CZECH UNIVERSITY OF LIFE SCIENCES PRAGUE

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Development of Screening Method for Assessment of Growth-Inhibitory Activity of Plant-Derived Volatile Compounds Against *Staphylococcus aureus*

Master´s thesis

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Declaration

I hereby declare that this thesis entitled "Development of screening method for assessment of growth-inhibitory activity of plant-derived volatile compounds against *Staphylococcus aureus*" is my own work and all the sources have been quoted and acknowledged by means of complete references.

In Prague,……

Bc. Anna Krkonošková

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Abstract

The thesis aimed to develop a new screening method for testing of natural volatile compounds for their *in vitro* antimicrobial effect in vapor phase by using microtiter plates as well as to made determination of minimal inhibitory concentrations (MICs) of selected compounds by this improved method against strains of *Staphylococcus aureus*. The MICs were determined by *in vitro* broth microdilution method (BMD), using 96-well microtiter plates according to Clinical and Laboratory Standards Institute (CLSI 2009) combined with improved agar diffusion assay for evaluation of the antimicrobial activity of the vapors. Bacterial growth in layer of agar was controlled using basic MTT colorimetric assay. Antibacterial activity of carvacrol, eugenol, thymol, and thymoquinone was determined against *S. aureus* (ATCC 29213), *S. aureus* (ATTC 43300), *S. aureus* clinical isolate 2, and *S. aureus* clinical isolate 3 using developed screening method. The most effective antibacterial agent was thymoquinone with the lowest MICs ranging from 8 to 32 μg/mL. Only the weak antibacterial effect was observed for eugenol with MICs 1024 - 2048 μg/mL. The potential of introduced method was proved by results obtained in liquid/vapour phase and via MTT assay. Developed method meets the requirements for fast antimicrobial screening of plant derived volatile compounds with low cost and labour effective manners. New prespectives of screening method, represent future developments for various applications in food perservation, medicine, and agriculture

Keywords: Vapor; natural compounds, Eucalyptol, Carvacrol, Thymol, Thymoquinone, *Staphylococcus aureus*, clinical isolates.

Abstrakt

Cílem práce bylo vyvinout novou metodu screeningu pro testování přírodních těkavých sloučenin pro jejich in vitro antimikrobiální účinek ve fázi výparu pomocí mikrotitračních destiček stejně jako pro stanovení minimálních inhibičních koncentrací (MIC) vybraných sloučenin touto zdokonalenou metodou proti kmenům Staphylococcus aureus. Hodnoty MIC byly stanoveny metodou mikrodiluce mikroorganismu in vitro (BMD) s použitím mikrotitračních destiček s 96 jamkami podle klinického a laboratorního standardu Institute (CLSI 2009) v kombinaci s vylepšenou analýzou difúze agaru pro hodnocení antimikrobiální aktivity par. Bakteriální růst ve vrstvě agaru byl kontrolován za použití základní kolorimetrické analýzy MTT. Antibakteriální aktivita karvacrolu, eugenolu, thymolu a thymoquinonu byla stanovena proti S. aureus (ATCC 29213), S. aureus (ATTC 43300), S. aureus klinickému izolátu 2 a S. aureus klinickému izolátu 3 za použití vyvinuté screeningové metody. Nejúčinnějším antibakteriálním činidlem byl tymochinon s nejnižšími MIC v rozmezí od 8 do 32 μg / ml. Pro eugenol byl pozorován pouze slabý antibakteriální účinek s MIC 1024 - 2048 μg / ml. Potenciál zavedené metody byl prokázán výsledky získanými v kapalné / plynné fázi a skrze MTT test. Vyvinutá metoda splňuje požadavky na rychlý antimikrobiální screening těkavých sloučenin odvozených z rostlin s nízkými náklady a efektivním způsobem práce. Nové předpoklady metody screeningu představují budoucí vývoj pro různé aplikace výparů při uchovávání potravin, a také v medicíně a zemědělství.

Klíčová slova: Výpar, přírodní sloučeniny, Eukalyptol, Karvakrol, Thymol, Thymoquinone, *Staphylococcus aureus*, klinické izoláty.

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1. Introduction

Conceptually, MRSA is a major pathogen in both dveloped and developing countries, causing nosocomial or hospital-acquired (HA) infections, health care-associated (HCA) and community-acquired (CA) infections. In view of the fact that *S. aureus* is exhibiting commensalism while occupying 30% of the healthy population. It is considerably virulent bacterial pathogen, due to production of lots of virulence factors, which represent a wide range of adhesins, extracellular enzymes, as well as toxins. Nevertheless, due to permanent emergence of new MRSA clones including high mortality rates related to *S. aureus* infections, there is constant need for new and sophisticated anistaphylococcal approaches, to combat increasing burden posed on human health (Lin & Peterson 2010). In addition, plant volatile agents show significant antibacterial effects in vapor phase against pathogenic *S. aureus* strains (Nedorostová et al. 2009), thus it can be potentially used in development of new various applications and procedures in medicine, such as; inhalation antimicrobial therapy (Inouye et al. 2001; Safdar et al. 2015; Houdková et al. 2017), decontamination devices of medical instruments and hospital environments (Bueno 2015); Laird et al. 2012), topical therapy of skin and soft tissue wounds (Muthayian et al. 2012; Muthayian et al. 2012). Another area represents food applications, especially as preservative agents of food (Lopéz et al. 2007; Fisher & Phillips 2006; Laird & Phillips 2011). Volatile plant-derived compounds have always been evaluated for *in vitro* antimicrobial activity based on slightly modified direct-contact antimicrobial tests, including predominantly broth dilution and agar diffusion methods. Although several authors use many different types of methods, no reliable screening test has yet been standardized to assess the antimicrobial activity of volatile substances in the vapor phase. In particular, it should meet the requirements for fast testing and at the same time be able to test a larger number of samples (Kloucek et al. 2012).

The aim of this study is the primary synchronization of two different antimicrobial methods and their expression in MICs, while taking into account volatility of plant derived volatile compounds.

2. Literature review

2.1 Staphylococcus aureus

The species *S. aureus*, is low G + C Gram-Positive bacterial organisms of the phylum Firmicutes, belonging to the genus *Staphylococcus*. *S. aureus* appears as an ovoid shaped coccus (Suzuki et al. 2012), with a size in the range of 0.5 to 1.0 μm in diameter (Foster 1996). Several staphylococcal cells can be arranged in grape-like clusters (Stratton 1982), due to formation of perpendicular planes within cell division of individual cocci (Lory 2014). Therefore, *S. aureus* is reproduced by binary fission (Chien et al. 2012). The surface of colonies covers yellow-orange carotenoid pigments, that produce typical gold coloration (Marshall & Wilmoth 1981). Cells are usually non-capsulated or have limited capsule formation (Götz et al. 2006), non-motile, non-flagellated, non-spore-forming, and the most firm in comparsion to others non-spore producing bacterias (Stratton 1982). In regard to respiration abilities, *S. aureus* is facultative anaerobe, capable of creating energy and grow by aerobic respiration, or switching to fermentation which produces predominantly lactic acid (Plata 2009). In infection diagnostic, several tests are performed for identification of staphylococcal colonies. Thus, *S. aureus* shows positive results for catalse enzyme, as well as for the enezyme coagulase, hemolysins on blood plates, thermostable deoxyribonuclease (Juliannelle 1922; Humphreys 1992; Foster 1996; Kateete 2010), and neutral results for oxidase reaction (Jurtshuk & McQuitty 1976).

The primary ecological niches yielding *S. aureus* are anterior nares of the nose, where bacterias are frequently isolated (Williams et al. 1959; Williams 1963; Kluytmans et al. 1997), and transmitted to skin by the direct contact (Moss et al., 1948) However, carriage of *S. aureus* include various skin sites, such as perineum, axillae, hand, chest, abdomen, thigh, leg and ankle (Williams 1963). Vagina (Guinan et al. 1982), intestine (Williams 1963), and pharynx (Wertheim et al. 2005) are sites with lover occurence. Thus, *S. aureus* as an opportunistic bacterium is constantly colonizing human microflora and can leads to disease when the hosts resistence is lowered (Singh et al. 2014). Therefore, bacterial strains are able to trigger the immune response and can cause serious infections, which are divided into three categories despite their

considerable variety. The first category includes superficial lesions in particular wound infections (Tenover & Gaynes 2000; De Sousa & De Lencastre 2004). Namely; impetigo (Bangert et al. 2012), abscess (Cheng et al 2011), furunculosis, folliculitis and cellulitis (Lina et al. 1999). Systematic and life threatening conditions represents the second type, it comprise; pneumonia, meningitis, endocarditis, brain abscesses, bacteremia and osteomyelitis. The third category covers toxinoses as for instance staphylococcal scalded skin syndrome (SSSS), Staphylococcal food-borne disease (SFD), and toxic shock syndrome (TSS) (Tenover & Gaynes, 2000; De Sousa & De Lencastre 2004).

Conceptually, MRSA is a major pathogen in both dveloped and developing countries, causing nosocomial or hospital-acquired (HA) infections, health care-associated (HCA) and community-acquired (CA) infections. It is also linked with high morbidity and moratility rates ranging from 15 to 60 %, and increasing invasion over yeras form 71 % to 76 %, thus a public health crisis emerged (Cluff et al. 1968; Julander 1985; Cosgrove et al. 2003; Kaplan 2005; Feng 2008; Bishara et al. 2012).

