination of interactions between EOs and/or plant volatile

agents simultaneously in liquid and vapour phase as well as comparison of MIC values and calculation of FICs in both liquid and solid media. Experiments were performed in white, 96-well immunoplates (total well volume = 400 µL) covered by tight-fitting lids with flanges designed to reduce evaporation (SPL Life Sciences, Naechon-Myeon, Republic of Korea). In the first part of the procedure, 30 µL of agar was pipetted into every flange on the lid (with the exception of the outermost wells) and inoculated with 5 µL of the bacterial suspension. Subsequently, both *O. vulgare* and *T. vulgaris* EOs were dissolved in DMSO and diluted in the broth medium to get the initial concentrations of 2048 µg/mL, with maximum DMSO content of 1%.

The preparation of plate assay and serial dilutions were performed by an automated pipetting platform, Freedom EVO 100, equipped with a four-channel liquid handling arm (Tecan, Mannedorf, CH). In combinations, six two-fold serial dilutions of oregano EO from horizontal rows were subsequently cross-diluted vertically by six two-fold serial dilutions of thyme EO. The final volume in each well was 100 µL, except for the outermost wells, which were left empty to prevent edge leakage effect. The plates were subsequently inoculated by bacterial suspensions using a 96-pin multi-blot replicator (National Institute of Public Health, Prague, CZ). Each plate also contained inoculated and non-inoculated broth, which served as growth and sterility controls, respectively. Oxacillin was used as a positive antibiotic control for verification of susceptibility of *S. aureus* strains in broth medium. The DMSO assayed as the negative control at a concentration of 1% did not inhibit any of *S. aureus* strains tested either in broth or agar media. After the inoculation, clamps (Lux Tool, Prague, CZ) were used to fasten the plate and lid together, with handmade wooden pads (size 8.5 × 13 × 2 mm) for better fixing, and microtiter plates were incubated for 24 h at 37 °C.

MIC values and combinatory effects in both liquid and the vapour phases (i.e., in plates and on lids) were evaluated by visual assessment of bacterial growth after colouring of metabolically active staphylococcal colonies with 25 µL of MTT dye in a concentration of 600 µg/mL when the interface of colour in broth and on agar changed from yellow and purple (relative to that of colours in control wells). The MIC values were defined as the lowest concentration that visually inhibited staphylococcal growth compared to the compound-free growth control and were expressed in µg/mL. The final MIC values presented in this work are the average of MICs obtained from three independent experiments that were performed in triplicate.

The combinatory effect of EOs was determined based on the value of ΣFIC. For the combination of agent A (*O. vulgare* EO) and agent B (*T. vulgaris* EO), the ΣFIC was calculated according to the following equation: $\Sigma FIC = FIC_A + FIC_B$, where $FIC_A = MIC_{A\ (in\ combination\ with\ B)} / MICA_{\ (alone)}$, and $FIC_B = MIC_{B\ (in\ combination\ with\ A)} / MICB_{\ (alone)}$ and evaluated according to EUCAST (2000). The ΣFIC index was interpreted as follows: synergistic interaction if $\Sigma FIC \leq 0.5$; additive effect if $\Sigma FIC > 0.5$ and ≤ 1; indifferent if $\Sigma FIC > 1$ and ≤ 2; and antagonistic if $\Sigma FIC \geq 2$.

5.2.5 GC/MS analysis

For determination of the main components of *O. vulgare* and *T. vulgaris* EOs, GC/MS analysis was performed using the dual-column/dual-detector gas chromatograph Agilent GC-7890B system equipped with autosampler Agilent 7693, two columns (fused-silica HP-5MS column (30 m × 0.25 mm, film thickness 0.25 μm) and a DB-HeawyWAX column (30 m × 0.25 mm, film thickness 0.25 μm)) and a flame ionization detector (FID) coupled with a single quadrupole mass selective Agilent MSD-5977B detector (Agilent Technologies, Santa Clara, CA, USA). Operational parameters were helium as carrier gas at 1 mL/min, injector temperature 250 °C for both columns. The oven temperature was raised for both columns from 50 °C to 280 °C. Initially, after an isothermic period of 3 min, the heating rate was 3 °C/min until the temperature reached 120 °C. Subsequently, the heating velocity increased to 5 °C/min until it reached 250 °C; and after 5 min of holding time on 250 °C, the heating rate increased to 15 °C/min until it reached 280 °C. Heating was followed by an isothermic period of 20 min. Both EOs were diluted in *n*-hexane for GC/MS at a concentration of 20 μL/mL, and for quantitative analysis, 1 μL of methyl octanoate was added as an internal standard. One μL of each EO solution was injected in split mode (split ratio 1:50). The mass detector was set to the following conditions: ionization energy 70 eV, ion source temperature 230 °C, scan time 1 s, mass range 40–600 m/z.

Identification of constituents was based on a comparison of their retention indices (RI) and retention times (RT) and spectra with the National Institute of Standards and Technology Library ver. 2.0.f (NIST, USA) (2020), as well as with authentic standards and literature (Umano & Shibamoto 1987; Stashenko et al 1996; Kaya et al. 1999; Ngassoum et al. 1999; Bassole et al. 2003; Avato et al. 2004; Lopes et al. 2004; Nebie et al. 2004; Lee et al. 2005; Adams 2007). The RI was calculated for compounds separated

by both HP5-5MS and DB-HeawyWAX columns using the retention times of n-alkanes series ranging from C_8 to C_{40} (Sigma-Aldrich, Prague, CZ). For each analysed EO, 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. Relative percentage contents of identified components have been determined using the FID data and indicated for both columns.

5.3 Results

5.3.1 Antimicrobial analysis

The detailed results of individual minimum inhibitory concentrations (MICs) of *O. vulgare* and *T. vulgaris* EOs against 12 strains of *S. aureus* including clinical isolates, as well as the MICs of their combinations with corresponding ΣFIC values are summarized in Table 5.1 and Table 5.2 for the vapour and liquid phases, respectively. Results show that *O. vulgare* EO exhibited an antistaphylococcal effect with MICs ranging from 427 to 796 μg/mL and from 512 to 1024 μg/mL in agar and broth media, respectively. Similar numbers were observed for *T. vulgaris* EO with MICs ranging from 427 to 796 μg/mL in the vapour phase and from 512 to 967 μg/mL in the liquid phase.

Considering their combinatory activity, EO of *O. vulgare* in combination with *T. vulgaris* EO produced an additive antimicrobial effect against all 12 strains tested. The combination profiles of four *S. aureus* strains are presented graphically in Figure 5.1. The isobole curves clearly show the additive effect against *S. aureus* strains tested, whereas the additive interactions can be read according to the curves indicating the borderline of additivity and synergy. In several cases (i.e., for one combination of these volatile agents in the vapour phase and four combinations in broth), they showed ΣFICs lower than 0.6, which can be considered a strong additive interaction, reaching values close to the synergistic effect. The most effective concentrations inhibiting the growth of *S. aureus* (SA) were found in the liquid phase against methicillin-resistant clinical isolate SA 2 at 512 μg/mL of *O. vulgare* EO and 32 μg/mL of *T. vulgaris* (ΣFIC = 0.53) and in the vapour phase against standard strain SA ATCC 29213 at 242 μg/mL of *O. vulgare* EO and 128 μg/mL of *T. vulgaris* EO (ΣFIC = 0.59). On average, the best FIC values were observed in both the liquid and vapour phases when the concentrations of *T. vulgaris* EO were 256

and 128 μg/mL. Based on the results, the optimum ratio of *T. vulgaris* and *O. vulgare* to achieve bacterial inhibition would be 0.5-2:1 in the vapour phase and 0.4-1.2:1 in the liquid phase.

