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Evaluation of *in vitro* growth-inhibitory potential of combinations of isopropylmethylphenol isomers against *Staphylococcus aureus* in liquid and vapour phase

BACHELOR'S THESIS

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Marcela Růžičková

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Abstract

Staphylococcus aureus is a causative agent of lower respiratory tract infections and represents a major global health burden, particularly in low-income countries due to limited access to healthcare and high treatment costs. With the increasing emergence of antibiotic resistance, new therapeutic approaches are being investigated, including the antimicrobial activity of volatile compounds. In addition, combinations of such compounds show considerable potential for enhancing bacterial growth inhibition. In this study, combinations of two natural isopropylmethylphenol isomers, namely thymol and carvacrol, and one synthetic isomer, 4-isopropyl-3-methylphenol, were tested for their combinatory antimicrobial potential against *S. aureus* using broth volatilization chequerboard method. All tested combinations exhibited some degree of additive effect, with the best results obtained for combinations of 4-isopropyl-3-methylphenol with either thymol or carvacrol. The lowest ∑FIC, indicating a strong additive effect, was 0.58 and was tested for combination of 4-isopropyl-3-methylphenol with thymol in vapour phase. This combination showed two ∑FIC values below 0.6 and one below 0.7, further supporting a strong additive combinatory effect. For the combination of 4-isopropyl-3-methylphenol and carvacrol, one ∑FIC value below 0.6 and three below 0.7 were observed in vapour phase, along with two values below 0.7 in the liquid phase. These findings may support the development of future inhalation therapies. However, further *in vivo* studies are required to confirm safety and efficacy before practical application.

Key words: volatile compounds, broth volatilization chequerboard method, *Staphylococcus aureus*, combinatory effect, antimicrobial potential

Contents

1. Introduction 1

2. Literature Review 2

2.1. S. aureus 2

2.1.1. Microbiology 2

2.1.2. Pathogenesis 3

2.1.3. Epidemiology 4

2.1.4. Treatment and antibiotic resistance 5

2.2. Combinatory antimicrobial effects 6

2.2.1. *In-vitro* evaluation of combinatory antimicrobial effects 7

2.3. Plant-derived volatile antimicrobial compounds 8

2.4. Isopropylmethylphenol derivates 9

2.4.1. Thymol 10

2.4.2. Carvacrol 11

2.4.3. 4-isopropyl-3-methylphenol 13

Aims of the Thesis 14

3. Material and Methods 15

3.1. Chemicals 15

3.2. Microorganisms and culture media 15

3.3. Broth volatilization chequerboard method 15

4. Results and discussion 20

5. Conclusion 23

6. References 24

List of tables

Table 1. *In vitro* growth-inhibitory potential of isoprophylmethylphenol isomers in combination against *S. aureus*. 22

List of figures

Figure 1. Chemical structure of isopropylmethylphenol isomers. 10

Figure 2. The 96-well microtiter plate lid: experimental layout with flat-bottom flanges 18

Figure 3. The 96-well microtiter plate: experimental layout 19

List of the abbreviations used in the thesis

MSSA	Methicillin-Susceptible *S. aureus*
MRSA	Methicillin-Resistant *S. aureus*
MIC	Minimal Inhibitory Concentration
MH	Muller-Hinton
MTT	Thiazolyl Blue Tetrazolium Bromide Dye
FIC	Fractional Inhibitory Concentration
AMR	Antimicrobial Resistance
MDR	Multi-drug Resistance
GRAS	Generally Recognized as Safe

1. Introduction

Lower respiratory tract diseases, including pneumonia, represent a global health threat. Pneumonia poses the highest risk to children under 5 years of age, with 81% of pneumonia-related deaths occurring within the first two years of life (Walker et al. 2013). It is recognized as the leading cause of death in countries of Southeast Asia (Ghimire et al. 2012). Low-income countries of this region experience a significantly higher death rates due to respiratory tract infections compared to middle- and high-income countries (Feddema et al. 2021). Limited access to healthcare, together with inadequate healthcare infrastructure, represents a major challenge. The increasing resistance of pathogenic bacteria to antibiotics leads to higher morbidity and mortality, longer hospitalizations, and higher treatment costs (Chinemerem et al. 2022). Although *Staphylococcus aureus* accounts for only about 3% of all cases of bacterial pneumonia (Kulkarni et al. 2022), it represents a significant threat as it has developed resistance to every therapy introduced to date (Reddy et al. 2017).

Plant-derived compounds are recognized for their antibacterial properties and are widely utilized in the pharmaceutical industry. Their antibacterial activity has been assessed in both liquid and vapor phase with promising results (Bolouri et al. 2022). Due to their volatility, plant-derived compounds can be easily administered via inhalation, targeting both the upper and lower respiratory tracts and limiting systemic exposure (Ács et al. 2018). The synergistic or additive effects of different antimicrobial agents further enhance their efficacy (Bassolé et al. 2012) and help overcome bacterial resistance mechanisms (Sharma et al. 2020).

2. Literature Review

2.1. S. aureus

The genus *Staphylococcus* belongs together with *Jeotgalicoccus, Macrococcus, Nosocomiicoccus*, and *Salinicoccus* to the family Staphylococcaceae (Becker et al. 2014). The genus S*taphylococcus* comprises about 60 species and subspecies (Lamers et al. 2012). Among those, the most significant are the species *Staphylococcus aureus* and *Staphylococcus epidermis* for their broad interactions with humans (Todar 2004). The term "*Staphylococcus*" was first used in 1882, and in 1884, German surgeon Rosenbach distinguished between the two most common species based on the colour of their pigmented colonies (yellow = *S. aureus*; white = *S. albus*) during experiments on bacterial recovery from abscesses. Pigment production was the most important feature for distinguishing pathogenic *S. aureus* from non-pathogenic *S. albus* (*epidermis*) until 1940, when R. W. Fairbrother introduced differentiation based on coagulase production, which is still widely used today (Becker et al. 2014). *S. epidermis* acts as a commensal organism to humans in most cases (Otto 2009), whereas *S. aureus* poses a great health threat, causing over 1 million deaths annually (Yan et al. 2025). It is the leading cause of skin and soft tissue infections, accounting for approximately 80% of all cases globally (Bhattacharya et al. 2024). It is a common causative agent of many other diseases, such as pneumonia, osteomyelitis, bacteremia, endocarditis, toxic shock syndrome, or food poisoning (Yamazaki et al. 2024; Yan et al. 2025).