2.2 Role in diseases

In view of the fact that *S. aureus* is exhibiting commensalism while occupying 30% of the healthy population. It is considerably virulent bacterial pathogen, due to production of lots of virulence factors, which represent a wide range of adhesins, extracellular enzymes, as well as toxins. Virulence factors are allowing colonization (Heilman 2011; Vandenesch et al. 2012, Sollid et al. 2014) and subsequently giving the oportunity to persist extreme conditions, inside of environment typical for human host (Liu 2009). Furthermore, virulence factors help bacteria to cause disease and often to evade host defenses (Peterson 1996). Therefore, staphylococcal strains can cause superficial lesions to person with the same strain mainly due to its nasal carriage (Williams et al. 1959), thereby creating one of the risk factors for *S. aureus* infection (Kluytmans et al. 1997; De Sousa & De Lencastre 2004). Mechanism of this phenomenon is the most widespread among people with type 1 diabetes (Rich and Lee, 2005), hemodialysis (Diawara et al. 2014), dermatologic conditions (Park et al 2013), AIDS (Larsen et al. 2012), and drug users (Basseti et al. 2004), their colonization rates

of nose and skin are always highest than does the general population (Tuazon & Sheagren 1974).

Moreover, the transmission of pathogenic bacterial strains affects mostly hospitalized patients, they are exposed to infection mainly because of their compromised immune system, and mainly due to repeated insertion of an indwelling catheter, injections (Lindsay & Holden 2004; Plata et al. 2009), continuous ambulatory peritoneal dialysis (Vychytil et al. 1998), surgeries (Kluytmans 1997), joint replacements (Song et al., 2013) and orthopaedic implants (Gosden et al., 1998), direct skin-to-skin contact (Miller & Diep 2008), and also by minor skin wounds (Decker et al. 1986) sometimes it may happen by a mere aspiration (APSR 2009) or by a contact with contaminated hospital reusable cleaning towels (Sifuentes et al. 2013).

Skin infection

S. aureus is globally identified as the most prevalent causative agent of direct skin and soft tissues infections (SSTIs) and syndrome. The most universal forms of SSTIs usually begin when physical skin barriers are impaired and pathogenic bacteria can enter the underlying tissues. Thus, the minor boils or abscesses are formed, in part, to contain the nidus of staphylococcal infection. However, SSTIs can lead to serious health conditions affecting bone and muscle, and also disperse to the heart valves, as inflammation of endocardium, as well as to the lungs. Therefore, clinical presentation of microbial SSTIs can differ from highly invasive to disseminated or superficial disease (McCaig 2006; Stryjewski & Chamber, 2008; Kobayashi et al. 2015). SSTIs are frequently manifested in various forms, including; impetigo, folliculitis, cellulitis, furuncles, abscess, pyomiositis, necrotizing fasciitis, skin abscesses, surgical-site infection carbuncles and furuncles. The skin is also affected by systemic syndrome mediated by toxins, including; Staphylococcal toxic-shock syndrome, very rare Purpura fulminans and Waterhouse-Friedrichsen syndrome, and also staphylococcal scalded-skin syndrome (Stryjewski & Chamber 2008). Staphylococcal scalded syndrome caused by exofoliative toxins, mostly affects neonates, adults and small children which are prone to hidden diseases, such as renal impairment (Ladhani et al. 1999). *S. aureus* also colonizes skin of patients with atopic dermatitis (Breuer et al. 2002).

Food poisoning

S. aureus is responsible for the most common human foodborne ilness. Food pisoning is caused by ingestion of contaminated food, such as processed meat or diary products, which contains staphylococcal enterotoxins. Contamination can be triggered mainly due to exposure of food to higher temperatures, as well as inadequate hygiene, and mistreatment (Loir et al. 2003; Argudín et al. 2010). Toxins are sometimes produced in digestive tracts after ingestion of food (Loir et al. 2003). The symptoms of food poisoning include; vomiting, abdominal cramps, diarrhea, and nausea. The outbreak of symptoms is within 30 minutes or up to 8 hours. Subsequent disappearance of the symptoms is recognized after 24 hours (Loir et al. 2003), and remission up to 48 hours (Foodsafety 2017). The most affected groups represent young children, elderly persons, pregnant women and their fetuses, and also people with weakened immunity. Especially for those people can be staphylococcal food poisoning very serious and potentially life-threatening (MFMER 2017).

Bacteremia

Staphylococcal bacteremia is also known as nosocomial bloodstream infection (Wisplinghoff et al. 2004; Naber 2009). It is diagnosed by a positive blood culture acquired from hospitalized patients during the course of their hospitalization, which is approximately after 48 hours or longer (Garner 1988; Siegman-Igra et al. 2002; Friedman et al. 2002). Therefore, bacteremia can be obtained in environment rich on resistant microorganisms found in hospital settings, and it relates primarily to instrumentation or to a procedure (Siegman-Igra et al. 2002). Especially, catheter related infections, such as skin osteomyelitis and soft-tissue infections; including cutaneous ulcerations and abscesses, as well as infections of lower repisratory tract, are the most common causes of bacteremia (Eiff et al. 2001). Other complications associated with identification of *S. aureus* bacteremia represent; infective endocarditis, septic arthritis, septic thrombophlebitis, meningitis, psoas abscess, and embolic stroke (Fowler et al. 2003). Pathogenicity of bacteremia is also influenced by virulence factors, such as superantigens *(*Argudín et al. 2010*),* which promote interaction with a mass of T-cells, to produce a forceful cytokine response via inflammation and improve susceptibility to endotoxin shock (Kotb 1995), which results in vasodilation, increased vascular permeability, and muscle relaxation (Moreland 2004*).*

Bone and joint infection

Osteomyelitis and septic arthritis are two main types of infection affecting the bone. It is condition involving the inflammatory destruction of bones and joints (Wright and Nair 2009). The primary acces of *S. aureus* to the joint or bone can be either direct; rupture of the overlying tissues including surgeries, open fractures, joint replacements; or haematogenous origin; as result of bacteremia, as well as contiguous; when the infection is disseminated from local tissue, regularly in diabetic foot infections (Mandal et al. 2002; Lew & Waldvogel 2004; Wright & Nair 2009). The accompanying manifestations of osteomyelitis and septic arthritis comprise high fever, constitutional symptoms, and very often bacteremia. Pain and loss of function are conspicuous, folowed by joint irritability, edema, bone tenderness, and warmth. Deep infection indicates bursitis, which can be manifested externally via erythema, usually as a consequence of septic arthritis or rupturing of a subperiosteal abscess directly from the bone into the soft tissues (Berendt & Byren, 2004). Arthritis and osteomyelitis can develop into a chronic condition and tissue damage. However, the endpoint of the extreme chronic condition represents ischemic bone necrosis and subsequent formation of sequestrum (Mandal et al. 2002; Lew & Waldvogel 2004).

Biofilm formation

S. aureus is usually attached to damaged tissues or medical implants and binds into a hydrated matrix, which is composed of proteins and polysaccharides and then begins to create a biofilm that appears like a thin slimy layer (Stewart & Costerton 2001). However, the sessile staphylococcal micro-communities are intrinsic to antimicrobial chemotherapy. Therefore, the persistent and chronic nature of several microbial infections is conditioned by biofilm formation (Costerton 1999). Clinical manifestations of biofilm formation caused by *S. aureus* (Wu et al., 2015), involving joint prosthesis (Song et al. 2013) include acute infection. It is represented by warmth, fever, erythema, swelling, and also infected joints. Chronic conditions are accompanied by mild pain, release of the prosthesis at the interface of bone-cement, and aslo creation of sinus tract with a spout (Pozo & Patel 2010). Other implants compromised by biofilm formation comprise heart valves, coronary stents, intraocular lenses, implantable neurological stimulators, fracture-fixation devices (Costerton et al. 2005). Moreover, biofilms are mostly present in chronic wounds (Percival et al. 2015).

Respiratory tract infections

S. aureus causes lower respiratory tract infections in adults, represented by nosocomially acquired pneumonia (Iwahara et al. 1994; González et al. 1999). It is infectious process, involving invasion and overgrowth of bacterial strains in lung parenchyma, inactivating immunological defenses, and initiating intra-alveolar exudates. Transmission of bacteria into the lower lungs occures usually via aspiration from upper respiratory tract (Alcón et al. 2005). Other associated factors, which are able to change normal flora of the respiratory tract comprise serious underlying diseases, particularly lung diseases, surgeries, advanced age, several catetherizations, recent antibiotic therapy (Iwahara et al. 1994; González et al. 1999) or mechanical ventilation (Chastre & Fagon 2001). Significant pulmonary infections and clinical syndromes, include; diffuse aspiration bronchiolitis (Hu et al. 2015), Mendelson´s syndrome (Dines et al. 1961), diffuse ventilator associated pneumonia (VAP) (Seligman et al. 2015), hypostatic pneumonia (APSR 2009), septic emboli pneumonia (González et al. 1999), and aspiration pneumonia (Son et al. 2017). In addition, missing function of the cystic fibrosis transmembrane regulator protein in patients diagnosed with cystic fibrosis (Boucher 2004), leads to mucus hypersecretion in lung epithelial cells, thus creates feasible conditions for *S. aureus* colonization and also acute airway obstruction caused by adhesive and hyperviscous mucus (Puchelle et al. 2002).

