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Anti-staphylococcal effect of plant-derived stilbenes

DISSERTATION THESIS

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CERTIFICATION

I, Tereza Žáková, declare that this thesis entitled "Anti-staphylococcal effect of plant-derived stilbenes" hereby submitted for the Ph.D. degree at the Czech University of Life Sciences Prague, Faculty of Tropical AgriSciences, is wholly my own independent work unless otherwise referenced or acknowledged.

Prague, 2019 .....

Ing. Tereza Žáková

TABLE OF CONTENTS

LIST OF TABLES i
LIST OF FIGURES i
LIST OF APPENDICES ii
LIST OF ABBREVIATIONS iii
ABSTRACT iv
ABSTRAKT vi
1 INTRODUCTION 1
2 LITERATURE REVIEW 3
2.1 *Staphylococcus aureus* 3
2.1.1 Microbiology 3
2.1.2 Pathogenesis 5
2.1.3 Epidemiology 6
2.1.4 Treatment and resistance 7
2.2 Antibacterial activity of plant-derived compounds 9
2.3 Stilbenes 10
2.3.1 Taxonomical distribution 12
2.3.2 Bioavailability 15
2.3.3 Biological activity 18
2.3.4 Antimicrobial activity 18
2.3.5 Anti-staphylococcal activity 19
2.3.6 Structure-bioactivity relationship 20
2.4 Encapsulation of plant-derived compounds 21
2.4.1 β-Cyclodextrin (β-CD) 24
2.4.2 Pharmaceutical and food applications 26
2.4.3 Encapsulation of biologically active natural compounds 27
3 HYPOTHESIS 29
4 OBJECTIVES 30
5 MATERIALS AND METHODS 31
5.1 Tested compounds and other chemicals 31

5.2 Microbial strains and culture medium 33
5.3 Inoculum preparation 34
5.4 Preparation of resveratrol complex with β-Cyclodextrin (β-CD-RSV)..... 34
5.5 HPLC analysis 34
5.6 Antibacterial assay 35
6 RESULTS AND DISCUSSION 37
6.1 Anti-staphylococcal effect of stilbenes 37
6.2 Relationship between structure and anti-staphylococcal effect of stilbenes.. 40
6.3 Anti-staphylococcal activity of β-CD-RSV complex 48
7 CONCLUSION 50
8 REFERENCES 51
9 APPENDICES I

LIST OF TABLES

Table 1 Taxonomical distribution of stilbenes.....14

Table 2 *S. aureus* strains tested.....33

Table 3 *In vitro* growth-inhibitory effect of stilbenes against *Staphylococcus aureus*...39

Table 4 Chemical structures of hydroxystilbenes tested and their growth-inhibitory effect against *S. aureus* strains 43

Table 5 Chemical structures of methoxylated stilbenes tested and their growth-inhibitory effect against *S. aureus* strains.....46

Table 6 *In vitro* growth-inhibitory effect of β-CD-RSV complex against *S. aureus*.....48

LIST OF FIGURES

Figure 1 Scanning electron micrograph of *S. aureus* 4

Figure 2 Basic structure of stilbenes.....11

Figure 3 Resveratrol structure 16

Figure 4 Piceatannol structure 16

Figure 5 Pterostilbene structure.....17

Figure 6 Rhapontigenin structure 17

Figure 7 Chemical structures of α-, β-, γ-CDs.....23

Figure 8 Optimized structure of resveratrol and β-CD.....26

Figure 9 Chemical structures of stilbenes tested 32

Figure 10 Calibration curve for resveratrol quantification.....35

Figure 11 Ortho and meta substitution 41

LIST OF APPENDICES

Appendix 1 *Curriculum vitae* I

Appendix 2 List of author's publications IV

LIST OF ABBREVIATIONS

AIDS	Acquired immune deficiency syndrome
ATCC	American type culture collection
β-CD	β-Cyclodextrin
β-CD-RSV	β-Cyclodextrin resveratrol
CA-MRSA	Community-associated methicillin-resistant *S. aureus*
CD	Cyclodextrin
CFU	Colony forming unit
CLSI	Clinical and Laboratory Standards Institute
EUCAST	European Committee on Antimicrobial Susceptibility Testing
GRAS	Generally recognized as safe
HA-MRSA	Health-care-associated methicillin-resistant *S. aureus*
HPLC	High-performance liquid chromatography
HP-β-CD	Hydroxypropyl-β-cyclodextrin
IUPAC	International Union of Pure and Applied Chemistry
KI	Clinical isolate of *S. aureus*
MIC	Minimun inhibitory concentration
MRSA	Methicillin-resistant *S. aureus*
MTT	Thiazolyl blue tetrazolium bromide
SEB	Enterotoxin B
spp.	*species pluralis*
TSST	Toxic shock syndrome toxin

ABSTRACT

Staphylococcus aureus is a deadly pathogen that was initially limited to hospital and healthcare facilities but has gradually become a growing problem in healthy children and adults. Stilbenes are polyphenol compounds of plant origin known to possess a variety of pharmacological properties such as antibacterial, antiviral, and antifungal effects. This study reports the *in vitro* growth-inhibitory potential of eight naturally occurring stilbenes against six standard strains and two clinical isolates of *S. aureus*, using a broth microdilution method, and expressing the results as minimum inhibitory concentrations (MICs). Pterostilbene (MICs 32 - 128 μg/ml), piceatannol (MICs 64 - 256 μg/ml) and pinostilbene (MIC 128 μg/ml) are among active compounds that possessed the strongest activity against all microorganisms tested, followed by 3′-hydroxypterostilbene, isorhapontigenin, oxyresveratrol and rhapontigenin with MICs 128 - 256 μg/ml. Resveratrol (MIC 256 μg/ml) exhibited only weak inhibitory effect against two standard *S. aureus* strains ATCC 25923 and ATCC 33592. Furthermore, structure–activity relationships were studied. Hydroxyl groups at ortho-position (B-3′, -4′) played crucial role for the inhibitory effect of hydroxystilbene piceatannol. Compounds with methoxy groups at the ring A (3′-hydroxypterostilbene, pinostilbene and pterostilbene) produced stronger effect against *S. aureus* than their analogues (isorhapontigenin and rhapontigenin) with methoxy groups at the ring B. Resveratrol has been described to possess several biological activities such as antioxidant, antimicrobial and anti-inflammatory. However, its efficacy is still limited owing to its low aqueous solubility. Cyclodextrins and their derivatives have the ability to encapsulate the bioactive compounds into its cavity and protect these from the environmental conditions, improve the solubility and bioavailability. Therefore, another goal of this study was the evaluation of resveratrol complex with β-cyclodextrine against standard strains of *S. aureus*. In our tests resveratrol complex showed moderate activity with the MIC value of 256 μg/ml for one standard *S. aureus* strain ATCC 33591. These findings provide arguments for further investigation of stilbenes as prospective structures for development of novel anti-staphylococcal agents. Moreover, stilbene encapsulation with β-cyclodextrine may increase their anti-staphylococcal potential.

Keywords: antimicrobial activity, natural antibacterial agents, minimum inhibitory concentration, stilbenes, *Staphylococcus aureus*, structure–activity relationships, β-cyclodextrine

ABSTRAKT

Staphylococcus aureus je smrtelný patogen, který se původně vyskytoval pouze v nemocnicích a zdravotnických zařízeních, ale postupně začal představovat čím dál větší problém u zdravých dětí a dospělých. Stilbeny jsou polyfenolové sloučeniny rostlinného původu, o nichž je známo, že mají řadu farmakologických vlastností, jako jsou antibakteriální, antivirové a protiplísňové účinky. Tato práce popisuje *in vitro* stanovení inhibičního efektu osmi přirozeně se vyskytujících stilbenů proti šesti standardním kmenům a dvěma klinickým izolátům *S. aureus* za použití mikrodiluční bujónové metody. Výsledky byly vyjádřeny jako minimální inhibiční koncentrace (MIC). Pterostilben (MIC 32 - 128 μg/ml), piceatannol (MIC 64 - 256 μg/ml) a pinostilben (MIC 128 μg/ml) patří mezi účinné látky, které vykazovaly nejsilnější účinek proti všem testovaným mikroorganismům, po nichž následovaly 3´-hydroxypterostilben, isorhapontigenin, oxyresveratrol a rapontigenin s MIC 128 - 256 μg/ml. Resveratrol (MIC 256 μg/ml) vykazoval pouze slabý inhibiční účinek proti dvěma standardním kmenům *S. aureus* ATCC 25923 a ATCC 33592. Dále byl zkoumán účinek na základě struktury látky. Hydroxylové skupiny v orto-poloze (B-3´, -4´) hrály klíčovou roli pro inhibiční účinek hydroxystilbenu piceatannolu. Sloučeniny s methoxy skupinami na kruhu A (3'-hydroxypterostilben, pinostilben a pterostilben) měly silnější účinek proti *S. aureus* než jejich analogy (isorhapontigenin a rapontigenin) s methoxy skupinami na kruhu B. U resveratrolu bylo popsáno mnoho biologických účinků jako antioxidační, antimikrobiální a protizánětlivé. Jeho účinnost je však vzhledem k nízké rozpustnosti ve vodě stále omezena. Cyklodextriny a jejich deriváty mají schopnost zapouzdřit bioaktivní sloučeniny do své dutiny a chránit je před okolními vlivy, zlepšit jejich rozpustnost a biologickou dostupnost. Dalším cílem této studie proto bylo vyhodnocení komplexu resveratrolu s β-cyklodextrinem proti standardním kmenům *S. aureus*. V našich testech vykazoval tento komplex střední aktivitu s hodnotou MIC 256 μg/ml pro jeden standardní kmen *S. aureus* ATCC 33591. Tato zjištění poskytují argumenty pro další zkoumání stilbenů jako perspektivních struktur pro vývoj nových anti-stafylokokových prostředků. Zapouzdření stilbenů s β-cyklodextrinem by navíc mohlo zvýšit jejich schopnost inhibovat růst *S. aureus*.