5.3.2 GC/MS analysis

The yields of *O. vulgare* and *T. vulgaris* EOs in the dried weight of plant materials (containing 14.42% and 13.68% of residual moisture) for *T. vulgaris* were 1.5% and 1.2% (*v/w*), respectively. The complete chemical compositions of oregano and thyme EOs are provided in Table 5.3; Table 5.4, respectively. In EOs isolated from *O. vulgare* and *T. vulgaris*, 19 and 28 components have been identified using an HP-5MS column, representing 99.78% and 99.26% of their respective total content. Using DB-HeawyWAX column, 25 and 34 compounds were determined, which constitute 99.90% and 99.53% of the volatile oil, respectively. In total, 26 compounds were identified in the EO of *O. vulgare*, whereas 37 compounds were found in the EO isolated from *T. vulgaris*. The analysis showed that the most monoterpene hydrocarbons and oxygenated monoterpenes were the main groups of chemicals in both EOs.

In EO extracted from *O. vulgare*, carvacrol was the predominant compound representing 77.92 %/82.60 % when measured using HP-5MS/DB-HeawyWAX columns, respectively. Other compounds detected in significantly lower amounts were *p*-cymene and *γ*-terpinene with percentage values 8.25 %/5.63 % and 4.52 %/3.33 %. In EO obtained from *T. vulgaris*, thymol was the most abundant component representing 42.34 %/48.46 %, followed by *p*-cymene and *γ*-terpinene representing 24.08 %/18.00 %, and 13.37 %/10.61 % when measured using HP-5MS/DB-HeawyWAX columns, respectively.

Table 5.1 *In vitro* inhibitory activity of interactions between *O. vulgare* and *T. vulgaris* essential oils against *S. aureus* in vapour phase

S. aureus strains	MICs alone (µg/mL)			OVEO in combination with listed TVEO concentrations (µg/mL)									
	OVEO	TVEO	O	+ TVEO 512		+ TVEO 256		+ TVEO 128		+ TVEO 64		+ TVEO 32	
				MIC	ΣFIC	MIC	ΣFIC	MIC	ΣFIC	MIC	ΣFIC	MIC	ΣFIC
SA ATCC 25923	427	427	ND	16	1.24	59	0.74	149	0.65	242	0.72	299	0.78
SA ATCC 29213	683	569	ND	16	0.94	158	0.70	242	0.59	398	0.70	484	0.79
SA ATCC 33591	626	569	ND	16	0.94	112	0.63	270	0.67	313	0.61	370	0.65
SA ATCC 33592	796	484	ND	16	1.09	149	0.72	370	0.73	512	0.78	512	0.72
SA ATCC 43300	512	512	ND	16	1.03	92	0.68	228	0.69	398	0.90	455	0.95
SA ATCC BAA 976	484	484	ND	16	1.10	82	0.70	228	0.74	341	0.84	341	0.78
SA 1	683	512	ND	16	1.02	92	0.64	242	0.63	341	0.63	455	0.76
SA 2	683	626	ND	16	0.86	178	0.67	313	0.67	427	0.76	484	0.78
SA 3	455	455	ND	16	1.20	62	0.73	185	0.72	270	0.76	341	0.85
SA 4	484	484	ND	16	1.10	92	0.73	194	0.67	348	0.85	356	0.80
SA 5	427	427	ND	16	1.24	44	0.70	149	0.64	270	0.77	370	0.96
SA 6	740	796	ND	43	0.75	341	0.81	427	0.76	512	0.79	512	0.74

ATCC: American type culture collection; MIC: minimum inhibitory concentration, the values are expressed as an average from three independent experiments, each performed in triplicate (rounded to integers); ND: not determined; O: Oxacillin; OVEO: *O. vulgare* essential oil; SA: *Staphylococcus aureus*; TVEO: *Thymus vulgaris* essential oil; ΣFIC: sum of fractional inhibitory concentrations; the combinatory effect is evaluated as follows: synergy ΣFIC ≤ 0.5; additive ΣFIC > 0.5 and ≤ 1; indifferent ΣFIC > 1 and ≤ 2 (rounded to 2 decimal places).

Table 5.2 *In vitro* inhibitory activity of interactions between *O. vulgare* and *T. vulgaris* essential oils against *S. aureus* in liquid phase

S. aureus strains	MICs alone (µg/mL)			OVEO in combination with listed TVEO concentrations (µg/mL)									
	OVEO	TVEO	O	+ TVEO 512		+ TVEO 256		+ TVEO 128		+ TVEO 64		+ TVEO 32	
				MIC	ΣFIC	MIC	ΣFIC	MIC	ΣFIC	MIC	ΣFIC	MIC	ΣFIC
SA ATCC 25923	512	512	0.6	16	1.03	156	0.81	313	0.86	512	1.13	512	1.06
SA ATCC 29213	740	569	0.4	16	0.94	270	0.81	341	0.69	455	0.73	512	0.76
SA ATCC 33591	910	683	555	16	0.77	370	0.78	484	0.73	512	0.66	512	0.61
SA ATCC 33592	740	512	164	16	1.02	121	0.67	341	0.72	512	0.88	512	0.81
SA ATCC 43300	626	512	36	16	1.03	185	0.79	313	0.75	512	0.96	512	0.90
SA ATCC BAA 976	569	512	64	16	1.03	142	0.75	284	0.75	427	0.89	512	0.98
SA 1	512	512	6	16	1.03	128	0.75	284	0.81	512	1.13	512	1.06
SA 2	1024	967	149	16	0.55	427	0.68	512	0.63	512	0.57	512	0.53
SA 3	512	512	455	16	1.03	116	0.73	256	0.75	398	0.90	512	1.06
SA 4	512	512	427	16	1.03	121	0.74	256	0.75	484	1.07	512	1.06
SA 5	512	512	1	16	1.03	107	0.71	313	0.86	512	1.13	512	1.06
SA 6	967	796	1	44	0.75	356	0.72	512	0.71	512	0.62	512	0.58

ATCC: American type culture collection; MIC: minimum inhibitory concentration, the values are expressed as an average from three independent experiments, each performed in triplicate (rounded to integers with exception of values lower than 1); O: Oxacillin; OVEO: *O. vulgare* essential oil; SA: *Staphylococcus aureus*; TVEO: *Thymus vulgaris* essential oil; ΣFIC: sum of fractional inhibitory concentrations; the combinatory effect is evaluated as follows: synergy ΣFIC ≤ 0.5; additive ΣFIC > 0.5 and ≤ 1; indifferent ΣFIC > 1 and ≤ 2 (rounded to 2 decimal places).

Table 5.3 Chemical composition of *O. vulgare* essential oil.