2.3 Treatment

Standard antibiotic therapy (chemotherapy)

In the 1940, benzylpenicillin was firstly introduced as an alternative to the treatment of infections caused by *S. aureus*. Nevertheless, in the 1960, *S. aureus* strains resistant to the methicillin, well known as the original member of the class, penicillinase-resistant penicillins, have increasingly spread through the world (Ayliffe 1997; Chambers 2001; Feng 2008). These strains have been termed as methicillin-resistant *S. aureus* (MRSA) and exhibit resistancy to all β-lactam antibiotics such as cloxacillin, dicloxacillin, nafcillin and oxacillin (Tournidge et al. 2008; Feng 2008). It is a result of mediation by a specific penicillin-binding protein PBP2a (or PBP2´), which has a lowered affinity for β-lactam agents (Brown & Reynolds, 1980; Hartman &

Tomasz 1984; Utsui & Yokota, 1985). Lately, these strains have become multi-resistant, showing resistance to gentamicin, tetracyclines, macrolides, lincosamides, as well resistence to sulfonamides and trimethoprim (Feng 2008). Nevertheless, due to permanent emergence of new MRSA clones including high mortality rates related to *S. aureus* infections, there is constant need for new and sophisticated anistaphylococcal approaches, to combat increasing burden posed on human health (Lin & Peterson 2010).

The treatment options of SSTIs caused by *S. aureus* are determined on the basis of severity. Therefore, several forms of SSTIs do not require any special treatment. Drainage, surgical incision and antibiotic therapy are in medical facilities included into routine medical procedures (Moran et al. 2006). Antimicrobial drugs prescribed for most CA SSTIs comprise dicloxacillin and cephalexin, however in areas with high incidence of CA-MRSA can no longer be adequate. Cefazolin and oxacillin are administered intravenously (Moran et al 2005), and for topical therapy are usually applied corticosteroids (Nilsson et al. 1992).

Several preventive strategies are recommended to avoid staphylococcal food poisoning, such as proper hand washing technique and cleaning all surfaces, adequate cooking temperature, separation of raw meat and ready-to-eatfoods, immediate freezing and prompt refrigeration. To reduce the risk of common food poisoning, vulnerable people should avoid the consumption of several foods, in particularly; raw meat and poultry, eggs, and unpasteurized milk (Food safety. org 2016). Several alternatives or modified methods for preservation of fresh food have been suggested, especially whey permeates, irradiation, organic acids, antioxidants, and modified atmosphere packaging (Rico et al. 2007).

The preventive measures for CA pneumonia in hospitalized patiens require routine respiratory culture and blood tests (Woodhead et al. 2011). CA pneumonia is treated with antimicrobial agents, namely; linezolid, clindamycin (Soavi et al. 2011), vancomycin in combination with rifampicin (Jung et al. 2010). Other successful treatment option includes intravenous immunoglobulin within adjunctive therapy (Woodhead et al. 2011). Symptoms of cystic fibrosis are mitigated by medications, such as mucolytics, ivacaftor, orkambi, bronchodilators (MFMER 2017) always combined

with chest physical therapy (CFF 2017), mask and tube for breathing, vibrating vest (MFMER 2017), and inhalation of antibiotics (CFF 2017).

Possible treatment for staphylococcal bacteremia, are still circumscribed to conventional antibiotic agents mainly; teicoplanin, vancomycin, tigecycline, daptomycin, linezolid, and telavancin. Other potential antimicrobial therapy includes oritavancin, dalbavancin, ceftaroline, and also ceftobiprole (Rasmussen et al. 2011).

Therapeutic approach to chronic osteomyelitis comprise palliative options including; débriment, drenaige and prescription of antibiotic agents (Walter et al. 2012), such as ampicillin, oxacillin, sulbactam, cefazolin, vancomycin, daptomycin, ciprofloxacin, levofloxacin, enoxacin, moxifloxacin, and linezolid (Spellberg and Lipsky 2011). Other curative options are represented by reconstructions; prosthetic implants, arthrodesis, callus distraction, vascularized pedicled graf or final amputation (Walter et al., 2012). Antimicrobial drugs intended for treatment of septic arthritis include clindamycin, and cephalosporin (Peltola 2012).

The treatment procedure of biofilm infection is determined by the identification, whether the infection has affected a foreign body or not. If a foreign body is not affected, thus the right combination of antimicrobial agents, given in high-doses for a longer time can potentially treat the infection (Wu et al. 2014). Presumed biofilm-active antibiotic include; vancomycin, tigecycline, daptomycin, and linezolid (Gbejuade et al. 2015). Nevertheless, if foreign bodies are affected, surgical removal is needed to acquire favorable outcomes (Wu et al. 2014) or another option is debridement with retention of prostheses (Song et al. 2013) by using ultrasonic instruments (McDonald & Nichter 1994).

In addition, plant volatile agents show significant antibacterial effects in vapor phase against pathogenic *S. aureus* strains (Nedorostová et al. 2009), thus it can be potentially used in development of new various applications and procedures in medicine, such as; inhalation antimicrobial therapy (Inouye et al. 2001; Safdar et al. 2015; Houdková et al. 2017), decontamination devices of medical instruments and hospital environments (Bueno 2015); Laird et al. 2012), topical therapy of skin and soft tissue wounds (Muthayian et al. 2012; Muthayian et al. 2012). Another area represents food applications, especially as preservative agents of food (Lopéz et al. 2007; Fisher & Phillips 2006; Laird & Phillips 2011).

2. 4 Volatile plant derived products

Plant-derived volatile compounds are low molecular weight lipophilic chemical structures with high vapour pressure, which play different ecological roles in plant bodies (Pichersky et al. 2006). Supposedly, their primary function in the life of the plant is to make possible interaction with environment, they simplify attraction of pollinators, and also seed dispersants; however, their main role is the protection against herbivores, parasites and pathogens (Doudareva 2013). Especially for insects, they may serve as pheromones influencing social behavior or impulses for finding prey or hosts. Thus, plant derived volatile compounds are emitted from roots to the soil and from flowers, fruits, buds and leaves into atmosphere (Maffei 2010). Nevertheless, for plant volatiles to be able to play such a comprehensive roles, they have to be released in complex mixtures, which compositions differ from one plant part to another (Baldwin 2010). Those blends of volatile and fragrant substances with an oily consistency derived from plants are known as essential oils, or aromatic plant essences. They can be liquid within 20° C to 25° C, up to some exceptions with solid or resinous structure, and typically have various coloring which ranges from blue to dark brownish red and from pale yellow to emerald green (Balz 1999). The synthesis and overall production of substances is localized in special structures such as glandular trichomes, oil cells, secretory canals and cavities (Fahn 1988). The most economically significant families producing essential oils are; Labiatae (*Mentha* spp.), Pinaceae (*Picea* spp., *Pinus* spp., *Cedrus* spp.), Rutaceae (*Citrus* spp.), Myrtaceae (*Corymbia* spp.), Rosaceae (*Rosa* spp.), Gramineae (*Vetiveria* spp., *Cymbopogon* sp.), Santalaceae (*Santalum* spp.), Lauraceae (*Litsea* spp.), and Oleaceae (*Jasminum* spp.), while many species, which also provide essential oil are Umbelliferae (Oyen & Dung 1999). During the years, there have been identified more than 1700 volatile compounds derived from 90 various plant families ranking amongs Angiospermae and Gymnospermae (Knudsen et al. 2006).

One of the characteristic features for compounds with high vapor pressure, if they are non-conjugated, is their ability to cross membranes without any obstructions and then may also evaporate into the atmosphere, if there are no obstacles to diffusion (Pichersky et al. 2006). Although, the co-dependency of biosynthesis - release rates play main role in emission of a specific volatile compound (Dudareva et al. 2004).

Furthermore, the total composition of volatile compounds in essential oils relies on plant variety, agro-ecological conditions and pre-treatments, but the final result is influenced by processing as well as selected extraction methods (Peter 2012). To these days, different methods are used to extract volatile components from various plant parts, namely; steam distillation, hydro-distillation, hydro-diffusion, cold expression (Li et al. 2014), solvent extraction (Mamede & Pastore 2006), and supercritical fluid extraction (Pourmortazavi & Hajimirsadeghi 2007).

The use of eessential oils is since medieval times popular mainly for their virucidal, insecticidal, fungicidal, bactericidal, cosmetical, medicinal and antiparasitical applications. For these characteristic, they are needful in many spheres, such as sanitary, agricultural, cosmetical, pharmaceutical, and food industries (Bakkali et al. 2008). From the holistic point of view, aromatherapy uses essential oils in form of therapeutic agents for the treatment of several diseases (Ali et al. 2015).