Klíčová slova: antimikrobiální aktivita, přírodní antibakteriální látky, minimální inhibiční koncentrace, stilbeny, *Staphylococcus aureus*, biologický účinek na základě struktury látky, β-cyklodextrin

1 INTRODUCTION

Staphylococcus aureus, previously known as a "golden staph", is one of the most adaptable human pathogens. The dispersion of certain successful lineages can be tracked across the globe (Zetola et al. 2005). It is a remarkably diverse bacterial pathogen as reflected in its capacity to cause various array of infections (e.g. skin and soft tissue infections, pneumonia, sepsis, suppurative diseases) and food poisoning (Foster 2004; Iwatsuki et al. 2006; Smeltzer 2016). Even though pharmacological industries continuously produced a number of new antibiotics, bacteria has gained resistance so fast to this treatment that it has become an alarming global problem. Acquiring resistance to more than one antibiotic has made this bacteria more dangerous, now known as "superbug" with multidrug-resistance (Gibbons 2004).

Plant-derived compounds are traditionally used as natural remedies against various infection diseases. In recent time, these active substances are very often investigated and serve as a huge source for pharmaceutical research how to overcome the complications in the anti-infectious therapy (Silva et al. 2015). Among these natural substances, plant stilbenes have received considerable interest over the past 20 years due to their biological activity (e.g. antioxidant, antiatherosclerotic, hypolipidemic, antidiabetic, cardioprotective, antiviral, anti-inflammatory and anticancer) and possible pharmacological applications (Basri et al. 2014). Stilbene compounds occur in wide range of plant species and edible plants from all over the world (Peng et al. 2008). In previous studies, antimicrobial effect of resveratrol, one of the most well-known and extensively studied stilbene, and its related structures (e.g. piceatannol, pterostilbene, *trans*-piceid, *trans*-ε-viniferin) have been described against various food and human pathogenic microorganisms such as *Acetobacter aceti*, *A. oeni*, *Bacillus cereus*, *B. subtilis*, *Dekkera bruxellensis*, *Escherichia coli*, *Listeria innocua*, *L. monocytogenes*, *Pseudomonas fluorescens*, *P. aeruginosa*, *Streptococcus spp*., *Zygosaccharomyces bailii* and *Z. rouxii* (Yim et al. 2010; Kumar et al. 2012; Pastorkova et al. 2013). The *in vitro* growth-inhibitory effect of (*trans*)-3-hydroxy-5-methoxystilbene, oxyresveratrol, pterostilbene and resveratrol has also previously been described against *S. aureus* and *S. epidermidis* (Xie et al. 2015; Joung et al. 2016). It is broadly recognized that the biological activity of a plant-derived compound depends on its chemical structure and are closely related

to the types of structural terminal groups, as well as to the number and locations of hydroxyls (Xie et al. 2015). In the case of resveratrol, it has been reported that the introduction of methoxy substitution in the place of hydroxyl groups improved the compound's anti-proliferative effect by apoptosis induction and cell cycle inhibition. The more methoxy groups added, the better the anti-tumor activity of the compound becomes (Chen et al. 2013).

Despite all these established biological activities and the potential health benefits, the poor water solubility of these compounds and their sensitivity to external agents such as air, light, and oxidative enzymes can constitute a serious problem for their bioavailability formulation and manipulation (Lucas-Abellan et al. 2008). Here is worthy of notice that the limited solubility of resveratrol was the critical point of all our research, many methods (pH adjustment, increased temperature, various solvents, etc.) have been used to improve it without appreciable impact. The encapsulation with cyclodextrins (CDs) species is one of new approaches in order to overcome these drawbacks during the developmental stage of drugs based on polyphenolics (Munin & Edwards-Levy 2011). In the pharmaceutical industry, CDs are used as drug carriers to enhance the solubility, stability and bioavailability of the bioactive molecules (Pinho et al. 2014).

With aim to bring new insights into relationship between antibacterial activity and the chemical structure of stilbene molecules, this dissertation reports the *in vitro* growth-inhibitory effect of selected stilbenes against strains of *S. aureus*, with special focus on structure-activity relationship. In addition, the thesis describes experiments focused on solubility improvement of resveratrol by encapsulation with CDs.

2 LITERATURE REVIEW

2.1 *Staphylococcus aureus*

Scottish surgeon Alexander Ogston first identified bacteria from the genus *Staphylococcus* (from Greek "staphylé", which means a bunch of grapes, and "kokkos", which means granule) as originators of suppuration in humans in 1880. This genus is taxonomically in the bacterial family *Staphylococcaceae*, which also includes three lesser-known genera (*Gamella*, *Macrococcus* and *Salinicoccus*). The genus *Staphylococcus* contains around 40 species, whereas most of them are exceptionally diverse bacterial pathogens. Staphylococci are widespread in nature, although they mainly occur living on the skin, skin glands, and mucous membranes of mammals and birds. They can also be found all over the world in a soil. Most of the species are facultative anaerobes and their growth is more fast and abundant under aerobic conditions. In general, they are divided into two groups (coagulase-positive and coagulase-negative) depending on the ability enzymatically coagulate plasma (Kloos & Bannerman 1999; Greenwood et al. 2002; Harris et al. 2002).

2.1.1 Microbiology

Gram-positive cocci are usually growing by aerobic respiration or by lactic acid producing fermentation. *S. aureus* is catalase-positive and oxidase-negative, non-motile and non-spore forming bacterium. Individuals have about 0.5–1.5 μm in diameter (Figure 1) and are characterized as microscopically visible round granules, they grow in grape-like clusters, pairs and occasionally in short chains (Götz et al. 2006; Harris & Richards 2006). It is able to tolerate high salt concentrations (as high as 15 %) and resistant to desiccation and to temperatures up to 50 °C for 30 minutes. It is often hemolytic and it can be easily cultivated on agar plates, where forms round entire, smooth, slightly raised shiny, cream-yellow to orange colonies; or in broth where create visible cloud and sediment. *S. aureus* and *S. intermedius* are coagulase positive. All other staphylococci are coagulase negative. Coagulase-negative staphylococci are normally less virulent and express fewer

virulence factors (Kloos & Bannerman 1999; Greenwood et al. 2002; Harris & Richards 2006; Bednar et al. 2009). The cell structure plays an important role in infectivity and pathogenicity of bacteria. Cells express on their surface proteins that promote attachment to host proteins such as laminin and fibronectin that form part of the extracellular matrix. Fibronectin is present on epithelial and endothelial surfaces as well as being a component of blood clots. In addition, most strains exhibit a fibrinogen/fibrin binding protein (the clumping factor) which promotes attachment to blood clots and wounded tissue. These include acquisition of specific adhesion factors, formation of biofilms, adaptation to an intracellular environment, production of a protective capsular polysaccharide or evasion of innate immune defenses (e.g. lysozyme) (Flannagan et al. 2015).

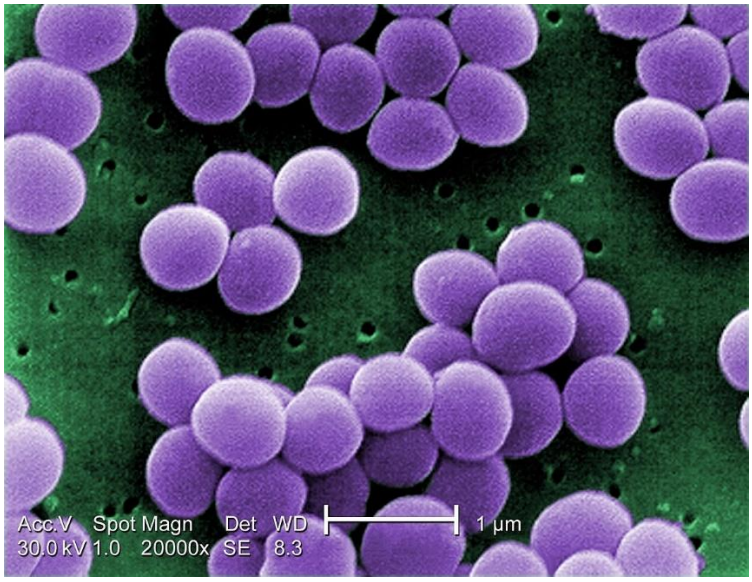


Figure 1 Scanning electron micrograph of *S. aureus*, the colors were added after the re-processing of the image (Image reproduced from Centers for Disease Control and Prevention's Public Health Image Library)

The staphylococcal cell wall is a complex network of surface proteins, capsular polysaccharides and wall teichoic acids covalently linked to peptidoglycan (Lowy 1998; Atilano et al. 2011; Mistretta et al. 2019). The peptidoglycan itself is composed of β-linked N-acetylglucosamine and N-acetylmuramic acid polysaccharide chains, which are cross-linked through peptide chains on alternating strands. It can determine the overall cellular shape, serves as attachment site for

virulence factors and adhesins, aids bacteria in undergoing morphological transformations in response to different stress-related factors, and its eventual fragility or instability may lead to cell lysis and death (Macheboeuf et al. 2006). The wall teichoic and lipoteichoic acids phosphate-rich glycopolymers are involved in the resistance of bacteria to environmental stress and regulation of bacterial division (Atilano et al. 2011). Murein and wall-associated surface proteins also compose the cell wall. The fibronectin-binding proteins are major ligands capable of the adherence of this bacterium to epithelial cells (Dmitriev et al. 2004; van Belkum et al. 2009).