	[1] RI		Component	[2] C	[3] Column		[4] Identification	
					HP-5MS	DB-H.WAX	HP-5MS	DB-H.WAX
	Obs.	Lit.			[%]	[%]		
1	922 [a]	924	*α*-Thujene	MH	1.17	0.77	GC/MS, RI	GC/MS
2	929 [a]	932	*α*-Pinene	MH	0.67	0.42	GC/MS, RI, Std	GC/MS
3	945 [a]	946	Camphene	MH	0.18	0.12	GC/MS, RI, Std	GC/MS
4	973 [a]	974	*β*-Pinene	MH	0.16	0.10	GC/MS, RI, Std	GC/MS
5	991 [a]	988	*β*-Myrcene	MH	1.87	1.23	GC/MS, RI	GC/MS
6	1005 [a]	1002	*α*-Phellandrene	MH	0.14	0.08	GC/MS, RI	GC/MS
7	1009 [a]	1008	3-Carene	MH	0.08	0.06	GC/MS, RI, Std	GC/MS
8	1017 [a]	1014	*α*-Terpinene	MH	0.85	0.63	GC/MS, RI, Std	GC/MS
9	1028 [a]	1025	p-Cymene	MH	8.25	5.63	GC/MS, RI, Std	GC/MS
10	1061 [a]	1054	*γ*-Terpinene	MH	4.52	3.33	GC/MS, RI, Std	GC/MS
11	1078 [a]	1068	trans-Sabinene hydrate	MO	0.30	0.11	GC/MS, RI	GC/MS
12	1110 [a]	1095	Linalool	MO	0.11	-	GC/MS, RI, Std	-
13	1185 [a]	1165	Borneol	MO	0.06	0.58	GC/MS, RI, Std	GC/MS
14	1190 [a]	1174	Terpinen-4-ol	MO	0.64	0.36	GC/MS, RI	GC/MS
15	1302 [a]	1289	Thymol	MO	0.26	0.47	GC/MS, RI, Std	GC/MS
16	1314 [a]	1298	Carvacrol	MO	77.92	82.60	GC/MS, RI, Std	GC/MS
17	1430 [a]	1418	*β*-Caryophyllene	SH	1.89	1.53	GC/MS, RI, Std	GC/MS
18	1466 [a]	1452	Humulene	SH	0.26	0.18	GC/MS, RI	GC/MS
19	1517 [a]	1505	*β*-Bisabolene	SH	0.45	0.35	GC/MS, RI	GC/MS
20	1181 [b]	1185 [c]	D-Limonene	MH	-	0.15	-	GC/MS, RI
21	1190 [b]	1195 [d]	*β*-Phellandrene	MH	-	0.15	-	GC/MS, RI
22	1438 [b]	1445 [e]	1-Octen-3-ol	O	-	0.22	-	GC/MS, RI

	[1] RI		Component	[2] C	[3] Column		[4] Identification	
					HP-5MS	DB-H.WAX	HP-5MS	DB-H.WAX
	Obs.	Lit.			[%]	[%]		
23	1450 [b]	1450 [f]	cis-Sabinene hydrate	MO	-	0.27	-	GC/MS, RI
24	1579 [b]	1583 [g]	Carvacrol methyl ether	O	-	0.36	-	GC/MS, RI
25	1848 [b]	1868 [h]	Carvacrol acetate	O	-	0.06	-	GC/MS, RI
26	1957 [b]	1953 [d]	Caryophyllene oxide	SO	-	0.14	-	GC/MS, RI
			Chemical classes					
			Monoterpene hydrocarbons		17.89	12.67		
			Oxygenated monoterpenes		79.29	84.39		
			Sesquiterpene hydrocarbons		2.60	2.06		
			Oxygenated sesquiterpenes		-	0.14		
			Others		-	0.64		
			Total identified [%]		**99.78**	**99.90**		

[1] RI = retention indices; Obs. = retention indices determined relative to a homologous series of n-alkanes (C_8-C_{40}) on [a]HP-5MS column and on [b]DB-HeawyWAX column, Lit. = literature RI values (Adams, 2007), [c]Nebie et al. (2004), [d] Lopes et al. (2004), [e] Umano & Shibamoto (1987), [f] Avato et al. (2004), [g] Lee et al (2005), [h] Kaya et al. (1999); [2] C = Class; MH - Monoterpene hydrocarbons, MO - Oxygenated monoterpenes, O - Others, SH - Sesquiterpene hydrocarbons, SO - Oxygenated sesquiterpenes; [3] Column = composition of essential oil detected on HP-5MS and DB-HeawyWAX columns; [%] = relative percentage content; - = not detected; [4] 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 matching literature database; Std = constituent identity confirmed by co-injection of authentic standards.

Table 5.4 Chemical composition of *T. vulgaris* essential oil.

	[1] RI		Component	[2] C	[3] Column		[4] Identification	
					HP-5MS	DB-H.WAX	HP-5MS	DB-H.WAX
	Obs.	Lit.			[%]	[%]		
1	922 [a]	924	*α*-Thujene	MH	0.93	0.55	GC/MS, RI	GC/MS
2	929 [a]	932	*α*-Pinene	MH	1.01	0.67	GC/MS, RI, Std	GC/MS
3	944 [a]	946	Camphene	MH	0.50	0.36	GC/MS, RI, Std	GC/MS
4	973 [a]	974	*β*-Pinene	MH	0.24	0.18	GC/MS, RI, Std	GC/MS
5	991 [a]	988	*β*-Myrcene	MH	2.71	1.35	GC/MS, RI	GC/MS
6	1005 [a]	1002	*α*-Phellandrene	MH	0.16	0.12	GC/MS, RI	GC/MS
7	1008 [a]	1008	3-Carene	MH	0.08	0.08	GC/MS, RI, Std	GC/MS
8	1017 [a]	1014	*α*-Terpinene	MH	1.96	1.51	GC/MS, RI, Std	GC/MS
9	1029 [a]	1025	p-Cymene	MH	24.08	18.00	GC/MS, RI, Std	GC/MS
10	1061 [a]	1054	*γ*-Terpinene	MH	13.37	10.61	GC/MS, RI, Std	GC/MS
11	1078 [a]	1068	trans-Sabinene hydrate	MO	0.59	0.24	GC/MS, RI	GC/MS
12	1090 [a]	1086	Isoterpinolene	MH	0.18	-	GC/MS, RI	-
13	1113 [a]	1095	Linalool	MO	2.84	2.87	GC/MS, RI, Std	GC/MS
14	1149 [a]	1141	Camphor	MO	0.28	0.30	GC/MS, RI, Std	GC/MS
15	1184 [a]	1165	Borneol	MO	0.47	1.14	GC/MS, RI, Std	GC/MS
16	1190 [a]	1174	Terpinen-4-ol	MO	0.91	-	GC/MS, RI	-
17	1244 [a]	1232	Thymol methyl ether	O	0.77	2.06	GC/MS, RI	GC/MS
18	1254 [a]	1244	Carvacrol methyl ether	O	0.64	1.47	GC/MS, RI	GC/MS
19	1289 [a]	1285	Bornyl acetate	O	0.17	0.13	GC/MS, RI, Std	GC/MS
20	1302 [a]	1289	Thymol	MO	42.34	48.46	GC/MS, RI, Std	GC/MS
21	1430 [a]	1417	*β*-Caryophyllene	SH	3.55	2.06	GC/MS, RI, Std	GC/MS
22	1466 [a]	1452	Humulene	SH	0.10	0.10	GC/MS, RI	GC/MS
23	1478 [a]	1475	Geranyl propionate	O	0.06	0.10	GC/MS, RI	GC/MS
24	1487 [a]	1478	*γ*-Muurolene	SH	0.17	0.23	GC/MS, RI	GC/MS
25	1517 [a]	1505	*β*-Bisabolene	SH	0.10	0.07	GC/MS, RI	GC/MS