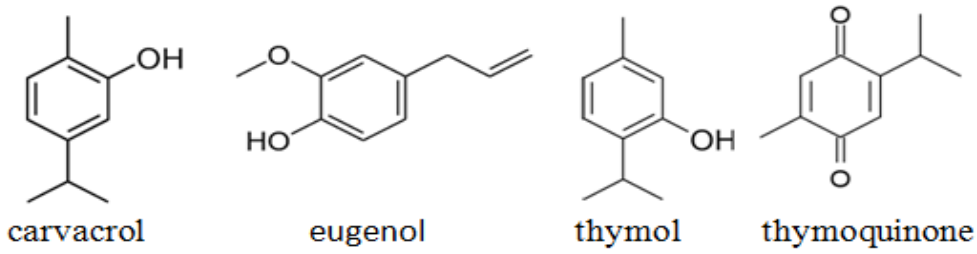

Figure 1. Chemical structures of compounds tested in this study (Sigma-Aldrich, 2017)

Carvacrol

Carvacrol (synonym; 5-isopropyl-2-methylphenol) (Figure 1.), a monoterpenic phenol isomeric with thymol, is one of the most common compounds present in various essential oils (Peixoto-Neves et al. 2010; Melo et al. 2011) It is colorless or yellowish thick fluid (Furia & Bellanca 1975), with a distinct woody, pungent and spicy scent (Khan et al., 2018). Oregano (*Origanum vulgare* L.), belonging to family (Lamiaceae) is the most valuable source of this naturally occurring volatile compound with the more than 90 % of total content in volatile oil (Khan et al. 2018). Savory (*Satureja thymbra* L.), common thyme (*Thymus vulgaris* L.), Greek origanum (*Origanum vulgare* L.

subsp. *hirtum*), thyme of kings (*Origanum vulgare* L. subsp. *glandulosum* Desf.), Turkish oregano (*Origanum onites* L.), wild bergamot (*Monarda fistulosa*), sweet marjoram *(Origanum majorana* L.*)*, lippia (*Lippia gracilis* Schauer), conehead thyme (*Coridothymus capitatus* L.), are other aromatic plant species and subspecies rich in carvacrol as their predominant component (Zamureenko et al. 1989; Lagouriet al. 1993; Juliani & Simon 2002; Baser et al. 2011; Neves et al. 2011; Fachini-Queiroz 2012, Béjaoui et al. 2013; Carroll et al. 2017; Özkan et al. 2017). Presently, carvacrol is mainly used as natural preservative to improve shelf-life of food (Oliveira et al. 2015), in cosmetic formulations as a fragrance ingredient and biocide (Andersen 2006). It also fulfills its purpose as a feed supplement in honeybees breeding, for the control of *Varroa jacobsoni* (Sammataro et al. 2009). In last years, extensive research has been developed in an attempt to form the biological effects of carvacrol for its possible use in clinical applications (Suntres 2013). Thus, carvacrol shows a great variety of beneficial biological effects, such as anti-genotoxic (Kumar et al. 2015), anti-tumor (Sobral et al. 2014), anti-platelet (Karkabounas et al. 2006), anti-depressant-like (Melo et al. 2011), anti-inflammatory, anti-ulcer (Silva et al. 2012), anti-nociceptive (Melo et al. 2012), anti-obesity (Cho et al. 2012), neuroprotective (Ultee et al., 2002) hepatoprotective (Palabiyik 2016), vasorelaxant (Peixoto-Neves et al. 2010), spasmolytic (Riviero-Cruz et al., 2011), and wound healing effect (Gunal et al. 2014). Carvacrol also possesses anti-oxidative effects, relating to chemical and sensory stability of processed sunflower seeds (Quiroga et al. 2014). In terms of biological properties, Carvacrol produces a broad spectrum of anti-microbial effects, which have attracted attention of many researchers around the world (Nostro and Papalia 2009). Carvacrol have been proved to possess antimicrobial effect against *S. aureus* ATCC 29213, with the MIC values 256 μg/mL in vapour phase (Houdková et al. 2017).

Eugenol

Eugenol is a phenylpropanoid (Fadilah et al. 2017) (Figure 1.), synonym; 2-methoxy-4-allylphenol (Baur et al. 2001), synthesized as product of shikimate pathway (Vogt 2010). Physical appearance of eugenol is as follows: pale yellow or clear, colorless liquid. The substance has a characteristically strong aromatic smell of clove and sharp, pungent taste (Yuwono et al. 2002). The richest source of eugenol is found in the clove tree (*Eugenia caryophyllata*), belonging to family *Myrtaceae* (Tripathi and Mishra, 2016). The total eugenol content for each oil is as follows; clove leaf oil 76.8 % (Jirovetz et al. 2006), clove bud oil 58.77% (Wenquiang et al. 2007) and clove stem oil, ranging between 97.20 % - 98.83 % (Sohilait 2015). Other aromatic plants having higher eugenol contents include; cinnamon tree (*Cinnamomum zeyllanicum* Blume), nutmeg (*Myristica fragrans* Houtt.), sweet basil (*Ocimum basilicum* L.), African basil (*Ocimum gratissimum* L.), Jamaican pepper (*Pimenta dioica* L.), and tulsi (*Ocimum sanctum* L.) (Raina et al. 2001; Dambolena et al. 2010; Zhang and Lokeshwar 2012; Saharkhiz et al. 2015; Taleuzzamana et al. 2017). Eugenol is widely used in dentistry, due to its beneficial antiseptic and anaelgesic effects, and as the primary ingredient of many dental materials, especially in combination with zinc oxide, is used for temporary fillings. This universal molecule is a key component in flavoring agents, perfumes, cosmetics (Raja et al. 2015; Sarkic & Stappen 2017), as well as fumigants, pesticides, and also food preservatives against micro-orgainsms in agriculture (Kamatou 2012). Furthemore, eugenol possesses versatile biological effects, namely; anti-inflammatory (Huang et al. 2015), anti-oxidant (Gülçin 2011), anti-cancer (Fadilah et al. 2017), neuro-protective (Said & Rabo 2017), anti-stress (Garabadu et al. 2010), anti-diabetic potential (Singh et al. 2016), anti-hypocholesterolemic (Venkadeswaran et al. 2014), antinociceptive (Park et al. 2010), fumigant toxicity (Brari & Thakur 2015), acaricidal (Pasay et al. 2010), and anti-fungal (Lee et al. 2007). Nevertheless, antimicrobial effects of Eugenol in vapor phase, against pathogenic strains of *S. aureus* 29213 has been evaluated, with MIC; 512 µg/mL (Houdková et al. 2017), 27 µg/mL (Goni et al. 2009).

Thymol

Thymol is a natural monoterphene phenol (Figure 1.), synonym; 2-isopropyl-5-methylphenol, isomeric with carvacrol (Surburg & Panten 2006, Peixoto-Neves 2010). Thymol has a colorless crystalline structure, and aromatic thyme-like odor (Surburg and Panten 2006). It is the major component of thyme (*Thymus vulgaris* L.) essential oil, which can contain up to 47.59% (Borugă 2014). Other species containing larger amounts of thymol are lippia (*Lippia gracilis* Schauer.; *Lippia sidoides* Cham), wild bergamot (*Monarda fistulosa* L.), scarlet beebalm (*Monarda didyma* L.), Greek oregano (*Origanum vulgare* L. spp. *hirtum*), thyme (*Thymus eriocalyx*; *Thymus daenensis subsp. Lancifolius*; *Thymus kotschyanus)*, and eyebright (*Euphrasia rostkoviana*) (Martino et al. 2009, Amiri et al. 2012; Marco et al. 2012; Bitu et al. 2014; Novy et al. 2015; Mattareli et al. 2017; Ricci et al. 2017). Thymol was used in ancient Egypt for mummification, mainly because of the good preservative qualities (Napoli et al. 2010). Nowadays, thymol vapors are used as fumigant in archival repositories for conservation of cultural properties (Strassberg 1978; Baer & Ellis, 1988). Moreover, thymol shows great potential in agricultural applications, especially for pathogen control in pre-plant cultivations, and also in post-harvest treatments of fruits and vegetables (Ji et al. 2005; Shin et al. 2014). Furthemore, thymol as a bioactive ingredient with antiseptic effects is added into toothpastes and mouth rinses, which supports the control of dental biofilm formation and prevents gingivitis (Kato et al. 1990; Mazzanobile & Ibrahim 1992; Amoian et al. 2017).Thymol possess a repertoire of biological activities, including; anti-cancer, vasorelaxant, anti-inflammatory, anti-spasmodic, anti-oxidant, anti-depressant like, antifungal, and immunomodulatory (Braga et al. 2006; Peixoto-Neves 2010; Amirghofran 2011; Engelbertz et al. 2012; Aman et al. 2013; Deng et al. 2015; De Castro et al. 2015; Kang et al. 2016). In addition, antimicrobial effects of Thymol in vapor phase, against pathogenic strains of *S. aureus* 29213 has been evaluated, with MIC 128 μg/mL (Houdková et al. 2017).

Thymoquinone

Thymoquinone is a naturally occuring quinone (Crooks et al. 2001), (Figure 1.), synonym; 2-isopropyl-5-methyl-benzoquinone (AbuKhader 2013), physically appearing in a crystal structure (Pagola 2004). Thymoquinone is found in seeds of black cumin (*Nigella sativa* L.), belonging to family *Ranunculaceae* (Kader & Eckl 2014). Overall content can reach up to 11.80% (Mohammed et al. 2016). Several medicinal plant species contain thymoquinone as their primary bioactive constituent, namely; wild bergamot (*Monarda fistulosa* L.), scarlet beebalm (*Monarda didyma* L.), purple bergamot (*Monarda media* L.) mountain savory (*Satureja montana* L.), common thyme (*Thymus vulgaris* L.) (Jukić & Miloš 2004; Grosso et al. 2009; Taborsky et al. 2012; Sovova et al., 2015). Thymoquinone exhibits outstanding hepatoprotective, anti-clastogenic, anti-inflammatory, anti-oxidant, and anti-tumor effects (Ivankovic et al. 2006; Badary 2007; Inci et al. 2012; Jafari et al. 2018). In addition, thymoquinone is well-known for significant antimicrobial effects on fungi, viruses, schisostoma, parasites, Gram-positive and Gram-negative bacteria (Forouzanfar et al. 2014). Thymoquinone have been proved to possess antimicrobial effect against *S. aureus* ATCC 29213, with the MIC values 16 μg/mL in vapour phase (Houdková et al. 2017).