2.1.1. Microbiology

S. aureus is a Gram-positive, round-shaped, nonmotile, facultative anaerobic bacterium, about 1 micrometer wide. The multiplication of the bacteria happens asexually by division in two planes, thus forming irregular "grape-like" clusters. It can survive under a wide range of conditions (Todar 2004). *S. aureus* is a facultatively anaerobic bacterium producing biofilm, which promotes its adhesion to host tissue cells (Murthy et al. 2024). *S. aureus* is catalase-positive, which serves as an important property for distinguishing between *Staphylococci* and *Streptococci*. *S. aureus* is also coagulase-positive, meaning it has the ability to secrete the enzyme coagulase, which converts

fibrinogen to fibrin. This allows for distinguishing between *S. aureus* and *S. epidermidis*. *S. aureus* is further urease-positive, phosphatase-positive, and oxidase-negative. The bacterium is capable of mannitol fermentation, producing lactic acid as the final product (Reddy et al. 2017). Aside from that, *S. aureus* produces carotenoids, which serve, as well as superoxide dismutase, for protection from cell oxidation. *S. aureus* does not form spores. The optimal pH for bacterial growth of *S. aureus* is 7.0–7.5, while its survival range is 4.2–9.3. The optimal growth temperature is 35–37°C, while the survival range is again very wide, from 10–45°C (Todar 2004).

2.1.2. Pathogenesis

S. aureus has been found as part of the natural human microbiome at various anatomical locales such as skin, conjunctiva, nose, face, hands, pharynx, mouth, large intestine, anterior urethra, or vagina (Todar 2004). The primary habitat of *S. aureus* in humans is the moist squamous epithelium of the anterior nares, where it can colonize without causing an infection. Approximately 20% of the healthy adult population are persistent nasal carriers and 60% carry *S. aureus* intermittently (Peacock et al. 2001; Aires De Sousa & Lencastre 2004; Foster 2004). Some subpopulations, such as patients with atopic dermatitis, human immunodeficiency virus, diabetes mellitus, or dialysis patients, showed even higher carrier rates (Reddy et al. 2017).

S. aureus is a very significant pathogen, as it is part of the natural microbiome of many and can be easily transmitted. Moreover, it can cause a broad range of infections, where essentially every tissue of the human body can be infected, and its virulence is very high (Todar 2004). The versatile characteristics of *S. aureus* are responsible for an easy switch from a commensal-like lifestyle to a pathogenic lifestyle, which happens when the balance between host defences (such as the status of immunity system) and pathogen factors (such as virulence and size of inoculum) is disturbed in the favour of the pathogen (Reddy et al. 2017). The virulence factors of *S. aureus* are:

- **surface proteins**, which promote colonization. They are the key property that determines the pathogen's ability to bind to host tissue cells. Those proteins are, for example, laminin and fibronectin. Another component of the cell wall is protein A, which acts against phagocytosis.

- **secreted proteins**, which allow for invasion and damage of host cells and tissues. Such proteins are mainly membrane-damaging toxins, among which the most potent is α-toxin. Other examples include δ-toxin, hemolysins, and leukotoxins, such as leucocidin, which induces the release of lysosomal contents into the cytoplasm of phagocytes, thereby protecting *S. aureus* from phagocytosis. Most strains also produce clumping factors that promote adhesion to blood clots.
- **toxins**, so-called superantigens, which can cause toxic shock. Such toxins are enterotoxins SE-A, -B, -C, -D, -G, and TSST-1 (toxic shock syndrome toxin). When disseminated systemically, they can stimulate large numbers of T-cells, leading to excessive cytokine release and the development of toxic shock syndrome. (Todar 2004).

The progression of infection is influenced by the quantity of microorganisms introduced into host tissue (Loebinger & Wilson 2012) or by a direct introduction to the via injuries, syringes, or medical instruments. The risk of infection is higher in individuals with a compromised immune system and in children whose immune systems are not fully developed. An individual can be infected by the strain of his own carrier or by cross-infection (Reddy et al. 2017). The high virulence of some strains accounts for *S. aureus* being the major cause of community- and hospital-acquired infections (Keim & Horswill 2023).

The infections, caused by *S. aureus* can be broadly divided into three groups: (i) superficial lesions, where *S. aureus* is the leading cause (e.g. impetigo, cellulitis, folliculitis, infected ulcers or wounds); (ii) systemic and life-threatening conditions (endocarditis, osteomyelitis, meningitis, bacteremia, brain abscess, pneumonia); (iii) toxinoses (food poisoning, scalded skin syndrome, toxic shock syndrome) (Aires De Sousa & Lencastre 2004; Krishna & Miller 2012; Reddy et al. 2017; Keim & Horswill 2023).

2.1.3. Epidemiology

In 2019, *S. aureus* was recognized as one of five leading pathogens causing infection-related deaths and ranked as the single leading pathogen in 135 countries. In individuals aged 15 years or older, it was the leading pathogen globally (Ikuta et al. 2022). *S. aureus* is the leading cause of skin and soft tissue infections, as well as in infective in

endocarditis (Reddy et al. 2017). It is the leading pathogen of Gram-positive caused nosocomial (hospital-acquired) infections (Todar 2004), and among these it is the major cause of community-acquired pneumonia (He & Wunderink 2020). Although *S. aureus* accounts only for 3-5% of all pneumonia cases compared to other Gram-negative bacteria, these infections are usually severe and frequently fatal (Torres et al. 2023).

2.1.4. Treatment and antibiotic resistance

The common treatment of infections caused by *S. aureus* is antibiotics. Infections caused by methicillin-susceptible *S. aureus* (MSSA) strains are usually treated with β-lactam drugs, such as cephalosporins, oxacillin, or nafcillin. Methicillin-resistant *S. aureus* (MRSA) caused infections are treated with the glycopeptide drug vancomycin, and in some countries, teicoplanin (David & Daum 2017).