	[1] RI		Component	[2] C	[3] Column		[4] Identification	
					HP-5MS	DB-H.WAX	HP-5MS	DB-H.WAX
	Obs.	Lit.			[%]	[%]		
26	1529 [a]	1513	γ-Cadinene	SH	0.30	-	GC/MS, RI	-
27	1535 [a]	1522	δ-Cadinene	SH	0.38	0.70	GC/MS, RI	GC/MS
28	1602 [a]	1582	Caryophyllene oxide	SO	0.37	0.40	GC/MS, RI, Std	GC/MS
29	1181 [b]	1185 [c]	D-Limonene	MH	-	0.31	-	GC/MS, RI
30	1192 [b]	1199 [c]	1,8-Cineole	MO	-	0.64	-	GC/MS, RI
31	1438 [b]	1445 [e]	1-Octen-3-ol	O	-	1.10	-	GC/MS, RI
32	1450 [b]	1450 [f]	cis-Sabinene hydrate	MO	-	0.68	-	GC/MS, RI
33	1471 [b]	1475 [d]	Copaene	SH	-	0.06	-	GC/MS, RI
34	1496 [b]	1531 [g]	Bourbonene	SH	-	0.07	-	GC/MS, RI
35	1799 [b]	1804 [h]	Calamenene	SH	-	0.07	-	GC/MS, RI
36	1824 [b]	1840 [i]	Thymol acetate	O	-	0.19	-	GC/MS, RI
37	2169 [b]	2186 [i]	Carvacrol	MO	-	2.65	-	GC/MS, RI
			Chemical classes					
			Monoterpene hydrocarbons		45.22	33.74		
			Oxygenated monoterpenes		47.43	56.98		
			Sesquiterpene hydrocarbons		4.60	3.36		
			Oxygenated sesquiterpenes		0.37	0.40		
			Others		1.64	5.05		
			Total identified [%]		**99.26**	**99.53**		

[1] RI = retention indices; Obs. = retention indices determined relative to a homologous series of n-alkanes (C_8-C_{40}) on [a]HP-5MS column and on [b]DB-HeawyWAX column, Lit. = literature RI values (Adams, 2007), [c] Nebie et al. (2004), [d] Lopes et al. (2004), [e] Umano & Shibamoto (1987), [f] Avato et al. (2004), [g] Ngassoum et al. (1999), [h] Stashenko et al. (1996), [i] Bassole et al. (2003) [2] C = Class; MH - Monoterpene hydrocarbons, MO - Oxygenated monoterpenes, O - Others, SH - Sesquiterpene hydrocarbons, SO - Oxygenated sesquiterpenes; [3] Column = composition of essential oil detected on HP-5MS and DB-HeawyWAX columns; [%] = relative percentage content; - = not detected; [4] 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 matching literature database; Std = constituent identity confirmed by co-injection of authentic standards.

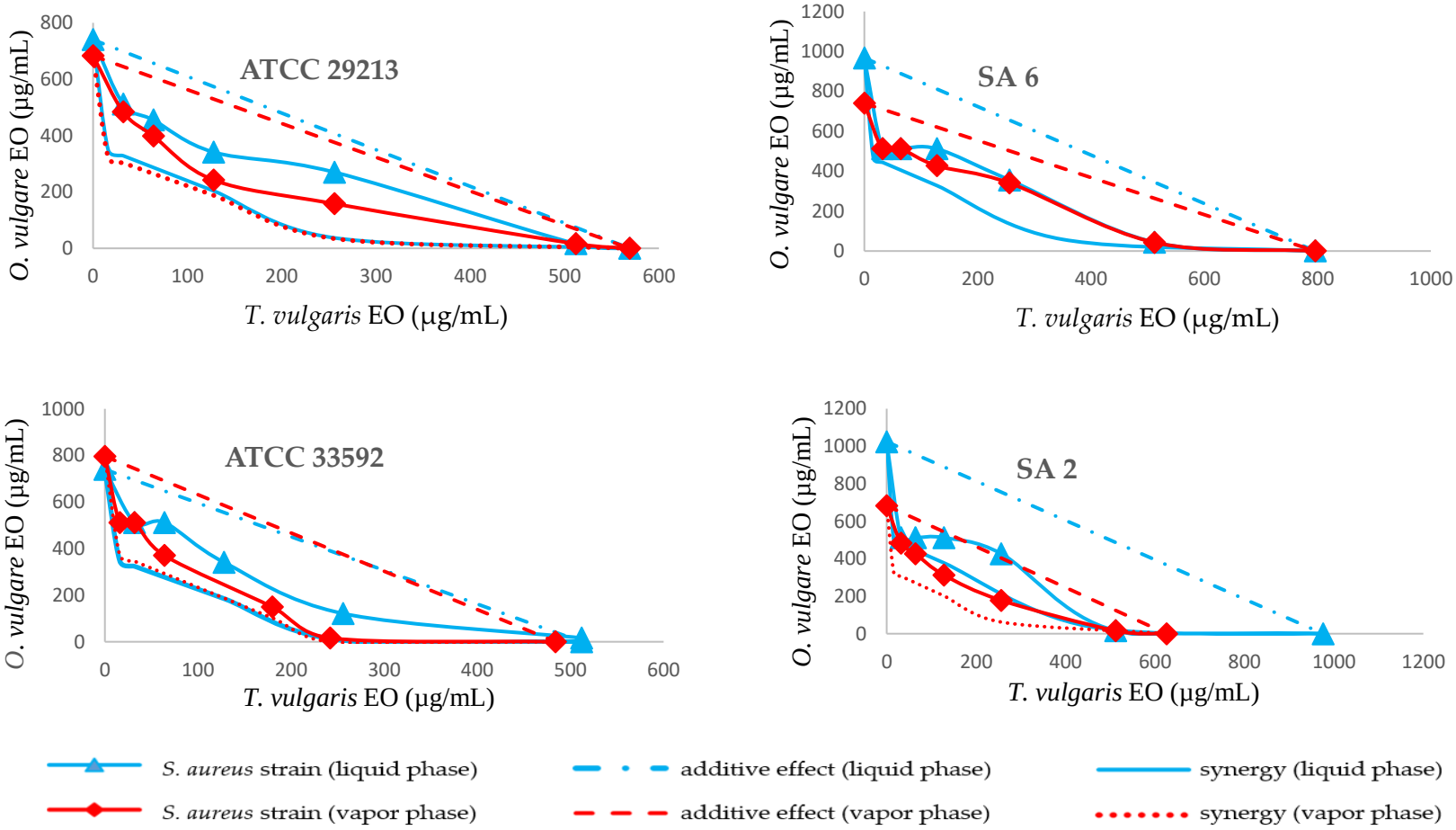

Figure 5.1 Isobolograms of the interactions between *O. vulgare* and *T. vulgaris* binary combinations against *S. aureus* strains in vapour and liquid phases. Additivity (ΣFIC > 0.5 and ≤ 1); synergy (ΣFIC ≤ 0.5)

Discussion

In our study, the *in vitro* growth inhibitory effect of both *O. vulgare* and *T. vulgaris* EOs was slightly stronger in the vapour phase than in a liquid medium since the MIC values were for the vast majority of the staphylococcal strains slightly lower on the agar media than in the broth. The only exceptions were standard strain ATCC 33592 and clinical isolate SA 1, where the antimicrobial effect of *O. vulgare* was stronger in the liquid phase, and staphylococcal strains ATCC 29213, ATCC 43000, SA 1, and SA 6, where the MICs of *T. vulgaris* EO were the same in both phases. Similar pattern showing that the vapour generated by EOs has a greater antimicrobial effect compared to EOs in liquid form applied by direct contact (in aqueous solutions or on solid agars) can be observed in several previous studies (Tullio et al. 2007; Tyagi & Malik 2011). This phenomenon can be explained by the fact that in the aqueous phase, lipophilic molecules associate to form micelles and thus restrain the attachment of EOs to microorganisms, whereas the vapour phase allows for free attachment (Inouye et al. 2003; Laird & Phillips 2012).