2.1.2 Pathogenesis

Commensal symbiont *S. aureus* is a part of the normal human microflora usually found on the skin and mucosa especially in anterior nares, axilla, perineum and pharynx (Lin et al. 2016; Mistretta et al. 2019), approximately 20 % of the general population always harbors it on the nasal mucosa without any pathogenic event (Iwatsuki et al. 2006); however it should be always considered as a potential pathogen. It can cause various array of diseases, ranging from the minor skin infections such a boils, impetigo, carbuncles, furuncles, abscesses and infections of the respiratory tract to a variety of very severe and life-threating illnesses such a serious blood stream infections (sepsis and bacteremia), meningitis, pneumonia endocarditis and osteomyelitis (Lina et al. 1999, Otto 2014). *S. aureus* is also the main causative agent of food intoxication (Kadariya et al. 2014). Among the array of toxin components being produced by this pathogen, enterotoxin B (SEB) and toxic shock syndrome toxin (TSST) are important virulence factors that play vital role in the pathogenicity of *S. aureus*. SEB is the primary cause of staphylococcal food poisoning and a potent mitogen, whereas TSST may lead to toxic shock syndrome, which is potentially fatal, especially young women are at a higher risk. Both TSST, and SEB belong to a family of super antigens, at very low concentrations, these super antigens induce polyclonal immune response. SEB can also set off a toxic shock (Krakauer 2010) and profound hypotension resulting in multi organ failure (Shylaja et al. 2012).

2.1.3 Epidemiology

The ecological niches of *S. aureus* strains are the anterior nares. Previous studies have shown that the nares are the most consistent area from which this organism can be isolated (Williams 1963). Over time, three patterns of carriage can be distinguished. Approximately 20 % of individuals almost always carry one type of strain (persistent carriers), a large proportion of the population harbors *S. aureus* intermittently (intermittent carriers), and the strains change with varying frequency. Finally, a minority of people almost never carries *S. aureus*. Persistent carriage is more common in children than in adults, and many people change their pattern of carriage between the age of 10 and 20 years. The reasons for these differences in colonization patterns are unknown. Younger people (under 20 years old) are the most frequent nasal carriers, whereas the least frequent are older people (Noble et al. 1964). In the general population, a mean carriage rate of 37.2 % was found (Kluytmans et al. 1997). Carriage rates increase in case of patients during hospitalization, those with insulin-dependent diabetes mellitus, those on hemodialysis, those on continuous ambulatory peritoneal dialysis, intravenous drug addicts, patients with *S. aureus* skin infections, and those with human immunodeficiency virus infection or AIDS (Kluytmans et al. 1997). However, infections in individuals with no exposures to healthcare settings are being reported with increasing frequency (Estivariz et al. 2007). The modes of infection transmission are two: exogenous, that is from external sources; and endogenous, that is from the carrier or infected person. In hospital settings, *S. aureus* may be transmitted from patient to patient via healthcare worker hands, contaminated equipment or through environmental contamination (Park et al. 2017). Skin infections have been reported to spread in settings where crowding, skin-to-skin contact, frequent skin abrasions, poor hygiene, and environmental contamination are prevalent. These factors have been associated with several groups experiencing outbreaks of disease including sports participants, prison inmates, military recruits, even children attending day care centers, men who have sex with men, and homeless people (Bamberger & Boyd 2005; Tenover 2006; Estivariz et al. 2007; Jenney et al. 2014).

2.1.4 Treatment and resistance

The control and treatment of skin and soft tissue infections treatment is not usually achieved via antibiotics alone. An important therapy that must be considered is surgical incision and drainage of an abscess. However, not all studies have shown that incision and drainage alone is successful. For these reasons, antibiotics, along with surgical incision and drainage, play a vital role in the management of *S. aureus* infections (Osmon et al. 2013). Selection of the best antibiotic is essential in order to ensure an effective method for the control and treatment (Nemerovski & Klein 2008). With respect to bacterial infections, the situation dramatically improved when penicillin became available for use in the early 1940s. However, the euphoria over the potential conquest of infectious diseases was short lived. Almost as soon as antibacterial drugs were deployed, bacteria responded by manifesting various forms of resistance. As antimicrobial usage increased, so did the level and complexity of the resistance mechanisms exhibited by bacterial pathogens (Tenover 2006). Initially, the problem of bacterial resistance to antimicrobial drugs was solved by the discovery of new classes of drugs, such as aminoglycosides, macrolides, and glycopeptides, as well as by the chemical modification of previously existing drugs (Gold & Moellering 1996). Two years after the introduction of penicillin for medical use, the first penicillin resistant *S. aureus* isolate was observed in a hospital. Later on, penicillin-resistant *S. aureus* strains were also observed in the community. Since 1960, around 80 % of all *S. aureus* strains are resistant to penicillin. In 1961, 2 years after the introduction of methicillin, a penicillinase-resistant penicillin, due to the acquisition of the mecA gene first methicillin-resistant *S. aureus* (MRSA) strains were described (Gibbons 2004). MRSA can be divided into two groups. The first group, called community-associated MRSA (CA-MRSA), is characterized by the presence of the toxin Panton-Valentine leukocidin (a gene that allows the production of a necrotizing cytotoxin), generally occurs in people who have not stayed in hospital for long time and the resistance was caused by mutations of *S. aureus* genome during the use of antibiotics against other infections or by transmission in everyday life (Al-Talib et al. 2009). These strains are more aggressive, but oppositely generally sufficiently sensitive to other antibiotics. The second group, called health-care-associated MRSA (HA-MRSA, initially known as epidemic-MRSA or

EMRSA), emerged in the 1960´s and is linked with hospitalization, surgery, hemodialysis, antibiotic treatment and exposure to invasive devices or procedures. These strains are less virulent but less sensitive to the antibiotics and during the last 45 years various HA-MRSA clones disseminated worldwide. In addition, since the 1990s, virulent CA-MRSA clones spread worldwide, first in the community, but later on also in healthcare facilities. At the moment, the distinction between CA-MRSA and HA-MRSA is beginning to fade (Lowy 1998; Lowy 2003; Deurenberg & Stobberingh 2008). The development of antibiotic resistance is multi factorial, including the specific nature of the relationship of bacteria to antibiotics, the usage of antibacterial agent, host characteristics and environmental factors.

In the majority of countries, tetracyclines and β-lactam antibiotics are the most frequently prescribed antimicrobial agents for the treatment of a number of bacterial infections, including those caused by staphylococci (Trzcinski et al. 2000).

Since the initial discovery of benzylpenicillin, numerous other β-lactam classes have been developed. They fall into four distinct structural classes that all have the four-membered lactam core moiety in common (penicillins, cephalosporins, carbapenems, and monobactams). Taken together, the multiple β-lactams constitute a comprehensive and structurally diverse set of compounds that display different pharmacological properties and are used for unique clinical indications (King et al. 2017). β-lactam agents inhibit synthesis of the bacterial cell wall by interfering with the enzymes requisite for the synthesis of the peptidoglycan layer (Typas et al. 2011).

The antibacterial effect of all ß-lactam antibiotics depends on the capacity of the antibiotic to diffuse through the cell membrane, the affinity of the antibiotic for its target proteins, and the stability of the antibiotic against bacterial degradation (Dever & Dermody 1991). There are three major mechanisms of bacterial resistance to the β-lactam antibiotics: (i) enzymatic degradation by β-lactamases, (ii) target modification of the PBPs resulting in a lack of β-lactam binding, and (iii) regulation of β-lactam entry and efflux (King 2017). Tetracyclines, relatively inexpensive antibiotics, are broad-spectrum antibiotics that have been widely used in human and veterinary medicine, as growth promoters in animal husbandry and even to treat

bacterial infections in plants (Trzcinski et al. 2000). Resistance to tetracycline is likewise caused by enzymatic modification, and alteration of this drug is followed by rapid efflux from the cytoplasm. Tetracycline efflux, unaccompanied by enzymatic modification, does not confer resistance to this drug (Yu et al. 2010). This situation has forced scientists to search for new antimicrobial substances from various sources as novel antimicrobial chemotherapeutic agents (Amenu 2014). Natural products have been used as antibiotics or as scaffolds for formulation of active semisynthetic derivatives, including examples of some well-known natural product classes, such as flavonoids, alkaloids, and quinones, among others. In addition, plant polyphenols possess some antimicrobial activities of varying potencies (Mora-Pale et al. 2015). Natural products from higher plants have traditionally been suggested as an important source of antimicrobial agents and have attracted extensive attention in fundamental and clinic applications. They are often effective in the treatment of infectious diseases while simultaneously mitigating many of the side effects that are often associated with synthetic antimicrobials (Su et al. 2014).