Antibiotic resistance

Before the discovery of penicillin in 1928, the mortality rate of systemic *S. aureus* infections was about 80%. Introduction of penicillin treatment in clinics in the early 1940's dramatically lowered the mortality rate. However, by 1942, the first penicillin-resistant strains emerged, and in the 1960s, 80% of clinical isolates showed resistance to penicillin. As a reaction, methicillin, a semisynthetic penicillin-based drug, was introduced in 1961. Clinical strains resistant to methicillin were reported shortly after, and infections caused by such strains were even more severe. Many different drugs have been developed, but *S. aureus* possesses a formidable ability to adapt, and therefore continues to develop new resistances (Lowy 2003; Reygaert 2013; Yamazaki et al. 2024). It has been reported that S. aureus strains have acquired resistance to virtually all commercially available antibiotics (Todar 2004).

Antimicrobial resistance (AMR) has emerged due to the overuse and misuse of antibiotics as microorganisms develop resistance to withstand environmental selection pressure (Abushaheen et al. 2020; Salam et al. 2023). AMR is either intrinsic (the bacterial species possesses a specific gene that can grant AMR, but it needs to be activated) or acquired (the bacteria's genetic information mutates via genes acquired from a different microorganism) (Reygaert 2013; Abushaheen et al. 2020). Genetic mutations in the bacterial genome enable the activation of diverse mechanisms that protect the bacteria from antimicrobial agents. These mechanisms include limiting drug uptake into

the bacterial cell, modifying the drug target, inactivating the drug, or actively pumping the drug out of the cell via active efflux (Reygaert 2013).

As an approach to address the rise in antibiotic resistance, nebulized antibiotics have been introduced for the treatment of severe respiratory infections. These are drugs converted into droplets that can be inhaled as aerosols directly into the lower respiratory tract. Their advantages are proposed to be a higher drug concentration at the site of infection and reduced systemic exposure. Some drawbacks of nebulized antibiotics include technical issues during administration and pulmonary complications such as cough, bronchospasm, wheezing, desaturation, and hypoxemia (Gorham et al. 2023).

Another approach for eradicating the increasing antibiotic resistance is the use of plant-derived compounds. The complexity of plant constituents, including polyphenols, alkaloids, and tannins, is studied for their health-beneficial effects. Phytochemicals are suggested to have a broad antimicrobial potential and the ability to modify antibiotic resistance (AlSheikh et al. 2020).

2.2. Combinatory antimicrobial effects

For the purpose of enhancing the treatment efficacy and overcoming the development of AMR, the use of combinations of drugs is studied as one of the strategies (Tyers & Wright 2019). Positive combinatory interactions between two compounds can be explained by four theoretical mechanisms of action: (i) sequential inhibition of several steps in a particular biochemical pathway, (ii) inhibition of enzymes that degrade of excrete antimicrobials, (iii) interaction of several antimicrobials with the cell wall, or (iv) interaction with the cell wall or membrane, leading to increased uptake of other antimicrobials. Another possible mechanism is that the two compounds have different modes of action that indirectly depend on each other (Hyldgaard et al. 2012).

Combinations of antibiotics, as well as combinations of antibiotics with non-antibiotic activity-enhancing compounds, have been investigated (Tyers & Wright 2019). Inhibitors of β-lactamases are commonly administered as co-drugs together with β-lactam-based antibiotics; other cell wall-active agents are combined with aminoglycosides; and combinations of agents inhibiting separate steps along the critical metabolic pathway also produce an enhanced bactericidal effect (Eliopoulos &

Moellering 1982). Co-amoxiclav is an example of a combinatory antimicrobial effect in practical use. The product, commercially available in different formulations, among which Augmentins® is the best known, is a complex of amoxicillin (semisynthetic β-lactam antibiotic) with clavulanic acid (β-lactamase inhibitor). Clavulanic acid has weak intrinsic antibiotic activity but is effective at inhibiting β-lactamases, thereby protecting substrate drugs from hydrolysis (Gordon 2010).

Secondary metabolites from plants are a promising source for combination therapy. Plants produce a wide range of phytochemicals with bactericidal potential. In most cases, these compounds are less potent than conventional antibiotics. However, they may exert antimicrobial effects through synergistic or combinatory interactions (Hemaiswarya et al. 2008). For example, *Berberis* medicinal plants produce alkaloids with strong antibacterial activity against *S. aureus*. However, these alkaloids can be extruded from the bacterial cell by the NorA multidrug resistance pump efflux. *Berberis* plants have been found to synthesize 5′-methoxyhydnocarpin, which acts as an inhibitor of the NorA multidrug resistance pump, indicating effective inhibition of the bacterial resistance mechanism to berberine antimicrobials (Stermitz et al. 2000). Dos Santos Barbosa et al. (2021) propose that multidrug resistance in *S. aureus* can be overcome by combination of plant-derived compounds with antibiotics. The study demonstrated that thymol and carvacrol act as competitive inhibitors of the NorA active efflux pump, increasing susceptibility of resistant *S. aureus* to conventional antibiotics (dos Santos Barbosa et al. 2021).

2.2.1. *In-vitro* evaluation of combinatory antimicrobial effects

Combinatory interactions between antimicrobials can be classified according to EUCAST (2000) into four possible outcomes:

- **Indifferent effect**: the effect of the combination is equal to the effect of the most active individual compound.
- **Additive effect**: the effect of the combination is equal to the sum of the effects of the individual compounds.
- **Synergic effect**: the effect of the combination exceeds the sum of the effects of the individual compounds.

- **Antagonistic effect**: the effect of the combination is reduced compared to the effect of the most active individual compound.

The most common assay for evaluating antimicrobial combinatory effects is the chequerboard method, where a two-dimensional array of serially diluted concentrations of two compounds is used to determine the minimal inhibitory concentrations (MICs). Subsequently, calculation of the fractional inhibitory concentration index (∑FIC) is carried out (Odds 2003). MIC is defined as the lowest concentration (μg/mL) that prevents bacterial growth under defined *in vitro* conditions within a defined period of time (EUSTAT 2000). ∑FIC is calculated according to the equation as follows: $FIC_A = MIC_{A+B}/MIC_A$, $FIC_B = MIC_{B+A}/MIC_B$, $\sum FIC = FIC_A+FIC_B$. The MIC_{A+B} value is the MIC of compound A in the presence of compound B, and vice versa for MIC_{B+A}. The combinatory effect is classified as synergic if ∑FIC is lower than 0.5, additive if ∑FIC is higher than 0.5 but lower than 1.0, or indifferent if ∑FIC ranges from 1.0 to 2.0. A value greater than 2.0 is considered an antagonistic effect, indicating that the two compounds act against each other (Magi et al. 2015).