Values of MICs observed in our study for *O. vulgare* and *T. vulgaris* EOs in the liquid phase were similar to numerous previously published data. For example, the investigation carried out by Boskovic et al. (2015) determined antibacterial effects of EOs isolated from *O. vulgare* against *S. aureus* ATCC 25923 (MIC values = 640 μg/mL) and MRSA ATCC 43300 (MIC values = 320 μg/mL) using broth microdilution method. In the study performed by Ozkalp et al. (2010), *O. vulgare* EO inhibited growth of *S. aureus* RSKK 96090 and MRSA with MIC values 64 and 250 μg/mL, respectively. Similarly, the results of antistaphylococcal activity of *T. vulgaris* EO obtained in this study correspond well with previous findings of Kot et al. (2019), who reported MIC values ranging from 90 to 780 μg/mL against 18 MRSA strains in the liquid phase, or with the results of Boskovic et al. (2015), who determined the antimicrobial effect of thyme EO against *S. aureus* ATCC 25923 (MIC values = 640 μg/mL) and MRSA ATCC 43300 (MIC values = 320 μg/mL). Moreover, our results are supported by research conducted by Stojkovic et al. (2013), where MICs of oregano and thyme EOs against *S. aureus* were equal to 0.5 μL/mL and 1 μL/mL, respectively.

If mixtures of EOs are used as antimicrobials, they may, according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (2000), show either an antagonistic, additive, indifferent, or synergistic effect, measured by assessment of the FIC values. Several authors have demonstrated additive effects as well as synergistic actions of *O. vulgare* EO in combination with *T. vulgaris* EO in the liquid phase. Our results correspond well with Gavaric et al. (2015), who reported additive antibacterial action of thyme and oregano against several bacteria, including *S. aureus* ATCC 25923 (FIC value = 1). Similarly, Gutierrez et al. (2009) confirmed the additive effect of these EO combinations against several spoilage organisms, such as *Enterobacter cloacae*, *Pseudomonas fluorescens,* and *Listeria innocua,* using a chequerboard method with FIC values ranging from 0.75 to 1. On the other hand, synergistic activity of oregano and thyme EO combinations have previously been reported as well; for example, in the study of Stojkovic et al. (2013), oregano combined with thyme EO produced synergy against *S. aureus* (FIC value 0.45). However, as the assayed EOs possess similar chemical composition, their combination may exhibit addition rather than a synergistic effect (de Souza, 2009).

The disc volatilization method is probably the most frequently used assay for the evaluation of *in vitro* growth inhibitory effect in the vapour phase. Both EOs have previously been tested individually against *S. aureus* in the gaseous phase. For example, an investigation carried out by Nedorostova et al. (2009) determined antibacterial effects of EOs isolated from *O. vulgare* and *T. vulgaris* against *S. aureus* ATCC 25923 using modified disc volatilization method, and the MIC value of both EOs was 0.017 μg/cm^3. Similarly, Kloucek et al. (2012) used a modified disc volatilization method to assess the antimicrobial activity of various EOs, including those of oregano and thyme. In this study, vapours of *O. vulgare* EO containing 92% of carvacrol inhibited growth of *S. aureus* ATCC 25923 with MIC values 62.5 μL/L, whereas three EO samples of *T. vulgaris* with different chemical composition exhibited antimicrobial activity against the same staphylococcal strain with the MIC ranging from 125 to 250 μL/L. However, a thyme EO where thymol was the predominant compound was not active at all. A study performed by Lopez et al. (2007) determined the growth inhibitory effects of *O. vulgare* and *T. vulgaris* vapours against *S. aureus* ATCC 29213 by a similar method and consequently calculated MIC causing apparent inhibition (17.5 μL/L and 87.3 μL/L, respectively) of the atmosphere above microorganisms. Subsequent research led by Reyes-Jurado et al.

(2019) assessed the MIC values of *T. vulgaris* EO vapours against *S. aureus* and MRSA as >5 μg/mL of air. However, although there has been increasing research interest in the antimicrobial activity of individual EO vapours in recent years, significantly fewer studies have been reported on their combinations. In the case of thyme and oregano EO vapours, to the best of our knowledge, the only study dealing with their combinatory effects in the gaseous phase was published by Cho et al. (2020), who reported synergistic activities of gaseous oregano and thyme EOs against *Listeria monocytogenes* by modified chequerboard assay (FIC = 0.375). Our study is the first report on *O. vulgare* and *T. vulgaris* interactions in the vapour phase against *S. aureus*.

The antimicrobial properties of *O. vulgare* and *T. vulgaris* have been attributed to their chemical compositions, which are primarily rich with monoterpene hydrocarbons and oxygenated monoterpenes. The principal terpenes identified in oregano and thyme are usually carvacrol, thymol, *γ*-terpinene, and *p*-cymene; while terpinen-4-ol, linalool, *β*-myrcene, *trans*-sabinene hydrate, and *β*-caryophyllene are also present. The proportion of these and other components in oils within the same species defines the chemotype (Leyva-Lopez et al. 2017). In our study, the chromatographic profiles of both EOs were analysed by GC/MS using two detectors and two capillary columns of different polarities to avoid the overlapping of signal peaks observed in the chromatogram and to achieve the best possible resolution of compounds. The internal standard was used for quantitative analysis. Compounds belonging to the classes with monoterpene hydrocarbons and oxygenated monoterpenes were the most numerous identified. Carvacrol was the most abundant compound in oregano EO, followed by *p*-cymene and *γ*-terpinene, and the oil is, thus, characterized as a carvacrol chemotype. This finding is in accordance with several previously published studies. For example, Stojkovic et al. (2013), Scalas et al. (2018), and Stoilova et al. (2008) reported carvacrol as the main component of oregano EO (contributing 64.50%, 62.61%, and 66.20% of the EO, respectively), *p*-cymene as the second the most abundant compound (10.90%; 12.36%, and 9.1%, respectively), and *γ*-terpinene as third most abundant component (10.80%, 7.60%, and 7.30%, respectively). Thymol, on the other hand, has been in our study detected as the most abundant constituent in thyme EO, also followed by its precursors, *p*-cymene and *γ*-terpinene; therefore, the present thyme oil belongs to thymol chemotype. This finding is also in accordance with numerous previously published studies (Grosso et al. 2010; Schmidt et al. 2012; Stokovic et al. 2013; Nikolic et al. 2014; de Carvalho et al. 2015; Boskovic