2.2 Antibacterial activity of plant-derived compounds

Since ancient times, plants have provided a source of inspiration for novel drug compounds, as plant-derived medicines have made large contributions to human health and well-being. There are numerous illustrations of plant-derived drugs (Cragg & Newman 2013). For example isoquinoline alkaloid emetine obtained from the underground part of *Cephaelis ipecacuanha*, and related species, has been used for many years as and amoebicidal drug as well as for the treatment of abscesses due to the spread of *Escherichia histolytica* infections. Another important drug of plant origin with a long history of use is quinine, alkaloid occurs naturally in the bark of cinchona trees (*Cinchona*), and artemisinin (*Artemisia annua*) and its derivatives, essential components of antimalarial treatment (White 2008). Currently, the widely prescribed drugs are analogs of quinine such as chloroquine. It is estimated that today, plant materials are present in, or have provided the models for 50 % western drugs. Many commercially proven drugs used in modern medicine were initially used in crude form in traditional or folk healing practices, or for other purposes that suggested potentially useful biological activity. Examples of these are bacteriostatic,

antifungicidal, and antibiotic action of allicin in garlic (*Allium sativum*), or the antimicrobial action of berberines in goldenseal (*Hydrastis canadensis*) (Lemar et al. 2002; Ettefagh et al. 2011). Recently, Kokoska et al. (2019) have published a comprehensive review on plant-derived antimicrobial agents currently applied in practice for the improvement of human health. It summarizes data on more than 40 plant-derived over-the-counter pharmaceuticals, dietary supplements, cosmetics, herbal medicines, and functional foods containing complex mixtures (e.g. *Glycyrrhiza glabra* extract, *Melaleuca alternifolia* essential oil, and *Pistacia lentiscus* resin) and pure compounds (e.g. benzoic acid, berberine, eucalyptol, salicylic acid and thymol). The effectiveness of these products is illustrated by results of clinical trials and supported by data on there *in vitro* antimicrobial activity.

The beneficial medicinal effects of plant materials typically result from the combinations of secondary products present in the plant. In plants, these compounds are mostly secondary metabolites such as alkaloids, terpenoids, steroids, and phenolic compounds, which are synthesized and widely distributed in all their parts (Abdallah 2011). These compounds are more complex and specific and are form in certain taxa such as family, genus and species, but heterogeneity of secondary compounds is found in wild species (Omojate et al. 2014). For purpose of this thesis, we focused on secondary metabolites stilbenes.

2.3 Stilbenes

Stilbenes are small molecular weight (200–400 g/mol), naturally occurring compounds found in a wide range of plant sources and fruits, aromatherapy products and dietary supplements. These molecules are synthesized via the phenylpropanoid pathway, and characterized by a 1,2-diphenylethylene backbone (structure is shown in Figure 2). More than 400 stilbenes have been described and they can appear as both monomers and complex oligomers. The monomeric stilbene structure is relatively simple and characterized by two benzene rings joined by an ethylene bridge. As a result of this ethylene bridge, stilbenes can occur as *cis*- and *trans*-isomers, of which the *trans*-isomer is the most common configuration (Silva et al. 2014). *Trans*-Resveratrol was first isolated from *Vitis vinifera*, and later from

those wines whose musts had been fermented together with the grape peel. *Cis*-resveratrol was not detected in grapes, but its presence has been demonstrated in wine (Palomino et al. 2000). It has been reported that *trans*-resveratrol is more stable that its *cis* isomer. Trela & Waterhouse (1996) performed trials conducted under a variety of commonly encountered laboratory conditions and showed that *trans*-resveratrol is stable for months, except in high-pH buffers, when protected from light. *Cis*-resveratrol was reported to be stable only near pH neutrality when completely protected from light. Interestingly, the *trans*-isomer has been reported to be biologically more active than the *cis*-isomer, probably due to its nonplanar conformation (Anisimova et al. 2011). Most plant stilbenes are derivatives of the basic unit *trans*-resveratrol (3,5,4′-trihydroxy-*trans*-stilbene), although other structures are found in particular plant families (Chong et al. 2009).

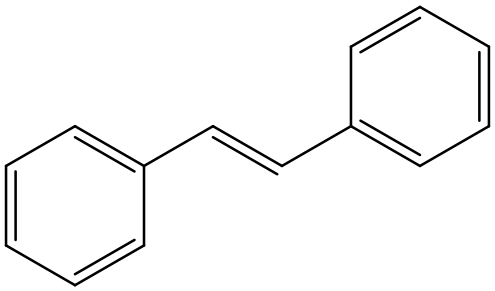


Figure 2 Basic structure of stilbenes

The major role attribute to stilbenes in several plant families is to act in plant resistance to fungal infections such as *Botrytis cinerea,* so they are widely considered phytoalexins that are defensive substances both synthesized by and accumulated in plants. In some plants, stilbenes are constitutively expressed and, furthermore, their synthesis can also be induced in response to a large range of biotic and abiotic stress factors and plant growth regulators (Tsai et al. 2017; Jeandet et al. 2014; Jeandet et al. 2010).Over the last 15 years, plant stilbenes have received considerable interest, due to their biological activities and possible pharmacological applications. Since resveratrol was postulated to be involved in the health benefits associated with a moderate consumption of red wine. This phenomenon is known as “French paradox”, concept correlating wine consumption and low incidence of coronary heart diseases,

despite high intake of dietary cholesterol and saturated fat, formulated by French epidemiologists (Renaud & Lorgeril 1992). However in recent years this theory has been relatively questioned (Lorgeril et al. 2002, Krobot et al. 1992). Nevertheless, resveratrol is one of the most extensively studied natural products. Hundreds of studies have reported that it can prevent or slow the progression of a wide variety of illnesses, including cancer, and cardiovascular diseases, as well as extend the lifespans of various organisms (Ferrieres 2004, Tsai et al. 2017). Nowadays there are dietary supplements containing resveratrol or other stilbenes structures (e.g. pterostilbene) marketed under various tradenames available on the market (Pezzuto 2019).

2.3.1 Taxonomical distribution

We can find stilbene compounds in wide range of several edible and non-edible plant species (Table 1) from all over the world including grape wine (*Vitis vinifera*), peanut (*Arachis hypogaea*), pistachio (*Pistacia vera*), sorghum (*Sorghum bicolor*), bilberries (*Vaccinium myrtillus*), blueberries (several *Vaccinium* species) and many tree species (*Pinus* and *Picea*) (Peng et al. 2008). Certain plants are used commercially as a source of stilbenes include many plants cultivated in Asia as folk medicines such as *Euphorbia lagascae*, *Melaleuca leucadendron*, *Polygonum cuspidatum*, *Rheum undulatum* and *Rhodomyrtus tomentosa* (McCormack & McFadden 2012; Shi et al. 2012). However their within a natural source may vary considerably depending on species, climate, location and time of year (Davidov-Pardo & McClements 2014). For example in case of resveratrol in wine, its content significantly differs between countries, cultivation areas, vintages, production years, temperature, pH value, and level of sulfur dioxide (Ferrieres 2004). Grapes and juice residues are important source of stilbenes when it is used in nutraceutical applications. Residues produced during wine making (grape pomaces), and other grape juice solids contain high polyphenol concentrations and are also attractive sources of many stilbene compounds not only resveratrol. The bark waste of conifer trees contains substantial amount of stilbene compounds such as pinosylvin, piceatannol, and *trans*-resveratrol. This enormous amount of industrial

byproducts represents a very attractive and inexpensive source of stilbenes with possible commercial applications (Karlund et al. 2015).

Table 1 Taxonomical distribution of stilbenes

Stilbene	**Occurrence**
Astringin	*Picea spp.*
Gnetol	*Gnetum gnemon*
Isorhapontigenin	*Picea spp.*
Oxyresveratrol	*Cyperaceae, Gnetaceae, Liliaceae, Moraceae, Smilacaceae*
Piceatannol	*Ananas comosus, Passiflora edulis, Picea, Rheum spp., Saccharum spp., Vaccinium corymbosum, Vitis vinifera*
Piceid	*Ananas comosus, Prunus dulcis, Vitis vinifera*
Pinosylvin	*Alnus spp., Pinus spp.*
Pterostilbene	*Pterocarpus marsupiu, Pterocarpus santalinus, Vaccinium corymbosum, Vitis vinifera*
Resveratrol	*Arachis hypogaea, Fallopia, Fragaria x ananassa, Humulus lupulus, Lycopersicon esculentum, Pistacia vera, Rheum spp., Saccharum spp., Theobroma cacao, Vaccinium corymbosum, Vaccinium macrocarpon, Vaccinium myrtillus, Vitis vinifera*
Rhapontigenin	*Rheum spp.*
Rhapontin	*Rheum spp.*
Trans-ε-viniferin	*Vitis vinifera*
3′-hydroxypterostilbene	*Sphaerophysa salsula*

Table adapted from Reinisalo et al.(2015), completed by data from El Khawand et al. (2018)

2.3.2 Bioavailability

Similarly as majority of phenolic compounds, stilbenes are considered as compounds with low bioavailability, which limits their potential benefits for health and their practical use. Nevertheless, the bioavailability depends on the route of administration but also relies on their absorption and metabolism. Those factors are mainly determined by the chemical structure of the compound (its basic structure, degree of glycosylation/acylation conjugation with other phenolic compounds, molecular size, degree of polymerization, solubility, etc.). That is the reason why bioavailability may considerably differ among the many different (even closely related) phenolics (Sirerol et al. 2016).