Another frequently used method for evaluating synergistic potential is the time-kill method. It also assesses the bactericidal activity, however, it is more time-consuming and labour-intensive (White et al. 1996).

2.3. Plant-derived volatile antimicrobial compounds

Plant-derived volatiles are compounds produced as secondary metabolites by various plants. They have a wide range of functions, such as attracting the pollinators or frugivores to disperse seeds, intra or inter plant communication, and protection against herbivores by either intoxicating them or attracting a predator. They have been proposed to play a role in preventing microbial attack (Hammerbacher et al. 2019). Given their properties, plant-derived volatiles have been widely studied as potential alternatives to synthetic antimicrobial agents in agriculture and in the food and pharmaceutical industries. The unique property of plant volatiles is their antimicrobial activity in vapour phase (Houdkova & Kokoska 2020), which makes them particularly suitable for application for the treatment of respiratory infections via inhalation, allowing direct delivery to the site of infection (Al-Harrasi et al. 2022).

2.4. Isopropylmethylphenol derivates

Isopropylmethylphenols are phenolic monoterpenes and important components of essential oils obtained from conifers and angiosperms (Ioppolo-Armanios et al. 1994; Farhadi et al. 2024). Their chemical structure consists of a phenolic ring with methyl and isopropyl substitutions. The chemical structures of thymol, carvacrol, and 4-isopropyl-3-methylphenol are shown in Figure 1. Naturally occurring isopropylmethylphenols are produced as plant secondary metabolites of plants via a biosynthetic pathway that involves geranyl pyrophosphate as a precursor and γ-terpinene and *p*-cymene as intermediates (Marinelli et al. 2018).

Thymol and carvacrol are the two main naturally occurring isopropylmethylphenol isomers. These compounds have been extensively studied for their activity against bacteria, filamentous fungi, and yeasts (Heckler et al. 2021). The key structural features responsible for their antimicrobial activity are the hydroxyl group (-OH) and delocalized electron cloud. Differences in the position of the hydroxyl group explain the variation in antimicrobial potency between thymol and carvacrol (Marinelli et al. 2018). These isomers have been further studied for their antibiofilm activity, as well as their ability to inhibit motility, membrane-bound adenosine triphosphatases, efflux pumps, and disruption of cell wall membrane (Farhadi et al. 2024).

Natural isopropylmethylphenols are used as components of agricultural fungicides (Houdkova & Kokoska 2020), as flavouring and preservative agents in the food industry, and in animal feed. They are also used in dental care, alongside their synthetic isomer 4-isopropyl-3-methylphenol. These compounds are being further investigated for their antimicrobial properties, addressing antibiotic resistance of pathogenic microorganisms (Kachur & Suntres 2020). The drawbacks of their use include toxicity at high doses and poor delivery due to their hydrophobicity, volatility, and instability when exposed to oxygen and light (Gago et al. 2025).

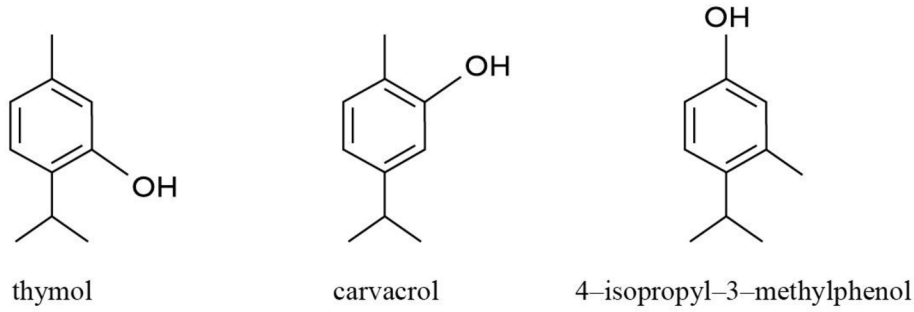

Figure 1. Chemical structure of isopropylmethylphenol isomers.

2.4.1. Thymol

Thymol (2-isopropyl-5-methylphenol) is a phenolic monoterpene with a hydroxyl group in the *meta* position (Marinelli et al. 2018), first identified in 1719 and later purified and named in 1853 (Ahmad et al. 2021). It is a colourless crystalline substance with an aromatic odour and strong flavour. Its melting point ranges from 49°C to 51°C. Thymol has poor solubility in water, but is highly soluble in alcohols, alkaline solutions, and other organic solvents (Nagoor Meeran et al. 2017).

Thymol possesses antimicrobial, anti-inflammatory, antispasmodic, antioxidant, anticancer, wound healing, analgesic, antifungal, antiseptic, and antitumor properties. Owing to these characteristics, thymol has been widely used in cosmetics and pharmaceuticals (Nagoor Meeran et al. 2017; Gago et al. 2025). It has also been classified as GRAS (Generally Recognized As Safe) and thus is commonly used as a food flavouring and preservative (EAFUS 2006).

The primary site of action of thymol is the polar headgroup region of the cell membrane, where it increases membrane fluidity. Its antibacterial activity also involves disruption of outer and inner membranes, interactions with membrane proteins, and effects on intracellular targets. Interactions with the cell membrane include affected permeability, loss of membrane potential, uptake of ethidium bromide, leakage of potassium ions, ATP, and carboxyfluorescein (Hyldgaard et al. 2012).

Thymol exhibits strong bactericidal activity against various bacteria, including *Acinetobacter baumannii, Pasteurella aerogenes, Salmonella typhimurium*, *Bacillus subtilis, Klebsiella aerogenes, Klebsiella pneumoniae, Serratia marcescens, Staphylococcus aureus, and Streptococcus agalactiae*. Thymol is generally used as an

antimicrobial agent in many commercially available products such as Listerine Antiseptic Mouthwash (Johnson & Johnson, New Brunswick, USA) and Cervitec Plus (Ivoclar Vivadent, Schaan, Liechtenstein) for oral care, as well as Bronchipret Saft (Bionorica, Neumarkt, Germany), which is recommended for relief of symptoms associated with acute respiratory tract inflammations (Kokoska et al. 2019). Furthermore, its antimicrobial potential in combination with other compounds has been reported with promising results. Strong synergy has been observed for combination of thymol with commercial antibiotics, namely streptomycin and gentamicin, against *S. aureus* (Gan et al. 2023). A similar strong synergic effect has also been reported for the combination with its isomer carvacrol against *Listeria innocua* in a study conducted by García-García et al. (2000).