et al. 2015), where thymol, *p*-cymene, and *γ*-terpinene were reported as the first, second, and third most abundant compounds, respectively. The number of components identified in our study (26 and 37 in total in oregano and thyme EOs, respectively) is within the range of numbers of compounds identified in other reported studies, as eight to 38 compounds have been reported for *O. vulgare* (Lukas et al. 2008; Stoilova et al. 2008; Stokovic et al. 2013; Boskovic et al. 2015; Scalas et al. 2018), and 16–50 for *T. vulgaris* (Hudaib et al. 2002; Grosso et al. 2010; Schmidt et al. 2012; Stokovic et al. 2013; Nikolic et al. 2014; Boskovic et al. 2015; de Carvalho et al. 2015; Scalas et al. 2018). Since the used plant material has been obtained from a commercial supplier, the age of the plants as well as their growing conditions, harvest time, transportation, and storage conditions are unknown. Therefore, the chemical composition of EOs analysed in this study can be influenced by all the above-mentioned factors (Figueiredo et al. 2008; Baser et al. 2010). The qualitative differences (numbers of components) between the two columns are in accordance with previously reported studies on GC/MS analysis of EOs using two columns. For example, Anderson and Parnell (2015), who compared cold-pressed orange oil profiles by GC/MS using polar (Zebron ZB-WAX column) and non-polar (Zebron ZB-1ms) GC columns, identified 22 and 29 components on non-polar and polar compounds, respectively. The higher number of volatile components identified on a polar column might have been caused, similarly as in our case, by the better resolution between compounds that were seen to co-elute on the non-polar column. Similarly, quantitative differences between the polar and non-polar columns have previously been reported as well. In our study, the main compound (thymol) in thyme EO showed the highest proportional difference between two columns (more than 6%), which can be, for example, compared to Fan et al. (2018), who analysed the composition of the EO from *Dendranthema indicum* var. *aromaticum* and detected *α*-thujone as the main compound with a difference of 4.88% between columns. Different amounts of the detected compounds are caused by different polarities and material of the used columns.

Since carvacrol and thymol have been found to be the most abundant compounds in our oregano and thyme EOs, respectively, the additive effects obtained by interactions between our volatile oils might be caused mainly by these two phenolic monoterpenoids. The presumption that the predominant component in both EOs is responsible for the antimicrobial activity of EOs can be supported by our previous research (Netopilova et al. 2018), whereas the range of MIC values of carvacrol (370–1593 μg/mL and 484–1024

μg/mL in agar and broth media) and thymol (341–1707 μg/mL and 355–1024 μg/mL in the vapour and liquid phases) were very similar to the MIC values of the *O. vulgare* (427–796 μg/mL and 512–1024 μg/mL in agar and broth media) and *T. vulgaris* (427–796 μg/mL in the vapour phase and from 512–967 μg/mL in liquid phase) EOs tested in this study. The occurrence of additive interaction between carvacrol and thymol could be related to the similarity in their molecular structures (they are isomers), suggesting a similar mechanism of action (Raquena et al. 2019). Both thymol and carvacrol are expected to cause functional and structural damages to the cytoplasmic membranes. The primary mechanism of antibacterial action of thymol is not fully known; however, it is believed to involve outer and inner membrane disruption and interaction with membrane proteins and intracellular targets. Similarly, the primary mechanism of action of carvacrol is its ability to position in the membrane where it increases permeability (Hyldgaard et al. 2012).

In various scientific articles is stated that the formation of thymol and carvacrol is thought to involve hydroxylation of γ-terpinene and p-cymene precursors. A pathway for the biosynthesis of thymol from the monoterpene γ-terpinene via an intermediate p-cymene was proposed in the late 1970s. However, according to Croccol (2011), this statement has never been validated and in his doctoral thesis is suggested that γ-terpinene is directly converted to thymol and carvacrol with p-cymene as a side product. One way or another, in both EOs tested in our study, the principal compounds, carvacrol, and thymol, were followed by their biosynthetic precursors p-cymene and γ-terpinene, which, together with the main compound comprised more than 90% and 77% of the oregano and thyme oils. Their interaction within the tested EOs is presumable and might also contribute to the additive effects. This statement can be supported by Ultee et al. (2000), who reported synergistic activity between carvacrol and cymene against *Bacillus cereus*, or by Delgado et al. (2004), who found synergistic effect against the same bacterium when cymene was combined with thymol. The additive antimicrobial effect of carvacrol and thymol has already been previously reported in several studies against different bacteria, including *S. aureus* in liquid (Lambert et al. 2001; Burt et al. 2005; Gavaric et al. 2015) as well as in the vapour phase (Netopilova et al. 2018). However, further research focused on a better understanding of antimicrobial interactions between major and minor components, which was suggested to play an important role in the synergistic activity of EO gases (Burt 2004) is warranted.

Although EOs of *O. vulgare* and *T. vulgaris* have acquired Generally Recognized as Safe (status from the Flavour and Extract Manufacturers Association and got approved by the US Food and Drug Administration (FDA) for safety food use (Kuttan & Liju 2017; FDA 2020)), there is limited published research on the safety of EO vapours *per se* (Laird & Phillips 2012). As EOs are complex blends of components, individual volatile compounds need to be assessed as potential allergens. Currently, 26 ingredients that may trigger allergic reactions, including, e.g., linalool and limonene, are listed in the seventh amendment of directive 76/768 CEE (directive 2003/15/CE); however, these are all based on skin contact and not inhalation (Heisterberg et al. 2011; Vostinaru et al. 2020). Regarding inhalation toxicity, which is a crucial aspect of inhalation administration, median lethal concentration (LC_{50}) values were determined neither for oregano nor for thyme EOs for the inhalation route. However, the data on their predominant compounds, carvacrol and thymol, might suggest their possible inhalation safety. The European Chemicals Agency reported that the LC_{50} of carvacrol in rats was estimated to be greater than 20 mg/L when rats were treated with the given test chemical via inhalation route for 6 h exposure period. Similarly, the reported LC_{50} value for thymol was 7.57 mg/L, when mice were exposed to a test chemical via inhalation by vapour for 2 h (ECHA 2020). Furthermore, neither data from literature nor results from chronic toxicity studies presented in the study by Xie et al. (2019) provide any evidence for chronic toxicity of inhaled thymol. In an acute oral toxicity study, the median lethal dose (LD_{50}) of carvacrol and thymol in rats was found to be 810 and 980 mg/kg of body weight (bw), respectively, and carvacrol-rich EO obtained from the leaves of *Origanum* spp. showed the oral LD_{50} to be 1850 mg/kg bw; therefore, they are all classified as category 4 (H302) according to the Classification, Labelling and Packaging Regulation N° 1272/2008 and the Globally Harmonized System of Classification and Labelling of Chemicals (ECHA 2020), which means that it might be “harmful if swallowed”. Moreover, thymol is FDA approved when used as a synthetic flavouring (21 CFR 172.515), a preservative and indirect food additive of adhesives (Xie et al. 2019) and is a common ingredient in many products such as perfumes, food flavourings, mouthwashes, pharmaceutical preparations, and cosmetics (EPA 2020). Similarly, carvacrol is generally considered safe for human consumption. It has been approved by FDA for its use in food and is included by the Council of Europe in the list of chemical flavourings that can be found in several food products, such as alcoholic beverages, baked goods, chewing gums, condiment relish, frozen dairy, gelatine

puddings, non-alcoholic beverages, and soft candies (Suntrez et al. 2015). Moreover, EO derived from *T. vulgaris* has been approved by the Committee on Herbal Medicinal Products of the European Medicines Agency as a traditional herbal medicinal product used for relief of cough associated with cold (EMA 2020).