2. 5 Methods for measuring vapours against bacterial strains

Volatile plant-derived compounds have always been evaluated for *in vitro* antimicrobial activity based on slightly modified direct-contact antimicrobial tests, including predominantly broth dilution and agar diffusion methods. Nevertheless, direct-contact assays have to deal with limiting factors, due to characteristic properties of volatile compounds, namely; lipophilicity, volatility and overall complexity of developed methods. The low solubility of the volatile compounds in liquid media is solved by the addition of solvents and emulsifiers, in particularly; Tween 80, DMSO and ethanol), which can affect activity (Kloucek et al. 2012). Volatility is able to induce complete evaporation of volatile compounds, especially during incubation time with dispersants (Kalemba & Kunicka 2003). Furthermore, volatile compounds begin to spread at different rates depending on their different vapor pressures to achieve an equilibrium state in a closed intact space (Kloucek et al. 2012). In addition, based on the

studies (Nový et al. 2011) active compounds in vapor phase are able to inhibit in concentration-dependent manner bacterial growth in adjoining wells and alter the results of antimicrobial assays acquired by the standard broth microdilution method. Thus, experimental measures for broth microdilution method should be arranged in order to take into account the true nature of volatile compounds and focused more on, multiple-compound antimicrobial screening. Although several authors use many different types of methods, no reliable screening test has yet been standardized to assess the antimicrobial activity of volatile substances in the vapor phase. In particular, it should meet the requirements for fast testing and at the same time be able to test a larger number of samples (Kloucek et al. 2012). Other non-negligible requirements for the new method are; to save labor for test preparation and also financial resources for the necessary testing materials. In addition, various methods for testing of the antimicrobial effects of plant volatiles have been developed with the main intention to study the prospectives of their volatile components for development of advanced inhalation therapeutics (Houdková et al. 2017).

Disc volatilization method

Disc volatilization method represents the less complex assay for antibacterial evaluation of plant derived volatile compounds in vapor phase. The advantage of this method is that it can be used as a basic screening for the primary selection of potential volatile compounds and their combinations (Bueno 2015).

The agar plate was inoculated by spreading the bacterial suspension to the entire surface and than allowed to dry. The inner surface of the upper lid was prepared as follow; on the inside was placed a sterile paper disc and subsequently, volatile compound in liquid form was added on each of the paper discs. Volatile compound was tested for various dilutions. Therefore, a suitable amount of sterile discs was used. The bottom plates inoculated with bacteria were promptly inverted on the top of the lid (Figure 2.), and firmly sealed by using sterile parafilm to reduce vapor leakage from volatile compound to the external environment. Finally, the petri dish was allowed to incubate for 24 hours at 30°C. The efficiency of the volatile compound was estimated by measuring the diameter of the resulting inhibition zone in the bacterial lawn (Tyagi and Malik 2012).

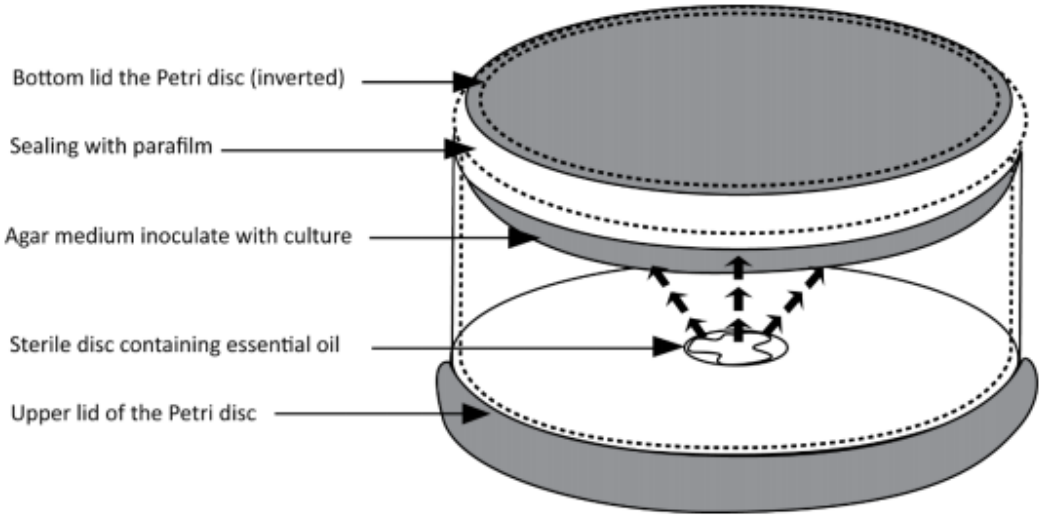

Figure 2. Disc volatilization method (Bueno 2015)

Modified disc volatilization method

Disc volatilization method was slightly modified via thin layer of the medium, which was firstly poured into the center of the lid on the petri dish. Than the sterile round-shaped filter papers to which the volatile substance has been added from each stock solution were laid on the media layer to prevent any adsorption of volatile substances into the plastic material of the lid (Nedorostová et al. 2009).

Micro-atmosphere diffusion method

Another slightly modified disc volatilization method represented as micro-atmosphere diffusion assay was used to evaluate antimicrobial activity.

The inner surface of the upper lid of a Petri dish was enriched for the micro-coverglass. It was attached by using a small portion of enamel. The sterile paper disc was humidified with volatile compound and positioned at the middle of the micro-coverglass (Mondello et al. 2009). Micro-coverglass served also as barrier against adsorption of volatile compound into the lid.

Agar vapor assay

The agar vapor assay practically put forward the same attributes as disc volatilization method. It also represents the primary screening for selection of potential

high volatile components. Moreover, assay offers the possibility to directly control the size of surface to exposure for evaporation (Bueno 2015).

The agar medium was inoculated by using microbial suspension. Subsequently, a paper disc soaked with volatile component of different dilutions has been positioned over agar medium in the bottom of Petri dish. Furthemore, a paper disc was sealed by a plastic ring from one side and closed with micro-glass (Figure 3.). Moreover, the petri dish was lately inverted and allowed to incubate for 24 hours at 30° C. Finally, the inhibition zone was measured via obtained MID of the volatile vapours (Bueno 2015).

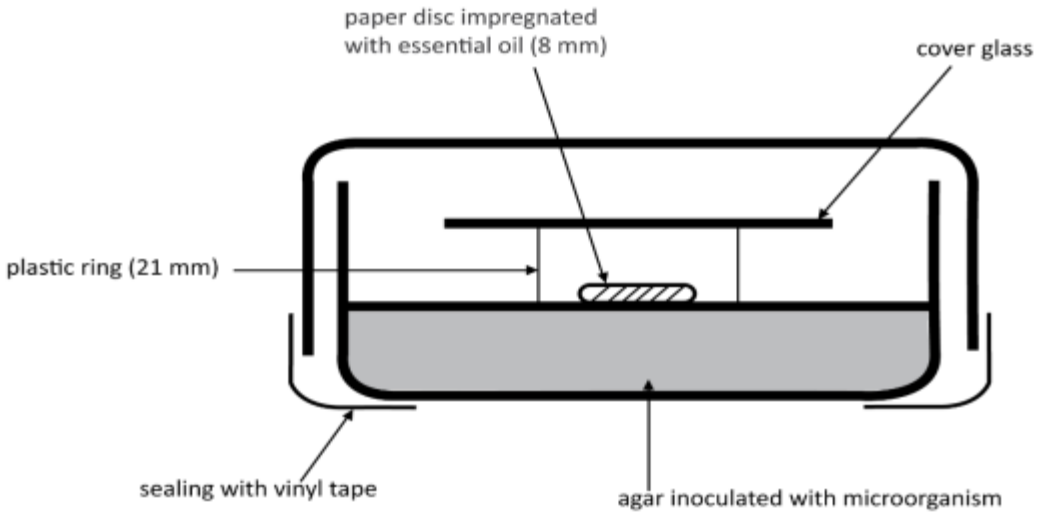

Figure 3. Agar vapour assay (Bueno 2015)

Airtight box

The airtight box assay was developped to improve the determination of vapour concentration of volatile compounds. Thus, the measurement of vapour activity was provided for larger air space capacity than previous common methodes performed in smaller Petri dishes (Inouye et al. 2001).

The evaluation of the antimicrobial activity of the vapor in the airtight space was preformed in plastic containers of defined volumes. The assay for airtight container was based on two feasible variants; agar plugs (Figure 4.) or culture media were added into the Petri dishes and subsequently inoculated by bacterial suspension, and finally positioned into containers. Aluminum foils have been used to protect volatile

compounds from possible absorption into plastic materials, as well as to protect plastic walls against undesirable contamination. A filter paper wetted with a volatile compound was attached to the top of the airtight container. The filter paper was changed for each different dilution. The containers were allowed to incubate for 24 hours at 37° C (Bueno 2015).