Thymol naturally occurs as a constituent of essential oils. The primary source is *Thymus vulgaris*, where thymol accounts for 10–64% of the essential oil. It is also present in many other plant species, not only within the Lamiaceae family (*Thymus zygis, Thymus glandulosus, Thymus hyemalis, Monarda fistulosa, Monarda punctata, Monarda didyma, Origanum compactum, Origanum dictamnus, Origanum onites, Origanum vulgare, Satureja spicigera, Satureja intermedia, Satureja mutica, Satureja sahendica, Coleus aromaticus, Zataria multiflora*), but also in Apiaceae (*Trachyspermum copticum, Lagoecia cuminoides, Carum copticum*), Verbenaceae (*Lippia multiflora, Lippia gracilis*), *Asteraceae* (*Centipeda minima*), Ranunculaceae (*Nigella sativa*), and Laminaceae (*Zataria multiflora*) (Salehi et al. 2018; Gholami-Ahangaran et al. 2022).

2.4.2. Carvacrol

Carvacrol is a phenolic monoterpene with a hydroxyl group in the *ortho* position. Its chemical structure is 2-methyl-5-(1-methylethyl)-phenol. It is also known as *p*-cymen-2-ol, 2-hydroxy-*p*-cymene, 5-isopropyl-2-methylphenol, and iso-thymol (Suntres et al. 2015; Marinelli et al. 2018). It is a colourless to pale yellow liquid with a boiling point of 238°C. Carvacrol is insoluble in water, but well soluble in lipids (Ahmad et al. 2021).

Carvacrol possesses antimicrobial, anti-inflammatory, antioxidant, antitumor, analgesic, anti-hepatotoxic, and insecticidal properties (Magi et al. 2015). It is active against food spoilage and pathogenic fungi, yeast, and bacteria. Carvacrol has been classified as GRAS and is generally used in the food industry. It also exhibits activity

against human, animal, and plant pathogenic microorganisms, and has been studied for its activity against drug-resistant and biofilm-forming strains. Its bactericidal activity has been attributed to its effects on the structural and functional properties of the cytoplasmic membrane (EAFUS 2006; Nostro & Papalia 2012). Stronger antimicrobial activity has been reported for Gram-negative than Gram-positive bacteria (Magi et al. 2015).

The primary mode of action is the ability of carvacrol to partition into the cell membrane, increasing its permeability. The hydroxyl group of carvacrol acts as a transmembrane carrier of cations, facilitating the influx of H+ ions into the cytoplasm and the efflux of K+ ions from the cell. Studies further suggest that carvacrol may interact with membrane proteins and periplasmic enzymes, and it may also have intracellular targets, but the evidence for this is limited (Hyldgaard et al. 2012).

The antimicrobial activity of carvacrol has been observed against a wide range of bacteria, including *Escherichia coli, Salmonella typhimurium, Staphylococcus aureus, Listeria monocytogenes, Pseudomonas aeruginosa, Proteus mirabilis, Bacillus subtilis, Lactobacillus plantarum, Mycobacterium avium, Enterococcus faecalis*, and *Bacillus cereus*. Carvacrol, similarly to thymol, shows the highest activity against *Salmonella typhimurium* with MIC 0.25 µl/mL (Ahmad et al. 2021). Previously conducted studies report that carvacrol can be effectively combined with other compounds, resulting in significant synergistic effects. Strong synergy has been observed for combination of carvacrol with erythromycin against Group A *streptococci* (Magi et al. 2015), as well as with its biosynthetic precursor, cymene, against foodborne pathogens such as *Bacillus cereus, Escherichia coli,* or *Vibrio cholerae* (Rattanachaikunsopon & Phumkhachorn 2010).

Carvacrol is a component of essential oils of various plant species, usually alongside thymol, e.g., *Thymus, Satureja*, and *Origanum* species (Mączka et al. 2023). Whithin the Lamiaceae family, it is commonly found in species *Coridothymus capitatus, Origanum compactum, Origanum dictamnus, Origanum majorana, Origanum minutiflorum, Origanum onites, Origanum scabrum, Origanum syriacum, Origanum vulgare, Thymus broussonetii, Thymus ciliatus, Thymus fallax, Thymus fedtschenkoi, Thymus fontanesii, Thymus Kotschyanus, Thymus leptobotrys, Thymus maroccanus, Thymus pubescens, Thymus revolutus, Thymus satureioides, Thymus schimperi, Thymus trauveterri, Thymus vulgaris, Thymus tosevii, Satureja boissieri, Satureja cuneifolia,*

Satureja montana, Satureja mutica, Satureja rechingeri, Satureja subspicata, Satureja khuzestanica, Satureja thymbra, and *Thymbra spiccata* (Marinelli et al. 2018).

2.4.3. 4-isopropyl-3-methylphenol

4-Isopropyl-3-methylphenol is a synthetic compound produced by the isopropylation of *m*-cresol via different catalysts, followed by refinement controls such as isomerization, distillation, extraction, and crystallization to obtain the final product (ChemicalBook 2020).

4-Isopropyl-3-methylphenol is a synthetic analogue of thymol and carvacrol, developed as an antimicrobial preservative. It is a colour- and odour-neutral substance (Kim et al. 2016). It exhibits antibacterial, antiseptic, anti-inflammatory, and acaricidal activity and is used in the food industry as a preservative agent. It has been further investigated for potential application in nonlinear optics (ChemicalBook 2020). The characteristics of antimicrobial activity are yet not fully understood, although Kim et al. (2016) suggested that they may be similar to those of its natural analogue, thymol. In a study involving fungi of the *Aspergillus* genus, 4-isopropyl-3-methylphenol was shown to affect redox homeostasis of the fungi. The study also observed that it induced fludioxonil tolerance in mutant fungi (Kim et al. 2016). MRSA *S. aureus* exhibits susceptibility to this compound at concentrations similar to those of carvacrol. Furthermore, 4-Isopropyl-3-methylphenol exhibits strong inhibitory activity against staphylococcal biofilm formation (Ohara et al. 2023; Korenaga et al. 2024), however, there are no reports on its activity in vapour phase.