Several studies deal with the issue of stilbenes bioavailability. Walle et al. (2004) studied the absorption, bioavailability, and metabolism of resveratrol (Figure 3) after oral and intravenous doses in six human volunteers. The absorption of a dietary relevant 25-mg oral dose was at least 70%, with peak plasma levels of resveratrol and metabolites of 491 ± 90 ng/ml (about 2 μM) and a plasma half-life of 9.2 ± 0.6 h. However, only trace amounts of unchanged resveratrol (<5 ng/ml) could be detected in plasma. Most of the oral dose was recovered in urine, and liquid chromatography/mass spectrometry analysis identified three metabolic pathways, i.e., sulfate and glucuronic acid conjugation of the phenolic groups and, interestingly, hydrogenation of the aliphatic double bond. The hydrogenation is likely produced by the intestinal microflora. Extremely rapid sulfate conjugation by the intestine/liver appears to be the rate-limiting step in resveratrol's bioavailability. Despite the low amounts of unchanged resveratrol detected in plasma by Walle et al. (2004), it is interesting that Wang et al. (2002) detected the resveratrol in brain when conducting the metabolic study on Mongolian gerbils demonstrating that resveratrol can cross the blood–brain barrier.

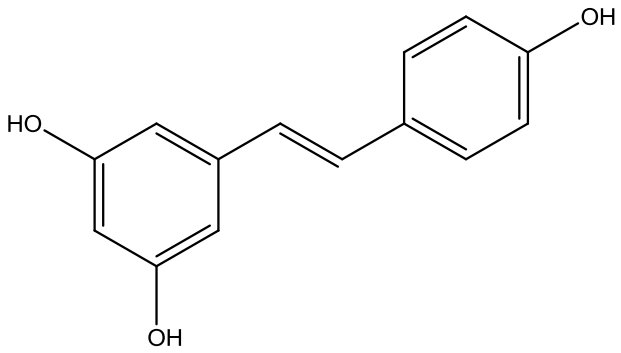


Figure 3 Resveratrol structure

Lesser-known metabolite of resveratrol is piceatannol (Figure 4). It is derived from resveratrol by cytochrome P450 enzyme CYP1B1 (Potter et al. 2002), but it is also found in sugar cane, berries, peanuts, the skin of grapes and red wines. The presence of an extra hydroxyl group in piceatannol structure makes it reactive and more potent antioxidant as compared to resveratrol. It has been also shown that piceatannol possesses stronger anticancer activity than resveratrol and higher ability to scavenge free radicals as compared to resveratrol (Kukreja et al. 2014). Hence, it could be hypothesized that biological activities ascribed to the resveratrol could be caused (at least partially) to the piceatannol.

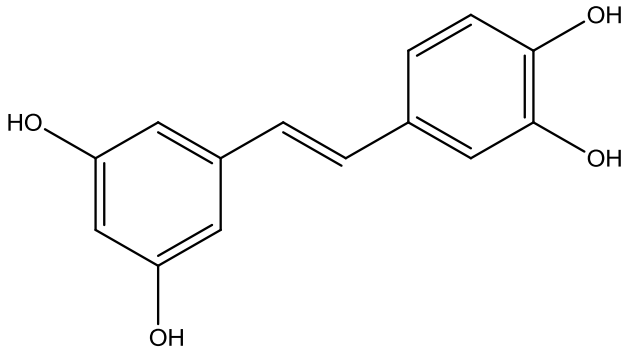


Figure 4 Piceatannol structure

Another important naturally-occurring dimethylether analog of resveratrol found mainly in *Vaccinum* berries is pterostilbene (Figure 5). Its metabolism in rats was studied by Kapetanovic et al. (2012). Following oral dosing, plasma levels of pterostilbene, and its metabolite pterostilbene sulfate were markedly greater than were plasma levels of resveratrol and resveratrol sulfate indicating that the *in vivo* biological activity of equimolar doses of pterostilbene may be greater than that of resveratrol.

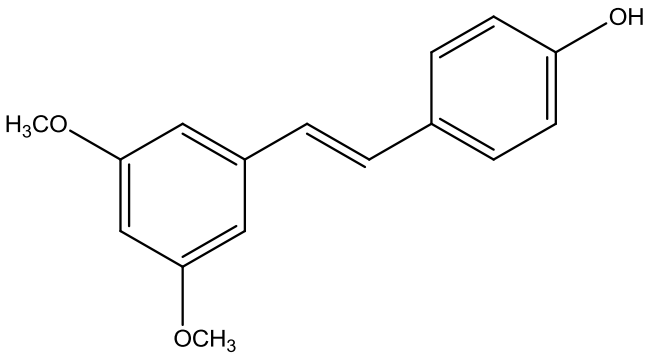


Figure 5 Pterostilbene structure

The metabolism of rhaponticin, compound found in *Rheum spp*., was studied by Zhao et al. (2012) in rats. The results showed that rhaponticin was rapidly distributed and eliminated from rat plasma. The absolute oral bioavailability of rhaponticin was calculated to be 0.03%. The plasma concentrations of rhapontigenin (Figure 6), a main metabolite of rhaponticin, rapidly increased and gradually eliminated after intravenous administration.

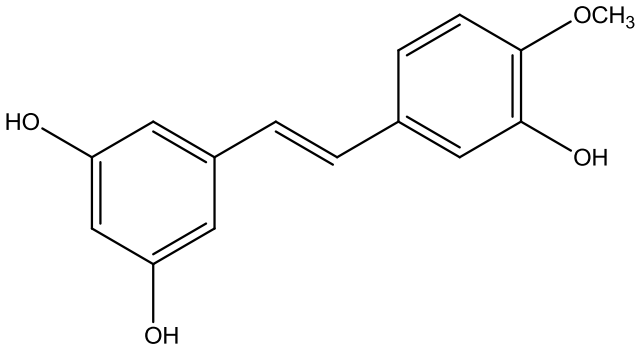


Figure 6 Rhapontigenin structure

In conclusion, the poor solubility, low bioavailability, limited stability, high rate of metabolic breakdown, and low target specificity have been considered as major obstacles to the use of stilbenes in major pharmacological applications (Reinisalo et al. 2015). Analysis of recent literature reveals an increasing number of formulations under study, which reflects the major interest in developing pharmaceutical forms able to improve stilbenes bioavailability as a step towards

applying its therapeutic potential *in vivo*. However, several research lines are currently underway to improve these properties.

2.3.3 Biological activity

Stilbenes comprise a class of plant polyphenols that have gained intense interest for their intricate structures and diverse biological activities (Silva et al. 2014). Historically, these compounds have been studied for antioxidant, antibacterial, antiviral and anticancer activities. However, recent efforts have been made to explore other potential uses such as therapeutic or preventive agents against non-communicable diseases, for example, diabetes mellitus, cardiovascular illnesses, and Parkinson's disease (Chatsumpun et al. 2016). According to Chaher et al. (2014) stilbenes could be of therapeutic value to prevent Alzheimer's disease. Natural hydroxystilbene oxyresveratrol is an antioxidant, anthelmintic, tyrosinase inhibitor and a cyclooxygenase inhibitor. Various studies have indicated that inhibits apoptotic cell death in transient cerebral ischemia, is hepatoprotective and is a potent free radical scavenger. It has been demonstrated to have an inhibitory effect on the herpes simplex and varicella zoster virus. In addition, the compound has been revealed to have neuroprotective effects (Joung et al. 2015). Piceatannol is getting more extensive attention because of its utilization to age-related diseases, such as anti-inflammatory, anticarcinogenic, antiviral, antioxidative, neuroprotective and estrogenic properties. It has been pronounced to be a stronger antioxidant than resveratrol (Piotrowska et al. 2012). Some recent studies have proved that isorhapontigenin also possesses antioxidative activity with the evidence of inhibiting oxidation of human low-density lipoprotein and other pro-oxidant system *in vitro* (Lu et al. 2015). Pterostilbene and rhapontigenin have phenolic constituents that provide antioxidative, antifungal, anti-inflammatory, anti-mutagenic and anticancer effects (Kim et al. 2013; Ishak et al. 2016).