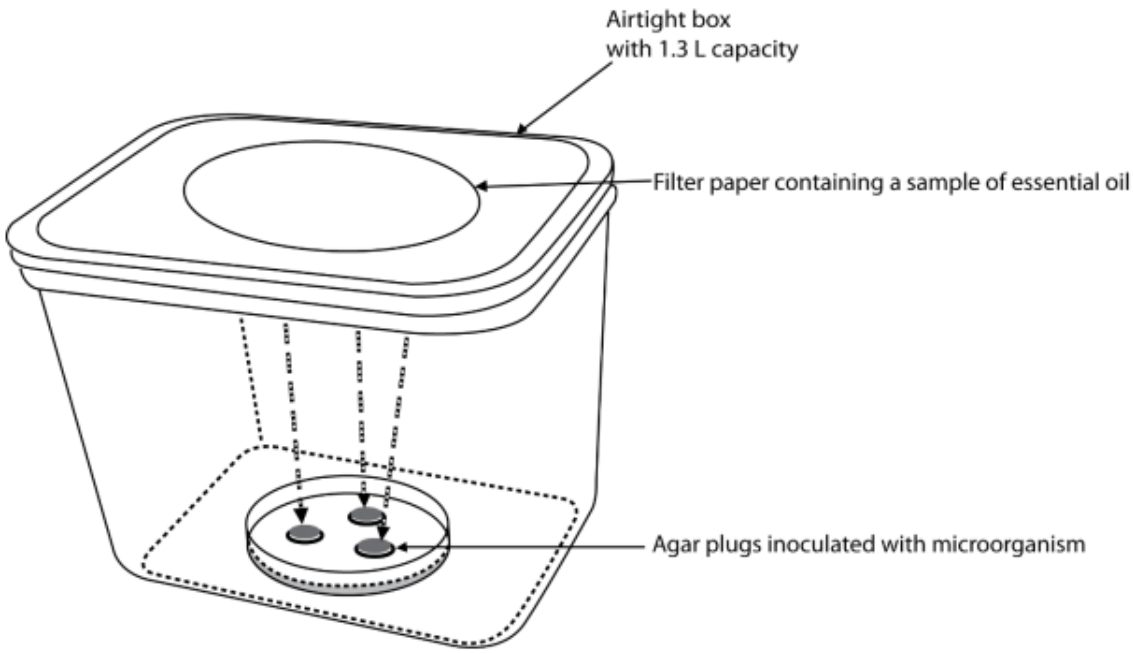

Figure 4. Airtight box (Bueno 2015)

Fast screening method

The fast screening method was introduced mainly for the quick screening of volatile compounds in the vapor phase against a large number of microbial organisms, the other interests of this method was to reduce labor and material costs (Klouček et al. 2012).

For the antimicrobial assay was used four sectioned Petri dish (Figure 3). The lid and the sections were saturated with agar and left to solidify. Three compartments were inoculated by spreading suspension, each with different microbe. The last compartment was left without inoculum as a contamination control. Volatile compounds were diluted in ethyl acetate in two-fold dilution manner to reach the desired volume for final solution, which was distributed on a sterile round-shaped filter paper. The filter paper

was left to dry for evaporation of the ethyl acetate, and than was immediately placed in a petri dish directly on the top of the walls separating the compartments. Therefore, the distance between the agar layer and the paper was minimized. At the end, the petri dishes were sealed with a hardened agar, and then incubated for bacteria and fungi for 18-24 hours at 37 ° C, as well as for 72 hours at 25 ° C (Klouček et al. 2012).

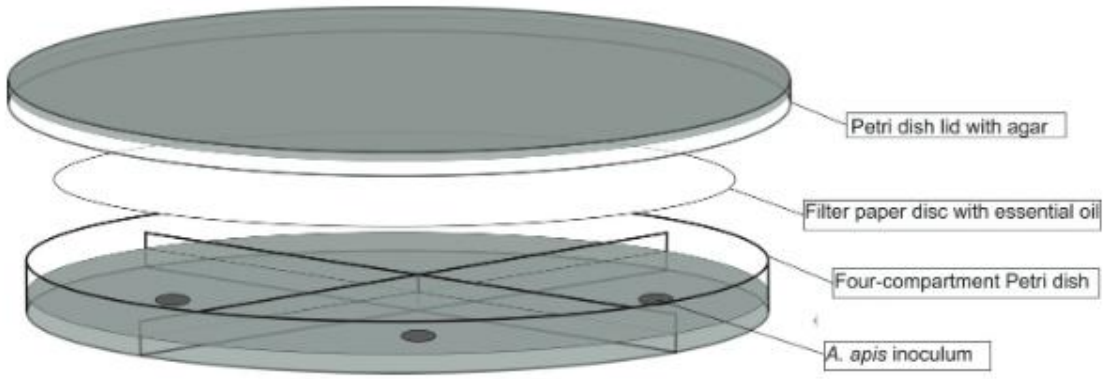

Figure 5. Fast screening method (Kloucek et al., 2012)

Experimental apparatus

The main objective of the presented method was to measure MIC and MLC values of volatile compounds in vapor phase against foodborne pathogenic and spoilage bacteria. A great advantage of the assay was the simultaneous evaluation of the vapor at different concentrations without the development of the concentration gradient (Seo et al. 2015).

Polycarbonate experimental apparatus was composed of a lower and upper chamber; each contained seven wells (Figure 5). The surface of upper well was designed to be identical to surface of lower well although; internal volumes of both wells were different. O-rings served as seal at the juncture of the lower and upper well rins as well as around the whole set of wells, in particularly, to prevent leakage of the vapors (Seo et al. 2015).

Sterilized and fluid medium was poured into upper wells of the sterilized experimental apparatus and led to solidifie for 30 minutes at 22°C. Microbial suspension was added on the solidified medium and placed in a laminar flow hood for 30 minutes at 22°C; one of the wells was left as a negative control, thus uninoculated. Furthermore, to the each of lower wells was placed a sterile paper disc, and also to each

well were added two-fold serial dilutions of liquid volatile compound in diethyl ether, although with the exception of the first and the second wells, which provided the negative and positive controls. The lower and upper chambres of the apparatus were firmly attached together by using bolts and nuts. The prepared apparatus was left for incubation at 30° C from 24 hours to 48 hours. Immediately after incubation, the bacterial growth was determined by assessing the resulting color alterations in medium (Seo et al. 2015).

Figure 6. Experimental apparatus (Seo et al., 2015)

Broth microdilution volatilization method

The screening method meets the expectations for fast and uncomplicated testing of volatile compounds for antimicrobial activity in the vapour and liquid phases at various concentrations. Another advantage was the development of MTT cytotoxicity assay, specially modified for assessing of plant volatiles on plates with a vapour barrier cover. The interest of the screening method was to confirm a concept that has a high potential, especially for further development of new approaches to inhalation therapy of respiratory diseases (Houdková et al. 2017).

Microtiter plates were covered by lids with flanges that were firmly attached to reduce possible evaporation. The agar was poured onto each flange on the lid and allowed to solidify. Subsequently, each of the flanges was inoculated with a microbial suspension. Samples of the volatile compounds were dissolved in a solvent and diluted in a suitable broth medium. Seven double serial dilutions of samples starting with the same volume were prepared for all compounds except a few exceptions. The final volume was the same in each well. The plates were further inoculated using a microbial suspension. Furthemore, some wells were left with an uninoculated broth to serve as a

purity control. Other inoculated wells were prepared as growth control. The outhermost wells remained empty to eliminate the edge effects. The microtiter plate was placed on a wooden pad and secured with clamps for more effective fixation of the lid and also to minimize evaporation. The prepared plates were allowed to incubate for 24 hours at 37 ° C Finally, (Houdková et al. 2017).

3. Aims of the Thesis

Throughout the comprehensive summarization of literature sources based on ability of volatile plant derived compounds to affect viability of bacterial pathogens through direct contact in non-vapor phase based on classical broth microdilution method. There is a potential to take its advantage to confirm transmission of anti bacterial agents via vapour and to facilitate practical researche on agar diffusion methode. However, the main aim of the thesis is the development a new method for testing of natural volatile compounds for their *in vitro* antimicrobial effect in vapor phase by using microtiter plates. Additionally, second objective is the determination of MIC´s of selected compounds by this improved method against *Staphylococcus aureus.*

4. Materials and Methods

4.1 Chemicals

Volatile plant-derived compounds (Figure 1), namely, carvacrol**,** eugenol, thymol and thymoquinone were purchased from Sigma–Aldrich (Prague, CZ). Oxacillin was received from Sigma-Aldrich (Prague, CZ). Dimethyl sulfoxide (DMSO), obtained from Penta (Prague, CZ), and deionized water were used as solvents. Thiazolyl blue tetrazolium bromide (98 %) (MTT) was purchased from commercial supplier (Sigma-Aldrich, Prague, CZ). Bacterial growth had been performed in bacterial culture media prepared from Cation-adjusted Mueller-Hinton broth (MHB) (Oxoid, Basinstoke, UK) and equilibrated for testing with Tris-buffered saline (Sigma-Aldrich, Prague, Czech Republic) to gain pH 7,6 and Mueller-Hinton agar (MHA) (Oxoid, Basinstoke, UK).

4.2 Bacterial strains

The antimicrobial effect in vapor phase was evaluated against representatives of the American Type Culture Collection (ATCC) and clinical isolates. Especially, the strains of methicillin-sensitive Staphylococcus aureus (ATCC 29213) (MSSA) and methicillin-resistant Staphylococcus aureus (ATCC 43300) (MRSA) were purchased from Oxoid (Basingstoke, UK), disposed at ready-to-use suspension (Culti-Loop). Two clinical isolates of S. aureus: CI 2 and CI 3 were obtained from University Hospital in Motol (Prague, CZ) on agar plate.