Aims of the Thesis

The aim of this thesis is to determine the *in-vitro* growth-inhibitory potential of isopropylmethylphenol isomers in combination against *Staphylococcus aureus* in liquid and vapour phase using the broth volatilization chequerboard method.

3. Material and Methods

3.1. Chemicals

Thymol (98.5%, CAS 89-83-8), carvacrol (98%, CAS 499-75-2), and 4-isopropyl-3-methylphenol (99%, CAS 3228-02-2) were purchased from Sigma-Aldrich (Prague, CZ) as well as thiazolyl blue tetrazolium bromide (MTT). Further, dimethyl sulfoxide (DMSO) was obtained from Penta (Prague, CZ)

3.2. Microorganisms and culture media

For the assay, *Staphylococcus aureus* ATCC 29213 was obtained from Oxoid (Basingstoke, UK) on ready-to-use bacteriological Culti-Loops. Cultivation and assay media were Mueller-Hinton (MH) broth and agar obtained from Oxoid (Basingstoke, UK). While bacteria were cultivated in pure MH broth medium, the antimicrobial assay was carried out using the MH broth with added buffer Trizma base (Sigma-Aldrich, Prague, CZ) and pH equilibrated to final value of 7.6. Bacterial strain from stock culture was cultivated in pure broth medium at 35°C for 24 h before testing. Prior to the assay, the turbidity of the bacterial suspension was adjusted to 0.5 McFarland standard using Densi-La-Meter II (Lachema, Brno, CZ) to get the final inoculum with the concentration of 10^7 CFU/mL.

3.3. Broth volatilization chequerboard method

The potential antibacterial combinatory effects of volatile compounds were examined using the broth volatilization chequerboard method (Netopilova et al. 2018). The experiments were performed in standard 96-well microtiter plates (volume of each well = 400 μL). The plates were covered with tightly fitting lids with flanges to reduce evaporation of the volatile compounds (SPL Life Sciences, Naechon Myeon, Republic of Korea).

Firstly, the volatile compounds were diluted in DMSO at a concentration of 204,800 μg/mL. Diluted volatiles were further pipetted into MH buffered broth in such a

manner to achieve the final concentration of 2,048 μg/mL. The maximum volume of DMSO in the solution was 1%, so it did not compromise the results. For the positive antibiotic control, oxacillin was diluted first in distilled water, and subsequently pipetted into MH buffered broth to achieve the final concentration of 4 μg/mL. For the lid, 30 μL of MH agar was pipetted into each flange, excluding the four outer corners. The agar was inoculated with 5 μL of bacterial suspension. Sterility and growth control, as well as positive antibiotic control, were present in each lid and plate according to the plate design shown in Figures 2 and 3.

For the plate, serial dilutions of dissolved compounds were performed by the automated pipetting platform Freedom EVO 100 equipped with a four-channel liquid handling arm (Tecan, Mannedorf, CH). Firstly, all wells, excluding A10, A11, and B12, were filled with 100 μL of MH buffered broth. Wells A2-H2 were additionally filled with 100 μL of solution with compound A at the concentration of 2,048 μg/mL and diluted horizontally in eight two-fold serial dilutions. Subsequently, a cross-dilution was performed, when wells A2-A9 were filled with 100 μL of solution with compound B at the concentration of 2,048 μg/mL, followed by vertical eight two-fold serial dilutions. Simultaneously, eight two-fold serial dilutions for each compound separately were performed. The highest concentration of both compounds tested separately was 2,048 μL/mL, and the lowest concentration was 16 μL/mL. The positive antibiotic control was prepared by six two-fold dilutions of oxacillin starting at 4 μL/mL. Prepared plates were inoculated with bacterial suspension (10^7 CFU/mL), excluding the sterility control wells. Inoculated plates and lids were fastened together with clamps (Lux Tool, Prague, CZ) and handmade wooden pads for tight fitting. Incubation was done at 35°C for 24 hours.

Evaluation of the growth-inhibitory activity in both liquid (plate) and vapour (lid) phase was done by visual examination of metabolically active colonies after colouring each well and flange with 25 μL of MTT dye (600 μg/mL). Colour ranged from yellow, indicating no or little bacterial growth, to deep purple, indicating no growth-inhibitory activity of the examined compounds. The interface of colour change was compared to the growth control and MICs for both compounds separately, and each combination were recorded as the lowest concentration exhibiting no bacterial growth, expressed in μg/mL.

Combinatory effects were evaluated according to The European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2000). Fractional inhibitory

concentration indices (∑FIC) for compounds A and B in combinations were calculated with the equation $\sum FIC = FIC_A + FIC_B$, where $FIC_A = MIC_{(A \text{ in combination with } B)}/MIC_{(A \text{ alone})}$ and $FIC_B = MIC_{(B \text{ in combination with } A)}/MIC_{(B \text{ alone})}$. The combinatory potential was determined as follows:

Synergy: $\sum FIC \leq 0.5$

Additive effect: $0.5 \leq \sum FIC < 1$

Indifferent effect: $1 \leq \sum FIC < 2$

Antagonism: $\sum FIC \geq 2$

Three different combinations were tested: combination I (compound A: thymol; compound B: carvacrol), combination II (compound A: 4-isopropyl-3-methylphenol; compound B: thymol), and combination III (compound A: 4-isopropyl-3-methylphenol; compound B: carvacrol). Combination I was carried out in three independent experiments in triplicate, whereas combinations II and III were performed in a single experiment in triplicate. In this work, all results are displayed as averages.