2.3.4 Antimicrobial activity

Compared to the foregoing paragraph, the antimicrobial properties of stilbenes have been less investigated. But plant phenolic antioxidants have been recognized as

antimicrobial agents that protect against bacterial pathogens, exhibiting significant activity against Gram-positive bacteria (Kim et al. 2013). In grapevines, stilbenes are constitutively accumulated at high concentrations in the heartwood where they act as phytoanticipins and can prevent the development of wood decay. In other tissues, they are accumulated in response to various microorganisms including pathogens: *Aspergilli*, *Botrytis cinerea*, *Cladosporium cucumerinum*, *Erysiphe necator*, *Fusarium solani*, *Phaeomoniella chlamydospora*, *Plasmopara viticola*, *Pyricularia oryzae*, *Rhizopus stolonifer*. Still resveratrol it has both antifungal and antibacterial properties, its practical use as an antimicrobial compound has been suggested (Plumed-Ferrer et al. 2013). Previous studies have shown that resveratrol is not the most active stilbene regarding antimicrobial activity, but it is the precursor of more active derivatives such as pterostilbene and viniferins (Chalal et al. 2014). Rhapontigenin, an aglycone of rhapontin, showed its antifungal activity against *Candida albicans*. Another stilbene compound pinosylvin present particularly in the heartwood and knotwood of trees of the *Pinus* family in the study of Valimaa et al. (2007) was active against both Gram-negative bacteria (*Salmonella*, *Escherichia coli*, *Pseudomonas fluorescens*) and Gram-positives like *Bacillus cereus* and *Listeria monocytogene*.

2.3.5 Anti-staphylococcal activity

In previous studies the *in vitro* growth-inhibitory effect of (*trans*)-3-hydroxy-5-methoxystilbene, oxyresveratrol, pterostilbene and resveratrol against *S. aureus* and *S. epidermis* have been described. The best results showed pterostilbene and (*trans*)-3-hydroxy-5-methoxystilbene, oxyresveratrol exhibited moderate activity and the weakest activity proved resveratrol (Kabir et al. 2008; Moran et al. 2014; Ishak et al. 2016; Joung et al. 2016). Some reports also showed that stilbene derivatives exhibited inhibitory activities against MRSA comparable with that of vancomycin (Peng et al. 2008). Previous research demonstrated an antimicrobial activity of *trans*-ε-viniferin isolated from *Vitis amurensis* against *S. mutans* and *S. sanguis*, establishing stilbenoid dimer as an active antimicrobial agent against some Gram-positive bacteria (Basri et al. 2014). However, the information of antimicrobial capacity of stilbenes against *S. aureus* remains limited.

2.3.6 Structure-bioactivity relationship

In general, the main problem regarding the use of polyphenols is the partial knowledge of their mechanisms of action and their low bioavailability, which as mentioned before is determined by their chemical structure. It is broadly recognized that the biological activity of a plant-derived compound depends on its chemical structure and that the biological properties of phenolics are closely related to the types of structural terminal groups, as well as to the number and locations of hydroxyls (Xie et al. 2015). These features regulate both absorption and excretion of phenolic compounds. As an example, 0.3 % of the intake of anthocyanins is excreted by urine, compared to 43 % of isoflavones, thus reflecting the importance of the chemical structure. There are a high number of reports in which the structure-activity relationships of polyphenols are studied. These studies intend to figure out, using structural analogs, which modifications may confer increased resistance to oxidation of the polyphenols, improving the interaction with domains of the target proteins and finally increasing the pharmacokinetics properties. Theoretically, part of these changes may help to direct certain polyphenols to target tissues. The main changes in structural analogs affect the number and position of hydroxylated and methylated groups, which also influence their metabolism. In fact, polyphenols metabolized to their secondary metabolites may even have more activity. Structure-activity studies have revealed that increasing the number of OH groups at their ortho position on the phenol ring of stilbenes could increase the free radical scavenging capacity, the cytotoxic activity, and the anti-inflammatory effects of these compounds. In the case of resveratrol, it has been reported that the introduction of methoxy substitution in the place of hydroxyl groups improved the compound's anti-proliferative effect by apoptosis induction and cell cycle inhibition. The more methoxy groups added, the better the anti-tumor activity of the compound becomes (Chen et al. 2013). Another research showed that structural modifications of the resveratrol increase its bioavailability, while preserving its beneficial properties in control of atherosclerosis and heart disease (Ferrer et al. 2005). In fact, polyhydroxylated analogs of resveratrol as hexahydroxystilbene turned out to be more potent and specific inhibitors of cyclooxygenases-2 activity than resveratrol both *in vivo* and *in vitro* (Murias et al. 2004). Moreover, this analog, which shows higher antiradical activity, also induces

apoptosis at concentrations than the parent compound. Whereas resveratrol generally shows a moderate antimicrobial activity, its analogues were proved to possess higher growth-inhibitory potency (Chalal et al. 2014). Additionally, comparing the effects of resveratrol to its reduced form, dihydroresveratrol, it seems that the chemical nature of the bonds between the phenolic moieties also influences the molecule's biological effects (Faragher et al. 2011). Pinosylvin differs from resveratrol in lacking one hydroxyl, which makes it more lipophilic but losing its antioxidant activity. Nevertheless, once inside the cell, it recovers the antioxidant activity. The methoxylated analogs have higher lipophilicity, which may favor their entry into cells and confer more resistance to degradation, thus improving pharmacokinetics. However, the number of methoxy and hydroxyl groups must be under equilibrium. The hydroxyl groups confer more solubility, which allows a better interaction with proteins, whereas the methoxylated group confer resistance to degradation although an excessive number of methoxylated groups may impair the interaction with the target protein (Sirerol et al. 2016). Despite several reports on anti-staphylococcal activity of stilbenes (Kabir et al. 2008; Peng et al. 2008; Basri et al. 2014; Moran et al. 2014; Ishak et al. 2016; Joung et al. 2016), there is lack of studies on the role of the functional groups at certain positions of resveratrol related structures in *S. aureus* growth-inhibitory effect.

2.4 Encapsulation of plant-derived compounds

Low aqueous solubility and rate of dissolution are two important factors encountered with formulation and development process of drugs and limit their therapeutic application. The administration of drugs through different routes especially those of which are poorly soluble represents a major challenge. Several techniques can enhance drug’s solubility, bioavailability, and dissolution properties such as solubilization, cosolvency, and solid dispersion; however, these methods suffer from various disadvantages such as low drug loading and its large dose. In an attempt to overcome these hurdles, many formulations based on microencapsulation, CDs, liposomes and nanoparticles have been described (Uekama et al. 1998). Encapsulation complexation came into existence as an option and presented a great interest (Duarte et al. 2015). Encapsulation is a technique in which one or more

ingredients are trapped within some form of matrix. This matrix may be solid or liquid, homogenous or heterogenous, and microscopic or macroscopic. The ingredient to be entrapped is usually referred to as the "active" or "core" material, whereas the material that forms the matrix is usually referred to as the "wall", "encapsulant", "shell", or "carrier" material (Madene et al. 2006). Particles with diameters from around 1 to 1000 μm can be referred to as microcapsules, while those with diameters from around 10 to 1000 nm as nanocapsules. The choice of an appropriate encapsulation technology, carrier material, wall material, and capsule properties is critical to developing a successful commercial application. The criteria for choosing the carrier and wall material are mainly based on their physicochemical properties such as solubility, molecular weight, glass transition temperature, diffusivity, film formation capacity, and emulsifier characteristics. Further, overall cost-in-use and health security must be taken into account. There are several encapsulation technologies such as emulsion-based systems, liposome/niosomes-based systems and molecular inclusion complexes (Davidov-Pardo & McClements 2014).

Molecular inclusion is a process in which a "guest" molecule is trapped within a "host" molecule due to physical forces (Marques 2010). A number of factors influence the efficacy of the inclusion process: the geometric compatibility, structure, charge and polarity of the host and guest molecules; solvent characteristics; and environmental conditions, such as temperature. Some of the potential advantages of using inclusion complexes to encapsulate bioactive compounds are: enhancement of water solubility; stabilization against oxidation or UV-light exposure; and controlled release (Del Valle 2004). Based on the number of published articles and patents, CDs are the most common host molecules used to form inclusion complexes. They are chemically and physically stable macromolecules produced by enzymatic degradation of starch. They are water-soluble, biocompatible in nature with hydrophilic outer surface and lipophilic cavity. They have the shape of truncated cone or torus rather than perfect cylinder because of the chair conformation of glucopyranose unit. CDs are classified as natural and derived CDs. Natural CDs comprise three well-known industrially produced (major and minor) cyclic

oligosaccharides (Thorsteinn & Brewster 1996). The most common natural CDs are α, β, and γ consisting of 6, 7, and 8 glucopyranose units, respectively (Figure 7).