The above-mentioned data suggest a low toxicological risk of carvacrol and thymol administration through an inhalation route. Moreover, the rich historical evidence of culinary, medicinal, and pharmaceutical uses of *O. vulgare* and *T. vulgaris* could support their use as safe herbal medicinal products. Therefore, due to the considerable antimicrobial activity as well as the presumable safety of *O. vulgare* and *T. vulgaris* EOs, it can be assumed that the results of oregano and thyme EO combinations could be potentially applied in the development of various pharmaceutical applications that are based on volatile antimicrobials. These combinations could decrease the minimum effective doses of the agents, thus reducing their possible adverse effects and treatment costs. However, further research to achieve a better understanding of the action mechanisms, further in vivo experiments, and clinical trials on *O. vulgare* in combination with *T. vulgaris* are still necessary to determine their pharmacodynamics and pharmacokinetics.

Perhaps even better results of antimicrobial testing could be achieved in the future if EOs are obtained by other methods, e.g., Gedikoglu et al (2019) tested antibacterial activity of *T. vulgaris* EO obtained by hydrodistillation and microwave-assisted extraction (MAE) against six bacteria. In this study, the EO of *T. vulgaris* that underwent MAE displayed significantly higher antibacterial activity against four bacteria than did the hydrodistilled EO. In another study, Benmoussa et al. (2016) tested antibacterial activity of *T. vulgaris* EOs obtained by four different means (microwave assisted hydrodistillation, solvent free microwave extraction (SFME), hydrodistillation and steam distillation) against 5 microorganisms. *T. vulgaris* EO obtained by SFME exhibited slightly higher antimicrobial activities than the other methods.

5.4 Conclusions

In summary, the present study reports the results of antistaphylococcal interactions between EOs obtained from *O. vulgare* and *T. vulgaris* that were tested by broth volatilization chequerboard assay. This combination of volatile oils exhibited additive

effects against all 12 *S. aureus* strains in both liquid and vapour phases, whereas the best results in the liquid phase were obtained against methicillin-resistant strain (SA 2). To the best of our knowledge, this is the first report on interactions between *O. vulgare* and *T. vulgaris* EOs against *S. aureus* in the gaseous phase. In addition, the results presented in the form of isobologram, a graphical diagram enabling precise and intuitive judgment of the additive effect produced by EOs combination, validates the accuracy of broth volatilization chequerboard method for evaluation of the combinatory antimicrobial effect of EOs in the vapour phase. These results can potentially serve as a base for further research focused on the development of various pharmaceutical applications that are based on volatile antimicrobials. However, since the MICs values obtained in the gaseous phase are only indicative and the real concentrations of evaporated EOs are lower, we believe that our results suggest a potential of thyme and oregano combination for application in the inhalation therapy against respiratory infections caused by *S. aureus.* However, further research focusing on *in vivo* evaluation will have to be carried out in order to verify its potential practical use.

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6 Conclusions

This study reports the development and verification of novel *in vitro* antimicrobial assay, named broth volatilization chequerboard method, which combines principles of classical chequerboard test and broth microdilution volatilization method. The new high-throughput screening assay overcomes some specific disadvantages of conventional standardised techniques that are commonly used for evaluation of antimicrobial combinatory potential as well as it allows determination of MIC and calculation of FIC values at different concentrations. Furthermore, the novel method is applicable in the development of new preparations for the inhalation therapy of respiratory diseases that are based on interactions between plant volatiles. Accuracy, appropriateness, and usability of our broth volatilization chequerboard method was verified on a combination of two plant-derived compounds, carvacrol and thymol (Chapter 3), on the interactions of a compound with an EO, 8-hydroxyquinoline and *Cinnamomum cassia* EO (Chapter 4) as well as on combination of two EOs, *Origanum vulgare* and *Thymus vulgaris* EOs (Chapter 5), which were tested against 12 standard strains and clinical isolates (both antibiotic-resistant and sensitive strains) of *S. aureus*. These three combinations of EOs and plant components confirmed suitability of newly developed approach for antibacterial combinatory testing. The analysis of chemical composition of EOs using dual column/dual detector GC/MS system accompanied the antistaphylococcal combinatory testing.

Newly developed broth volatilization chequerboard method was crucial point of this study. Our findings suggest that the new technique is suitable for fast screening and simple determination of growth-inhibitory effect of interactions between two plant-derived volatile agents (EOs and their components) simultaneously in the liquid and the vapour phase. The main advantages of this method include simplicity, rapidity, the cost and labour effectiveness and possibility of automation of assay plate preparation. This method is also suitable for testing of a broad range of concentrations in one 96-well microtiter plate, so it considerably saves the consumption of material and it is suitable for high-throughput screening of volatile agents with no need of special apparatus. Since the previously developed techniques for the combinatory antimicrobial testing in the gaseous phase were mostly techniques that were based on disc volatilization method providing only qualitative results, another advantage of our method is the possibility of indicating the degree of bacterial susceptibility via quantitative data (MIC endpoints) for proper

determination of combinatory effects expressed as FICs. Despite the fact that interpretation of FIC data slightly above or below the critical theoretical cut-off of 1.0 as additive interaction seem to put a positive spin on findings (Odds 2003), the EUCAST scale that includes additive effect (EUCAST 2000) was recommended for evaluation our results of broth volatilization chequerboard method because final concentrations of antimicrobial agents used for calculation of MIC values are in fact lower due to their spontaneous transitions between liquid and gaseous systems.

Furthermore, depending on the evaporation temperature and vapour pressure, the final concentrations of volatile antimicrobials and their combinations may be also affected by loses caused by the evaporation of volatile compounds during preparation of the test. For this reason, observed MICs (which also means the FIC values) of volatile antimicrobial agents in vapour phase should be considered as indicative values only. Nevertheless, the indicative FIC values obtained by broth volatilization chequerboard assay are still suitable for interpretation of screening experiments focused on identification of combinatory interactions of volatile antimicrobial agents in vapour phase and if required, the exact concentrations can be determined e.g. by using combination of solid-phase microextraction/head-space techniques and gas chromatography/mass spectrometry analysis. Another weakness of this method is the necessary use of clamps to fast plate and lid together. Moreover, small volume of agar that is applied on the lid may limit the bacterial growth. Since our novel broth volatilization chequerboard technique is performed microplates, which are serially produced and have not been designed for the antimicrobial testing in the vapour phase, development of microplate lid-based device could overcome these difficulties.

According to the results of growth-inhibitory effects of plant-derived volatile compounds and EOs (carvacrol, thymol, 8- hydroxyquinoline and *C. cassia*, *O. vulgare*, *T. vulgaris* EOs), each volatile agent produced a certain level of antistaphylococcal activity against standard strains and clinical isolates., whereas 8-hydroxyquinoline was found as the most active antibacterial agent. The results of *in vitro* antistaphylococcal combinatory effects of all three different interactions between volatile agents (combinations of carvacrol and thymol, 8-hydroxyquinoline and *C. cassia* EO, and *O. vulgare* and *T. vulgaris* EOs) have shown to produce additive antimicrobial effects against all tested strains of *S. aureus* in both phases. In several cases, they reached ΣFIC

values lower than 0.6, which can be considered as a strong additive interaction. Giant chequerboard method proposed by Horrevorts et al. (1987), which is constructed by compiling the results of series of component chequerboards (Hsieh et al. 1993), might however bring more accurate results and perhaps even the identification of synergistic effects.