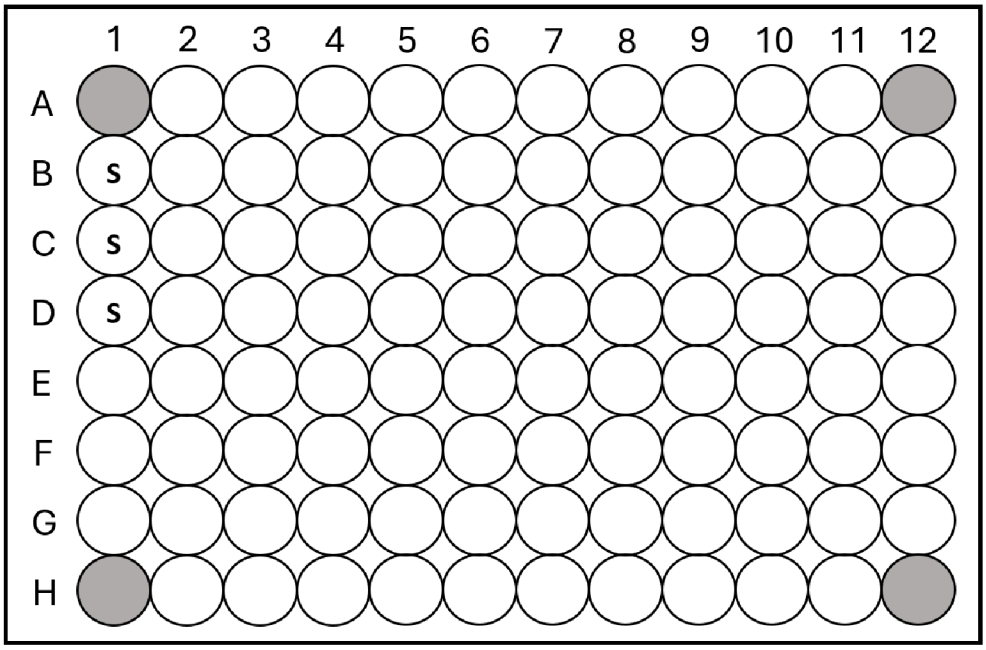

Figure 2. The 96-well microtiter plate lid: experimental layout with flat-bottom flanges. Grey-coloured wells: empty wells, not utilized; S: sterility control indicating 0% bacterial growth; white-coloured wells: agar inoculated with bacterial suspension (Netopilova et at. 2018).

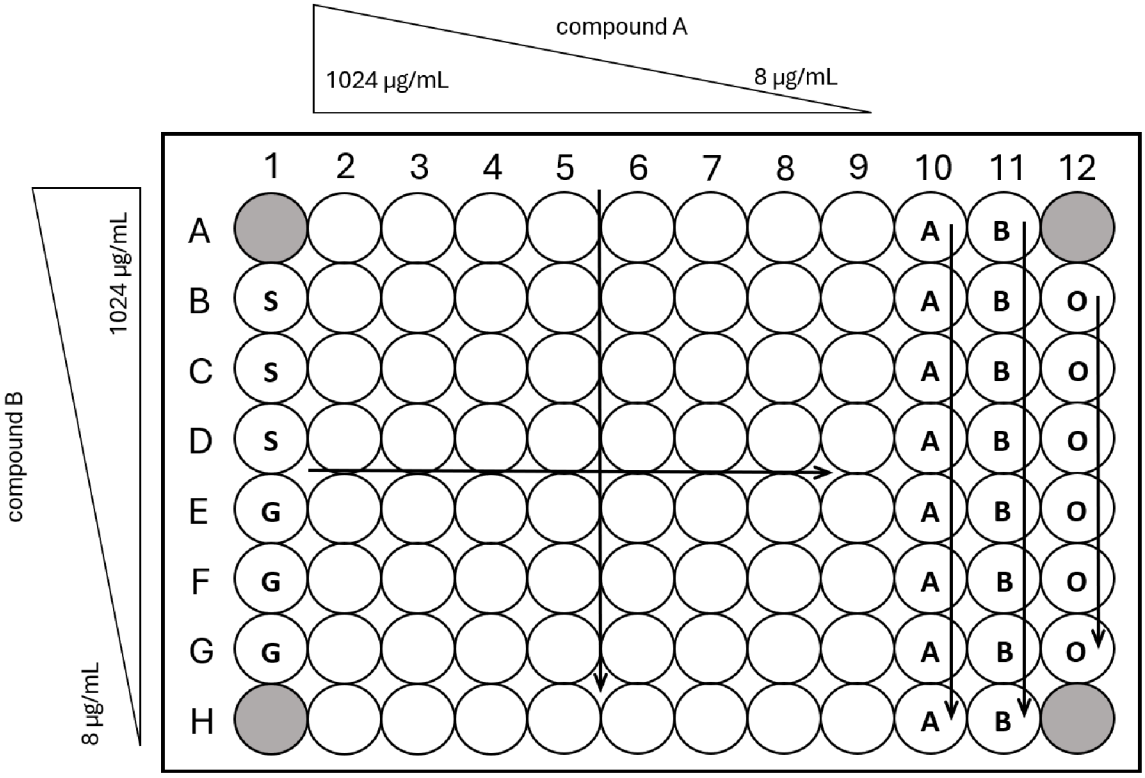

Figure 3. The 96-well microtiter plate: experimental layout. A: compound A alone in two-fold dilutions (starting at concentration 2,048 µg/mL); B: compound B alone in two-fold dilutions (starting at concentration 2,048 µg/mL); G: growth control (medium inoculated with bacterial suspension; 100% growth of bacteria); O: oxacillin (positive antibiotic control) in two-fold dilutions; S: sterility control (non-infected medium; 0% growth of bacteria); grey-coloured wells: wells not used (Netopilova et al. 2018).

4. Results and discussion

This study evaluated the growth-inhibitory potential of three isopropylmethylphenol isomers, namely thymol, carvacrol, and 4-isopropyl-3-methylphenol, against *S. aureus* in liquid and vapour phase. The combinatory antimicrobial effect was determined for combinations of thymol and carvacrol, 4-isopropyl-3-methylphenol and thymol, and 4-isopropyl-3-methylphenol and carvacrol. All tested compounds showed some degree of antibacterial activity both alone and in combinations. Additive effects were found in all combinations in liquid and in vapour phase, where the strongest additive effect was observed for the combination of 4-isopropyl-3-methylphenol with thymol and the combination of 4-isopropyl-3-methylphenol with carvacrol in vapour phase. The detailed results of individual MICs of each compound and corresponding ∑FIC are shown in Table 1.