4.3 Antimicrobial assay

4.3.1 Broth microdilution assay

Overnight cultivated cultures of individual microorganisms were suspended in 10 ml of MHB, where a turbidity of bacterial suspension was modified to 0.5 McFarland standard, approx. 1.5 x 10^8 bacterial/ml, via Densi–La-Meter II (Lachema, CZ) a spectrophotomectric device, designed for standardization of inocula (McFarland 1907; Hindler et al. 1992). The minimum inhibitory concentrations (MICs) were determined by *in vitro* broth microdilution method (BMD), using 96-well microtiter plates according to Clinical and Laboratory Standards Institute (CLSI 2009) and modified on the basis of recommendations proposed for the more effective assessment

of the anti - infective potential of natural products (Cos et. al. 2006) Briefly, The stock solution was prepared at 100x higher concentration (than the starting concentration) then was diluted in Cation-adjusted MHB equilibrated with Tris-buffered saline to attain required starting concentration. Afterwards, two-fold serial dilution of each sample was prepared at concentration ranging between 0.0625 and 2048 µg/ml for volatile compounds, which varied depending on the strain sensitivity. Therefore, sensitive strains were tested at oxacillin concentrations 0.00098-2 µg/ml and methicillin- resistant strains at concentrations 0.125-256 µg/ml. After dilution, microtiter plates were inoculated with bacterial suspension to reach final density of 5 x 105 a colony forming units/ml then were microtiter plates placed into incubator for 24 hours at 37 °C. Subsequently, the turbidity of each well was measured on an Tecan microplate reader, Infinite M200 (TECAN, Mannedorf, CH) at 405 nm and MICs obtained by standard broth microdilution method was expressed as the lowest concentration of compound that inhibits the growth by ≥ 80 % of the test bacteria compared with that of the agent-free growth control. The results used in this study were expressed as the average of MICs obtained from three independent experiments that were performed in triplicate. *S. aureus* ATCC 29213 and ATCC 43300 were used as a quality control strains for antibiotic susceptibility testing.

5. Results

5.1 Antimicrobial assay results

5.1.1 Improved agar diffusion assay

Moreover, in view of the fact, that selected compounds are highly volatile, the antimicrobial activity of the vapors was evaluated by improved agar diffusion assay as follows: the lid of microtiter plate (Picture 1.) (Thermo Fisher Scientific, DK) was inoculated with the non-unified drops of bacterial suspension (Picture 2.) which were applied by syringe to the middle of each condensation ring. Thus they were spread mechanically over the surface by the needle. Then on the bacterial strain was covered by a layer of warm MHA medium in amount of 30l (Picture 3.). After solidification and drying of agar, the lid was placed on bottom part of microtiter plate which had been prepared as described above in the section on the BMD. Finally, microtiter plate was fixed together with single wooden plate (Picture 4.) by using vices to improve those conditions:

- Catch vapors from each concentrations
- Avoid affection from adjoining wells

After 24 h cultivation at 37°C in incubator, the bacterial growth was checked visually.

5.1.2 MTT assay

The lid of microtiter plate was also controlled using basic colorimetric assay. Firstly, MTT was dissolved in deionized water then 25 µl of yellowish solution had been applied on individual condensation rings. After coloring, the MTT assay was evaluated visually for confirmation of cell metabolic activity in each sample (Picture 5.).

5.1.3 Evaluation of Broth micro dilution assay

It was found that plant derived volatile compounds proved certain degree of antibacterial activity in the broth microdilution assay, the efectiveness varied substantially rating from 8 to 2048 µg/mL in broth, as shown in Table 1. The strongest antibacterial activity in liquid phase was acquired for thymoquinone with the low MIC values 8 µg/mL against S. aureus ATCC 43300, and S. aureus Clinical isolate 3 as well

as with MICs16 μg/mL against S. aureus ATCC 29213, and Clinical isolate 2. Carvacrol and thymol possessed moderate inhibitory effect against all bacterial strains ranging 256 - 512 μg/mL. The weak antibacterial activity was obtained for eugenol with MICs 1024 and 2048 μg/mL.

5.1.4 Evaluation of improved Agar diffusion assay

It was found that plant derived volatile compounds proved certain degree of antibacterial activity via improved agar diffusion assay, the efectiveness varied substantially ranging from 16 to 1024 μg/mL on agar, as shown in Table 1. The highest inhibitory effect in vapour phase was observed for thymoquinone against *S. aureus* ATCC 43300 and *S. aureus* ATCC 29213, with low concentration 16 μg/mL. Moreover, thymoquinone also showed strong antibacterial activity with MICs 32 μg/mL for both; Clinical isolate 2 and Clinical isolate 3. Moderate inhibitory effect was observed for carvacrol (256 μg/mL) and thymol (128 μg/mL). Eugenol expressed weak antibacterial activity with MIC values 1024 μg/mL.

5.1.5 Evaluation of MTT Colorimetric assay

It was found that MTT colorimetric assay proved inhibitory effect on agar after application ofsolution. Therefore, improved agar diffusion assay was evaluated based on results of MTT colorimetric assay. All the results for the MTT Colorimteric assay are summarized in Table 1.

Antibacterial activity of plant-derived volatile compounds and antimicrobial agents in liquid and vapour phase against *Staphylococcus aureus* strains

Tab. 1

	Bacterium/growth medium/MIC (µg/mL) *Staphylococcus aureus*							
	ATCC 43300 (MRSA)		ATCC 29213 (MSSA)		Clinical isolate 2		Clinical isolate 3	
	Broth	Agar	Broth	Agar	Broth	Agar	Broth	Agar
Compound								
Carvacrol	512	256	512	256	512	256	512	256
Eugenol	1024	1024	1024	1024	2048	1024	1024	1024
Thymol	256	128	256	128	512	128	256	128
Thymoquinone	8	16	16	16	16	32	8	32
Positive antibiotic control								
Oxacillin	32	> 256	0.25	> 2	128	> 256	0.25	> 2

MIC: minimum inhibitory concentration

MTT colorimetric assay was applied thus microbial growth in Agar media was detected

6. Discussion

Nový et al. (2014) reported lower MIC 8 μg/mL of thymoquinone in liquid phase against *S. aureus* ATCC 29213 than this study. However, their research demonstrated the ability of thymoquinone to pass from vapor phase into liquid medium and create false positive results in adjoining wells. MIC 16 μg/mL observed by Houdková et al. (2017) are similar with this study. Thus the improved agar diffusion method proved that is possible to create conditions, which avoid the evaporation of compound from the well and the results of broth microdilution assay stay non-altered. Furthermore, Rondevaldová et al. (2015) covered microtiter plates with EVA capmat^{TM} and expressed MIC 64 μg/mL, which was higher than in our study. However, uncovered microtiter plates without EVA capmat^{TM} proved altered effect. *S. aureus* ATCC 43300 with MICs 8 μg/mL observed by Nový et al. (2014) showed corresponding results with our findings. MIC 8 μg/mL in our study was also expressed against Clinical isolate 3. At this point, further studies of this phenomenon are necessary. Eugenol expressed weak antimicrobial activity with MIC 1024 μg/mL against strain ATCC 29213 (Houdková et al. 2017), thus showed similar results with this study. Abdelhamid and El-Hosseiny (2017) also reported moderate antibacterial activity with MIC 256 μg/mL for thymol against strain of *S. aureus* ATCC 43300, observed values were similar with our results. However, based on the literature individual clinical isolates as well as quality control strains expressed differences in susceptibility to antimicrobial agents. Those differences in susceptibility of each individual strain could be responsible for plenty of diverse results obtained in this study (Houdková et al., 2017).

In view of the fact that inhibitory effect of volatile compounds in the liquid phase has been reported by many researchers. It is possible to acquire reliable sources. On the other hand, there are only a several reports on their antimicrobial activity in vapor phase (Houdková et al., 2017). Although, some of the assays has been developed for determination of antimicrobial effect in vapor phase, no standardized screening assay has been officially recognized (CLSI 2009). However, the most reliable method for evaluation of antibacterial efficiency in vapour phase represents new developed broth microdilution volatilization method introduced by Houdková (2017). The results showed that thymoquinone possessed strong antimicrobial activity in vapour phase with

MIC 16 μg/mL against *S. aureus*. In this study was obtained the same value. Other results acquired by Houdková, were expressed as follow; thymol (128 μg/mL), eugenol (512 μg/mL), carvacrol (256 μg/mL). Our study proved the similar values for all compounds, although the eugenol showed weak antimicrobial activity in agar with MIC 1024 μg/mL, due to differences in susceptibility of individual strains to antimicrobial agents.

Some of the plant derived volatile compounds exhibited charasteristic phenomena ; the distinct differences of MIC between the liquid and vapour phase (Table 1). In our study was observed, that for all tested bacterial strains thymol and carvacrol showed two times higher MIC values on broth than in agar. It was observed also for eugenol against Clinical isolate 2, and the main factor is low water solubility and high volatility, which is typicall for phenol terpenoids (Fadilah et al. 2017; Kloucek et al. 2012; Peixoto-Neves et al. 2010; Melo et al. 2011). Moreover, thymoquinone as the sole representative ofquinones (Crooks et al. 2001) showed reversible condition, higher values in agar than in broth in all of these bacterial strains; Clinical isolate 3, Clinical isolate 2 and ATCC 43300, due to low vapour presure showed limited volatility.

The growth-inhibitory activity of plant derived volatile compounds against strains of *Staphylococcus aureus* in liquid and vapor phase using a new developed screening method as a combination of a broth microdilution assay and improved agar diffusion assay enriched for basic colorimetric assay, proved the validity of this method based on afore mentioned results. Antibacterial activity of plant derived volatile compound has been always evaluated according to previously developed methodes with separated liquid and vapor phase (Bueno 2015). Moreover, the main advantages of the new developed method were as follow; the rapid comparsion of results obtained from liquid and vapour phase, the posibility to modified 96-well microtiter plate onto plate with lower number of wells, evaporation was avoided due to tightly fixed lids together, material as well as labour were reduced. However, the main disadvantages of this method included; the vapours were not equally distributed within one well, there were cases where agar fell into the well.