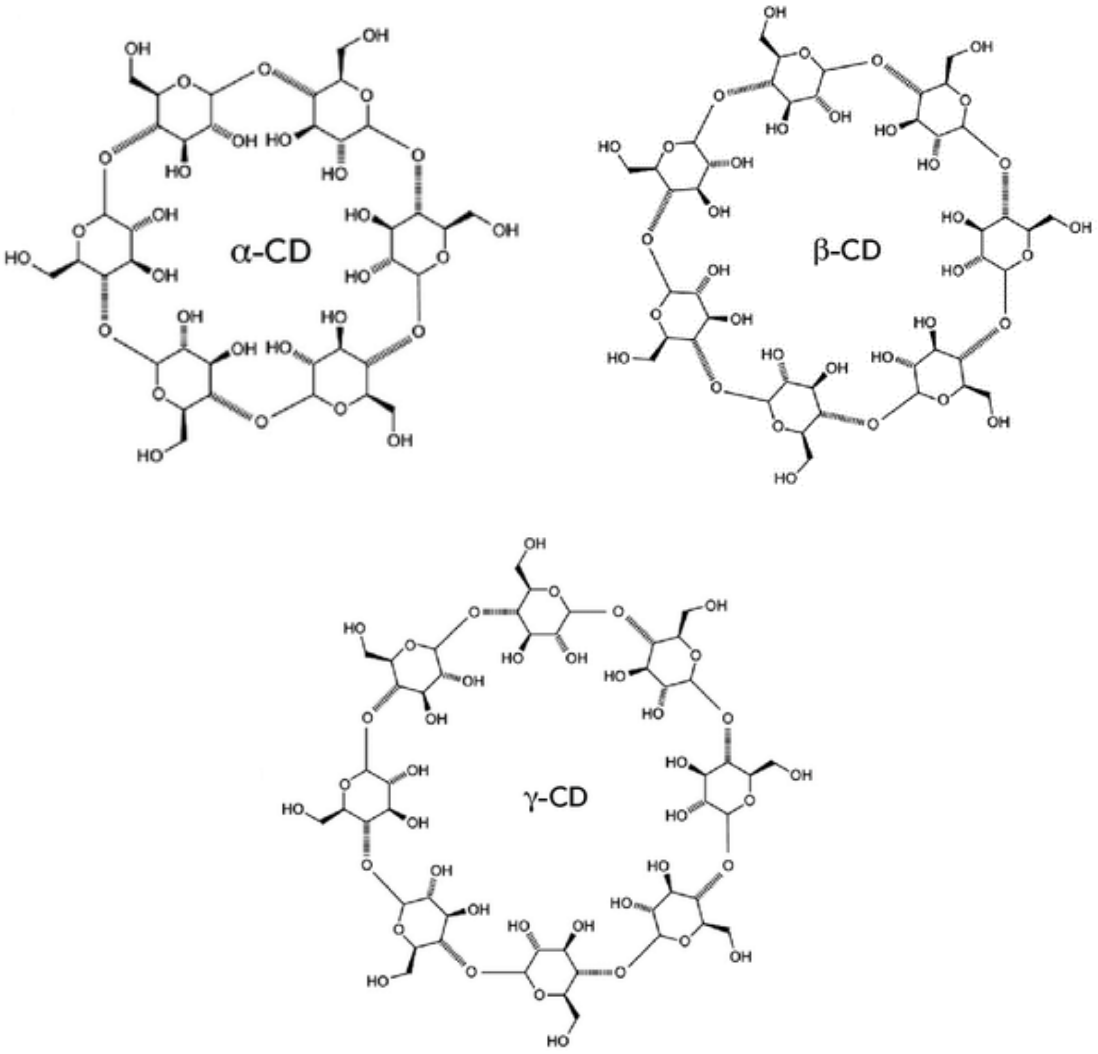


Figure 7 Chemical structures of α-, β-, γ-CDs

These three CDs are generally recognized as safe (GRAS) by the United States Food Drug Administration. They are crystalline, homogeneous, and nonhygroscopic substances. Amongst these, β-CD is ideal for complexation due to perfect cavity size, efficient drug complexation and loading, availability, and relatively low price (Karande & Mitragotri 2009). Various hydrophilic, hydrophobic, and ionic derivatives have been developed and utilized to improve the

physicochemical and biopharmaceutical properties of drug and inclusion capacity of natural CDs. Hydroxypropyl-β-CD (HP-β-CD), randomly methylated-β-cyclodextrin, and sulfobutylether-β-cyclodextrin are mostly preferred for complexation (García-Rodriguez et al. 2001; Donnelly & De Pauw 2004). Polymerized CDs are high molecular weight compounds, either water soluble or insoluble. They offer the advantage of amorphous state and complexation without toxic effects. The examples of polymerized CDs are soluble anionic β-CD polymer, soluble γ-CD polymer, and epichlorohydrin β-CD polymer (Gidwani & Vyas 2014). Due to superior solubilizing and complexing abilities exhibited these are nowadays most preferred for complexation. Inclusion complexes are formed when the "guest" molecule usually a drug is partially or fully included inside the "host's cavity". Owing to the hydrophobic cavity, CDs as host offer the guest a suitable environment for interaction. The outer sphere of CDs is compatible with water, which allows hydrogen bonding cohesive interactions. Due to this feature, CDs form inclusion complexes with a wide variety of hydrophobic compounds and change the physicochemical and biological properties of guest molecules (Cortes et al. 2001; Del Valle 2004). These changes may enhance the therapeutic potential of drugs by diminishing their decomposition before they enter tissues and by altering how they enter tissue. The ability of CDs to form an inclusion complex is a function of steric as well as thermodynamic factors. The driving force for complexation involves the removal of water molecule from hydrophobic cavity and formation of Vander Waal forces, hydrophobic, and hydrogen bond interactions. This hydrophobic cavity forms inclusion complexes with a wide range of organic and inorganic guest molecules, altering their physicochemical behavior and reducing their undesirable effects. In the pharmaceutical, cosmetics and food industries, CDs have been used as complexing agents to increase the water solubility of various compounds, such as drugs, vitamins and food colorants (Lucas-Abellan et al. 2008).

2.4.1 β-Cyclodextrin (β-CD)

β-CD has been generally used in the initial stages of pharmaceutical applications due to easily accessible and suitable cavity size for a wide range of drugs. The cavity size of β-CD is more suitable than other CDs to encapsulate a wide range of molecules.

The use of β-CD on drug solubility, bioavailability, safety, stability, and as a carrier in drug formulation may be attained by the formation of inclusion complexes with drug molecules; in fact, the use of β-CD already has a long history in pharmacy. β-CD consists of seven glucopyranose residues and is only moderately soluble in water because of intermolecular hydrogen bonding. It has a hydrophilic outer surface and a lipophilic central cavity to accommodate a variety of lipophilic drugs, resulting in increased solubility of the incorporated drug, enhanced permeation for macromolecular drugs and inhibition of certain protease activities (Vikas et al. 2018). Stilbene compound resveratrol displays a hydrophobic behavior, and is also extremely affected by exposure to oxygen, light, and oxidative enzymes, reducing its bioactivity. The use of CDs to protect resveratrol and to increase its solubility, stability and bioactivity was applied in several studies (Pinho et al. 2014). Molecular modeling of the inclusion of resveratrol in β-CD, suggested that resveratrol and β-CD form an axial inclusion complex, and that part of the A-ring and the B-ring of resveratrol are placed in the cavity of β-CD while the hydroxyl groups are projected outside (Lu et al. 2009) as show in Figure 8. Different methods have been used to determine the complexation constants of resveratrol and CDs. For example, measurements of the retention time using HPLC can be used to determine the complexation constant. Typically, resveratrol and CDs form 1:1 molar complexes, meaning that one molecule of resveratrol is included in one molecule of CD. It has been reported that the water solubility of resveratrol can be increased from around two times (Bertacche et al. 2006) to around 100 times (Davidov-Pardo & McClements 2014) using β-CD. Among the GRAS CDs, β-CD is most effective at complexing resveratrol (Lucas-Abellan et al. 2008). It also happens to be the most commonly used CD in the food industry due to its wide availability and relatively low cost (Davidov-Pardo & McClements 2014).

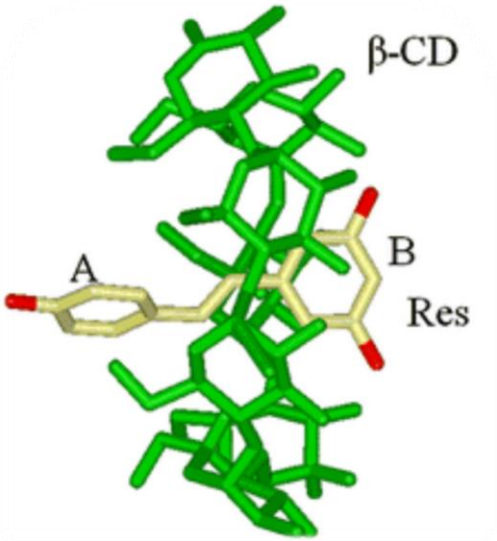


Figure 8 Optimized structure of resveratrol and β-CD (Figure reproduced from Lu et al. 2009)