Thymol produced moderate to weak antistaphylococcal effect with MICs ranging from 569 µg/mL to 683 µg/mL in broth and 484 µg/mL to 683 µg/mL in agar. These values correspond well with MICs detected by Netopilova et al. (2018), who observed MICs 512 and 355 µg/ml in liquid and vapour phase, respectively. Nevertheless, Houdkova et al. (2017) reported stronger antimicrobial activity with MIC values of 256 µg/mL in broth and 128 µg/mL on agar. Netopilova et al. (2018) proposed that such differences can be caused by different methods used, while in the broth volatilization chequerboard method, higher evaporation may occur due to longer time of plate preparation. Similarly, carvacrol exhibited MIC ranging from 569 µg/mL to 853 µg/mL in liquid phase and 597 µg/mL to 683 µg/mL on agar, which corresponds well with the results of Netopilova et al. (2018), who reported 512 µg/mL in liquid phase and 370 µg/mL in vapour phase. The weakest antistaphylococcal activity was observed for 4-isopropyl-3-methylphenol with MICs ranging from 512 µg/mL to 853 µg/mL in liquid phase and 427 µg/mL to 1195 µg/mL in vapour phase. These results are consistent with a study by Kim et al. (2022), who reported MIC of 512 µg/mL in liquid phase for wild-type strains of *S. aureus* and also observed reduced MICs for combination with oxacillin (Kim et al. 2022). In another previously reported study (Ohara et al. 2023), this compound produced antifungal and antimicrobial effects against *Candida* spp. and multiple intraoral pathogenic microorganisms, including *S. aureus*. It also showed strong biofilm formation inhibitory activity in combination with β-thujaplicin, which supports our findings on

combinatory effects of 4-isopropyl-3-methylphenol. This compound also inhibited the growth of *Aspergillus brasiliensis,* and a weak additive effect was observed in combination with octylgallate (Kim et al. 2016). According to our best knowledge, this is the first report on the antibacterial effect of 4-Isopropyl-3-methylphenol in vapour phase.

As far as combinations of compounds tested in this study are considered, the combination of 4-isopropyl-3-methylphenol with thymol showed the best results. This combination produced a strong additive effect with ∑FIC ranging from 0.58 to 0.91 in vapour phase. The lowest ∑FIC recorded was for combination of 683 µg/mL of 4-isopropyl-3-methylphenol and 8 µg/mL of thymol. Only weak additive effect was observed in liquid phase where the lowest ∑FIC was 0.88. Similar results were recorded for combination of 4-isopropyl-3-methylphenol with carvacrol. Results indicated strong additive effect in both liquid and vapour phase. Additive effect was reported for all the combinations in vapour phase with ∑FIC ranging from 0.59 to 0.81. The strongest additive potential was reported for combination of 171 µg/mL of 4-isopropyl-3-methylphenol with 128 µg/mL of carvacrol. The lowest ∑FIC for liquid phase was 0.63 for 171 µg/mL of 4-isopropyl-3-methylphenol combined with 256 µg/mL of carvacrol. According to our best knowledge, this is the first report on the combinatory antibacterial effect of 4-isopropyl-3-methylphenol with thymol and carvacrol.

The combination of thymol and carvacrol showed additive to indifferent combinatory effect. In liquid phase, results were slightly better than in vapour phase. Three cross-diluted combinations showed additive effect in broth medium, ranging from ∑FIC 0.71 to ∑FIC 0.92, while only two combinations indicated additive effect in vapour phase with ∑FIC of 0.83 and 0.84. The rest of the combinations indicated indifferent antimicrobial combinatory potential compared to when tested alone, with ∑FIC ranging from 1.01 to 1.16. These results correspond fully with findings of Netopilova et al. (2018), who recorded the lowest ∑FIC value for SA ATCC 29213 of 0.83 in liquid phase and 0.91 in vapour phase.

Table 1. *In vitro* growth-inhibitory potential of isoprophylmethylphenol isomers in combination against *S. aureus*.

	Combination	MICs alone (µg/mL)				Compound A in combination with listed concentration of compound B (µg/mL).													
		T	C	IPMP	O	+ B 512		+ B 256		+ B 128		+ B 64		+ B 32		+ B 16		+ B 8	
						MIC	∑FIC	MIC	∑FIC	MIC	∑FIC	MIC	∑FIC	MIC	∑FIC	MIC	∑FIC	MIC	∑FIC
liquid	I[1]	569	569		3	n.d.	n.d.	142	0.71	313	0.78	455	0.92	569	1.09	626	1.16	569	1.07
	II[2]	683		853	2	192	0.98	427	0.88	683	0.99	853	1.09	853	1.05	1024	1.25	1024	1.25
	III[3]		853	512	4	n.d.	n.d.	171	0.63	256	0.65	427	0.91	512	1.04	427	0.87	512	1.04
vapour	I[1]	484	597			n.d.	n.d.	188	0.84	292	0.83	427	1.01	484	1.08	512	1.12	484	1.05
	II[2]	683		1195		192	0.91	512	0.8	683	0.76	683	0.67	853	0.76	683	0.59	683	0.58
	III[3]		683	427		n.d.	n.d.	n.d.	n.d.	171	0.59	256	0.69	256	0.65	256	0.62	341	0.81

[1]Combination I: compound A: thymol; compound B: carvacrol
[2]Combination II: compound A: 4-isopropyl-3-methylphenol; compound B: thymol
[3]Combination III: compound A: 4-isopropyl-3-methylphenol; compound B: carvacrol

5. Conclusion

In this study, three different combinations of isopropylmethylphenol isomers were tested against *S. aureus* in liquid and vapour phase using broth volatilization chequerboard method with the aim to determine their potential combinatory effects. Values of MICs and FICs were obtained. All tested compounds exhibited additive to indifferent effects in combination compared to when tested alone. Overall results indicated stronger potential antimicrobial additive effects in vapour than in liquid phase. The strongest additive potentials were recorded for combinations of 4-isopropyl-3-methylphenol with thymol and carvacrol in vapour phase. The weakest results were observed for the combination of thymol and carvacrol, which differed from the other two combinations in exhibiting better additive effects in liquid phase than on agar. In this study, the overall better combinatory antimicrobial effects of volatile compounds were observed for vapour phase, which is why we suggest further testing of volatiles in combinations. These results provide theoretical background for development of future pharmaceutical applications, especially inhalation therapies. However, further research focused on safety and pharmacological efficacy using other *in vivo* models and *in vivo* experiments will be necessary before possible practical use of these combinations.

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