7. Conclusions

The new developed screening method based on combination of broth microdilution method and improved agar diffusion assay with MTT colorimetric assay showed valuable results, confirmed the satisfying potential for rapid and simple simultaneous determination of antimicrobial potential of plant derived volatile compounds in the vapour and the liquid phase at different concentrations. Effectiveness of individual compounds, which were tested for MIC, especially tyhmoquinone which showed the most effective inhibitory activity in vapour phase as well as in liquid phase against Clinical isolate 3 and S. aureus ACTT 43300 the most. The potential of this screening methode include; low cost and labour effective high-through put screening of volatile agents without the use of the most complex apparatus.The future utilization of this developed method are in preparations for future developments in broad areas of human endeavor such as medicnal, food, agricultural applications.

Based on the results obtained in this study,

8. References

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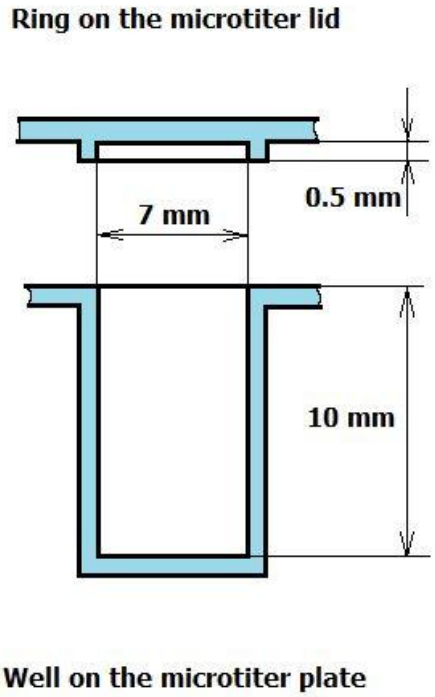
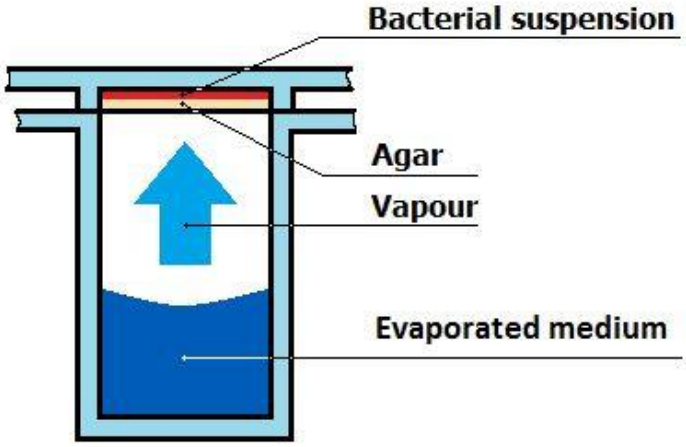
9. Appendices

List of pictures:

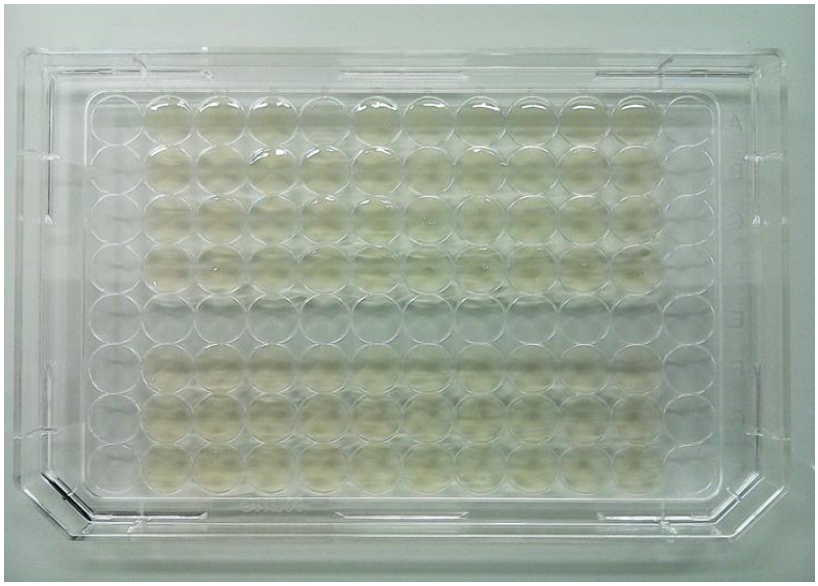


Picture 1. Detail schema of microtiter well and lid



Picture 2. Detail schema of improved agar diffusion assay

Picture 3. Inoculated microtiter plate after agar application



Picture 4. Fixed microtiter plate on a wooden plate

Picture 5. MTT assay

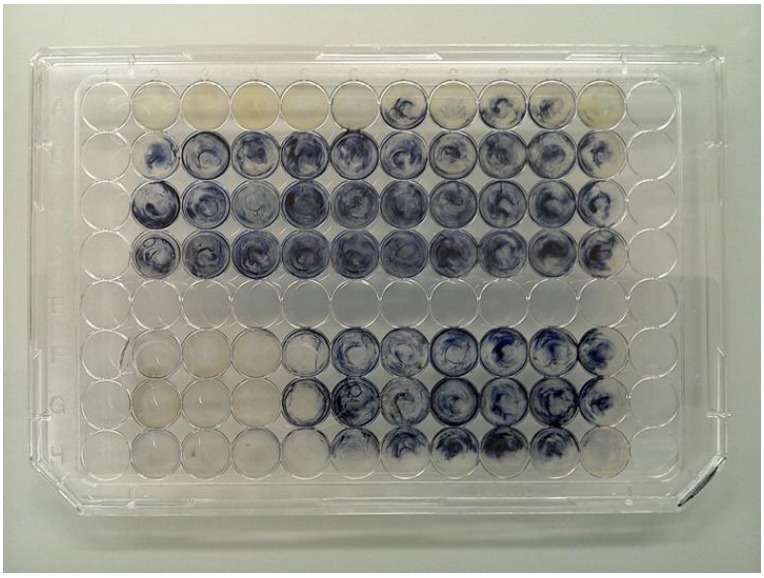


Table 1a: *In vitro* inhibitory activity of Volatile-plant-derived compounds (VOC´s) and oxacillin against *S. aureus*, combined with *in vitro* inhibitory activity of VOC´s and oxacillin against S. aureus in vapor phase captured in agar and confirmed by MTT assay

		MIC (µg/ml)		MTT assay/Vapor-Agar		MIC (µg/ml)		MTT assay/ Vapor-Agar	
S. aureus strain	Test No.	Carvacrol	Ox	Carvacrol	Ox	Eugenol	Ox	Eugenol	Ox
ATCC 43300 (MRSA)	1	512	32	512	>256	1024	32	1024	>256
	2	512	32	256	>256	2048	64	1024	>256
	3	512	32	256	>256	1024	16	1024	>256
	Mean	512	32	256	>256	1024	32	1024	>256
ATCC 29213 (MSSA)	1	512	0.125	256	>2	512	0.25	512	>2
	2	512	0.25	256	>2	1024	0.25	1024	>2
	3	512	0.25	512	>2	1024	0.25	1024	>2
	Mean	512	0	256	>2	1024	0.25	1024	>2
Clinical isolate 2	1	512	128	512	>256	2048	128	1024	>256
	2	512	128	256	>256	2048	128	1024	>256
	3	512	64	256	>256	2048	128	1024	>256
	Mean	512	128	256	>256	2048	128	1024	>256
Clinical isolate 3	1	512	0.5	256	>2	1024	0.5	512	>2
	2	512	0.25	256	>2	1024	0.25	1024	>2
	3	512	0.25	256	>2	1024	0.25	1024	>2
	Mean	512	0.25	256	>2	1024	0.25	1024	>2

The results used in this study will be expressed as the average of MICs obtained from three independent experiments that will be performed in triplicate.

Table 1b: *In vitro* inhibitory activity of Volatile-plant-derived compounds (VOC´s) and oxacillin against *S. aureus*, combined with *in vitro* inhibitory activity of VOC´s and oxacillin against S. aureus in vapor phase captured in agar and confirmed by MTT assay

		MIC (µg/ml)		MTT assay/Vapor-Agar		MIC (µg/ml)		MTT assay/Vapor-Agar	
S. aureus strain	Test No.	Thymol	Ox	Thymol	Ox	Thymoq.	Ox	Thymoq.	Ox
ATCC 43300 (MRSA)	1	256	64	128	>256	8	32	8	>256
	2	256	16	256	>256	8	4	8	>256
	3	256	32	128	>256	8	16	32	256
	Mean	256	32	128	>256	8	17	16	>256
ATCC 29213 (MSSA)	1	256	0.5	128	>2	16	0.5	8	2
	2	256	0.25	128	>2	16	0.125	16	2
	3	256	0.25	128	>2	8	0.25	32	2
	Mean	256	0.25	128	>2	16	0.25	16	2
Clinical isolate 2	1	256	256	128	>256	32	256	32	>256
	2	512	256	128	>256	16	256	16	>256
	3	512	256	128	>256	16	128	32	256
	Mean	512	256	128	>256	16	256	32	>256
Clinical isolate 3	1	256	0.5	128	2	16	1	32	>2
	2	256	0.5	128	>2	8	0.125	16	2
	3	256	0.25	128	>2	8	0.25	32	>2
	Mean	256	0.5	128	>2	8	0	32	>2

The results used in this study will be expressed as the average of MICs obtained from three independent experiments that will be performed in triplicate.