The chemical compositions of EOs obtained by hydrodistilation from *C. cassia* bark and *O. vulgare* and *T. vulgaris* aerial parts were analysed using GC/MS equipped by dual column/dual detector system, which provides complementary information and higher quality identification of detected components. All samples were separated on two capillary columns of different polarity (polar DB-HeawyWAX and non-polar HP-5MS) to overcome possible limitations of difficult identification and quantification of plant EOs that are composed of components showing similar chromatographic retention behaviour. This approach increases the resolution of the analysis leading to the more complex volatile sample separation and higher identification probability. Two detectors, flame ionization detector providing us data useful for components' quantification and mass spectrometer being effective especially in identification of samples' chemical structures via molecular weight determination, were used.

Apart from the invention of the new volatilization chequerboard method suitable for antimicrobial combinatory testing in vapour phase, this study brings, to the best of our knowledge, first reports on antistaphylococcal additive interactions of thymol with carvacrol and *O. vulgare* with *T. vulgaris* EOs in the vapour phase and on additive effects of combinations of 8-hydroxyquinoline and *C. cassia* EO in both phases. These findings suggest vaporous combinations of carvacrol and thymol, 8-hydroxyquinoline and *C. cassia* EO, and *O. vulgare* and *T. vulgaris* EOs as promising substances for the further research especially focused on the development of new antistaphylococcal agents. These results can be potentially applied in development of various pharmaceutical applications that are based on volatile antimicrobials and can be used through inhalation therapy against respiratory infections caused by *S. aureus*. However, further research focused on various aspects of the combinatory action in vapour phase as well as the *in vivo* evaluation will be needed prior to their possible pharmacological application.

The future practical applications of proof-of concept approach consisting of broth volatilization chequerboard method accompanied by dual column/dual detector GC

analysis could be potentially applied not only in development of various pharmaceutical applications that are based on antimicrobial combinatory effect of volatile compounds but it could also be applicable in the shelf-life extension and preservation of food products, in the protection of agriculture products, documents, and exhibits, as well as in development of various disinfection and sterilization agents in healthcare facilities. However, further optimization and modification of this technique should be considered for other possibilities of its use.

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8 Appendices

List of the Appendices:

Appendix 1: *Curriculum vitae*

PERSONALIA

Name	**Ing. Marie Straková**
	(born Netopilová)
Address	V Zahrádkách 263, 252 45, Zvole
	Czech Republic
Mobile phone number	+420 721 179 696
E-mail	netopilova@ftz.czu.cz
Date of birth	18 February 1991
Nationality	Czech

EDUCATION

2016 – present	**Doctoral Study**
	Czech University of Life Sciences Prague
	Faculty of Tropical AgriSciences
	Study Programme: Tropical Agrobiology and Bioresource Management
	Thesis: Evaluation of growth-inhibitory interactions between essential oils and their volatile compounds against *Staphylococcus aureus* in vapour phase
2013 – 2016	**Master's degree**
	Czech University of Life Sciences Prague
	Faculty of Tropical AgriSciences
	Study Programme: Tropical Crop Management and Ecology
	Thesis: Determination of antimicrobial activities of some Kyrgyz medicinal plants
2010 – 2013	**Bachelor's degree**
	Czech University of Life Sciences Prague
	Faculty of Tropical AgriSciences
	Study Programme: Sustainable Development in Tropics and Subtropics
	Thesis: Influence of abiotic factors on the yield of yacon's tuberous roots

ABROAD EXPERIENCES

10/2018 – 10/2019	**12 months internship in Belgium (Erasmus+ Traineeship)** Ghent University, Faculty of Bioscience Engineering, Department of Plants and Crops - Laboratory training focused on antimicrobial activity - Data processing and preparation of manuscript - Presentation of results of ongoing PhD research - Participation in teaching: seminar focused on antimicrobial activity - Help and cooperation in several ethnobotanical activities with other Ph.D. students from Department of Plants and Crops
04/2018 – 06/2018	**Student mobility in Philippines** Visayas State University, Baybay - plant sample collecting, essential oils distillation
04/2017 – 05/2017	**Student mobility in Cambodia** Royal University of Agriculture, Phnom Penh - plant sample collecting, essential oils distillation
09/2014 – 01/2015	**5 months of study placement (Erasmus+)** Universidad Politécnica de Madrid, Madrid, Spain - Erasmus Program (winter semester)

PROJECT PARTICIPATION

2020	Evaluation of biological effects and chemical analysis of compounds from tropical plants (IGA 20205001)
2019	Biological activity and chemical composition of compounds obtained from medicinal and edible tropical plants (IGA 20195003)
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)

LANGUAGE SKILLS

Czech: Mother tongue

English: Proficient user

Spanish: Basic user

French: Basic user

Russian: Basic user

Appendix 2: List of author's publications

Publications in scientific journals:

Netopilova M, Houdkova M, Urbanova K, Rondevaldova J, Kokoska L. 2021. Validation of qualitative broth volatilization chequerboard method for testing of essential oils: dual-column GC–FID/MS analysis and *in vitro* combinatory antimicrobial effect of *Origanum vulgare* and *Thymus vulgaris* against *Staphylococcus aureus* in liquid and vapour phases. Plants **10**:393 (IF = 2.762)

Netopilova M, Houdkova M, Urbanova K, Rondevaldova J, van Damme P, Kokoska L. 2020. *In vitro* antimicrobial combinatory effect of *Cinnamomum cassia* essential oil with 8-hydroxyquinoline against *Staphylococcus aureus* in liquid and vapour phase. Journal of Applied Microbiology **129**:906-915. (IF = 3.066)

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:

Netopilova M, Houdkova M, Urbanova K, Rondevaldova J, van Damme P, Kokoska L. 2019. *In vitro* anti-staphylococcal combinatory effect of *Cinnamomum cassia* essential oil and 8-hydroxyquinoline evaluated simultaneously in liquid and vapour phase. *67th International Congress and Annual Meeting of the Society for Medicinal Plant and Natural Product Research (GA conference 2019),* Innsbruck, Austria, 1.-5. 9. 2019. Planta Medica 2019, 85 (18): 1534-1535. Poster presentation. Abstract: P-331.

Osei-Owusu H, Kudera T, **Netopilova M**, Rondevaldova J, Kokoska L. 2019. Combinatory effect of plant compounds and their derivatives with conventional antibiotics on diarrhoea causing bacteria. *67th International Congress and Annual Meeting of the Society for Medicinal Plant and Natural Product Research (GA conference 2019),* Innsbruck, Austria, 1.-5. 9. 2019. Planta Medica 2019, 85 (18): 1523. Poster presentation. Abstract: P-293.

Netopilova M, Houdkova M, Rondevaldova J, Kokoska L. 2018. *In vitro* antimicrobial effect of carvacrol and thymol combination against *Staphylococcus aureus* using new broth volatilization chequerboard method. 22nd *International Congress "Phytopharm 2018"*, Horgen, Switzerland, 25.-27.7. 2018. Book of Abstracts, short lecture, p. 73.

Netopilova M, Houdkova M, Rondevaldova J, Kokoska L. 2017. Determination of combinatory effect of thymol and carvacrol against *Staphylococcus aureus* using new broth volatilization chequerboard method. *48th International Symposium on Essential Oils*, Pecs, Hungary, 10.-13.9. 2017. Book of Abstracts, Poster P-86, p. 147.