2.4.2 Pharmaceutical and food applications

Given the fact that these oligosaccharides possess negligible toxicity and are pharmacologically inactive excipients for both pharmaceutical and food industry, where they are used as food additives E459 (Duarte et al. 2015). CDs are used in food formulations for flavor protection throughout many rigorous food-processing methods of freezing, thawing, microwaving, and are used for flavor preservation to a greater extent and longer period (Vyas et al. 2008). Also they have been used for the removal of cholesterol from animal products such as eggs and dairy products, removal of bitter components from citrus fruit juices, removal of phenolic compounds, which cause undesirable enzymatic browning, and enhancement of flavor in various alcoholic beverages (e.g. whisky and beer). Aqueous solubility and bitter taste of flavonoids and terpenoids, the plant components which are rich of antioxidant and antimicrobial properties, can be improved by CD complexation (Cheirsilp & Rakmai 2017). They are currently used also in the development of controlled release active packaging systems (Duarte et al. 2015). CDs have the capabilities to encapsulate the guest molecule into its cavity and improve the solubility, stability, and release profile of the drugs (e.g. thermal and chemical stability, photostability, reduce odors and tastes). Mechanism involved in the

transportation of drug-CD complex might be through extraction of lipophilic components from the membrane or by disruption of the cell membrane. Many of the synthetic drugs like pilocarpine, cetirizine, dipivefrine with complexation of HP-β-CD, α-CD, β-CD, γ-CD, have shown decreased irritation improved stability and bioavailability (Loftsson & Masson 2001; Suvarna et al. 2017). The addition of α- or β- CD increases the water solubility of several poorly water-soluble substances to improve bioavailability and increase the pharmacological effect allowing a reduction in the dose of the drug administered. CDs also have been used successfully in aqueous dermal formulations, nasal drug delivery systems and several eye drop solutions. Furthermore, they can be applied to reduce the effects of bitter or irritant tasting and bad smelling drugs as well as for controlled release of drugs such as ciprofloxacin, triclosan, vancomycin and chlorhexidine digluconate. The low water soluble anticancer drug candidates were also encapsulated in β-CD to enhance water solubility (Cheirsilp & Rakmai 2017). Encapsulation of drugs with CDs for ophthalmic delivery enhances the permeability across the biological membrane of cornea, which actually helps in masking the irritation caused by other additives of formulation. In 1976, the first time a formulation of CD and prostaglandin (Prostarmon-E™ sublingual tablets) was marketed by Japan. In 1977, Piroxicam/β-CD (Brexin® tablets) pharmaceutical product was to be the first formulation which was marketed in Europe and itraconazole/2-hydroxypropyl-β-CD oral solution (Sporanox®) was the first US-approved product (Vikas et al. 2018).

2.4.3 Encapsulation of biologically active natural compounds

An extensive amount of research reports have been published regarding the use of CDs for complexation of phytochemicals, which can be further formulated according to advanced drug delivery technologies. The phytomolecules with solubility enhancement investigations reported in literature range from various flavonoids, phenolic derivatives, coumestans to triterpenes. It encompasses a isoflavone like genistein; flavonoids like alpinetin, baicalein and rutin; flavonoid glycoside like diosmin; flavanolols like ampelopsin, galangin, myricetin, naringenin and quercetin; flavones like apigenin, chrysin and luteolin; flavanone like hesperidin; phenolic compound like curcumin, gallic acid, mangiferin and paeonol; sesquiterpene lactone

like artemisinin; dihydroartemisinin a semisynthetic derivative of artemisinin; a triphenolic compound like *trans*-resveratrol; pentacyclic lupane-type triterpenes like betulin and betulinic acid. All of these phytochemicals are associated with scientifically proven therapeutic potentials like antioxidant, anticancer and many others. But their therapeutic efficacy is limited due to severe solubility limitations. (Suvarna et al. 2017). Even though complexation of stilbenes with CDs and evaluation of their antimicrobial potential is not as frequent, there are some reports regarding it. For example, the use of CDs to protect resveratrol and its analogues and to increase their biological activity was applied in several studies (Pinho et al. 2014). Moringin conjugated with α-CD, a complex with an improved solubility and stability in aqueous solutions, was able to exert antimicrobial activity against the *S. aureus* reference strains (Romeo et al. 2018). In study of Silva et al. (2014) revealed that pterostilbene and pinosylvin and their inclusion complexes with modified CDs (HP-β-CD and HP-γ-CD) had antimicrobial activity against *Campylobacter jejuni* and *C. coli* reference strains and clinical isolates. Another research with aim to increase stilbenes (resveratrol, pterostilbene and pinosylvin) aqueous solubility and stability using hydropropyl-CDs was conducted by Silva et al. (2014). Photostability studies revealed that CDs were able to increase stilbene photostability at 4 $^{\circ}$C (Silva et al. 2014). Increasing resveratrol aqueous solubility using methylated-β-CD and its antimicrobial activity against *Campylobacter spp*. was assessed and its antioxidant activity was also evaluated in research of Duarte et al. (2015).

3 HYPOTHESIS

Natural compounds, e.g. resveratrol, have been reported as biologically active. Among number of such compounds previously tested for their biological activities, some stilbenes have been reported to possess antimicrobial effect but screening for their growth-inhibitory potential against human pathogens remains limited. It is also known, that the presence and position of substituent groups in the molecules are important factors for their antibacterial potency. Therefore, we suppose that systematic evaluation of antibacterial effect of various stilbene structures will lead to identification of the relationship between their chemical structure and effectiveness against human bacterial pathogens. However, stilbenes utilization is limited due to their poor water solubility. One of the methods in pharmaceutical industry to overcome it is the use of CDs. On that account, one can expect that CD encapsulation with resveratrol will increase its antibacterial activity.

4 OBJECTIVES

The objective of this study is to investigate *in vitro* antimicrobial activity of natural plant stilbenes against strains of *S. aureus*.

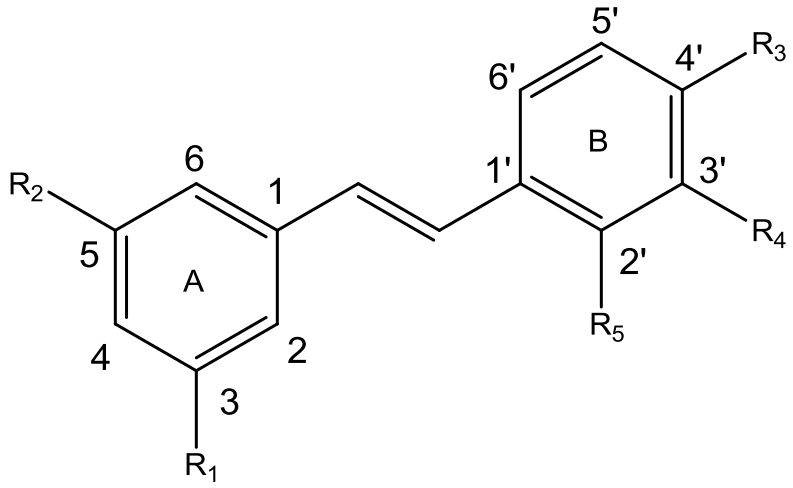
The specific aims of the study are as follow:

1. Determination of Minimum inhibitory concentration (MIC) values of stilbenes using broth microdilution method.
2. To identify relationship between structure of stilbene molecules and their antibacterial effect.
3. Encapsulation of resveratrol with CD and assessment of its anti-staphylococcal activity.

5 MATERIALS AND METHODS

5.1 Tested compounds and other chemicals

Isorhapontigenin (purity > 95 %), piceatannol (purity > 98 %), pinostilbene (purity > 97 %), pterostilbene (purity > 98 %), resveratrol (purity > 98 %), and rhapontigenin (purity > 98 %) were purchased from TCI EUROPE N.V. (Zwijndrecht, BE), oxyresveratrol (purity ≥ 97 %) and β-Cyclodextrin (β-CD) (purity ≥ 97%) were obtained from Sigma-Aldrich (Prague, CZ). 3´-hydroxypterostilbene (purity 97 %) was received as a gift sample from the Sabinsa Corporation- NJ (New Jersey, USA). Chemical structures of stilbenes tested are shown in Figure 9. The dimethyl sulfoxide (Lach-ner, Neratovice, CZ) has been used as solvent for stilbenes and β-CD-RSV complex, whereas oxacillin (purity ≥ 81.5 %) and thiazolyl blue tetrazolium bromide (MTT) (purity 98 %) (Sigma-Aldrich, Prague, CZ) were dissolved in deionized water. The potency of the powder was incorporated in the formula for preparation of stock solutions according to EUCAST (2003).



Name	R1	R2	R3	R4	R5
3´-hydroxypterostilbene	OCH_3	OCH_3	OH	OH	H
Isorhapontigenin	OH	OH	OH	OCH_3	H
Oxyresveratrol	OH	OH	OH	H	OH
Piceatannol	OH	OH	OH	OH	H
Pinostilbene	OH	OCH_3	OH	H	H
Pterostilbene	OCH_3	OCH_3	OH	H	H
Resveratrol	OH	OH	OH	H	H
Rhapontigenin	OH	OH	OCH_3	OH	H

Figure 9 Chemical structures of stilbenes tested

5.2 Microbial strains and culture medium

The antimicrobial activity was evaluated against six American Type Culture Collection (ATCC) strains of *S. aureus* and two clinical isolates of *S. aureus* listed in Table 2.

Table 2 *S. aureus* strains tested

S. aureus	
Standard strains	Purchased from
ATCC 25923 ATCC 29213 ATCC 43300 ATCC 33591 ATCC 33592 ATCC BAA 976	(Oxoid, Basingstoke, UK)
Clinical isolates	Obtained from
KI1 KI2	(The Motol University Hospital, Prague, CZ)

ATCC: American type culture collection, KI: clinical isolates

Mueller–Hinton broth (Oxoid, Basingstoke, UK) was used as the cultivation medium for *S. aureus*. The identification of clinical isolates was performed by Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry as it is described in Rondevaldova et al. (2017). The subcultures of all microorganisms were maintained at 4 °C until use.

5.3 Inoculum preparation

Overnight cultures of each *S. aureus* strain were directly suspended in 10 ml of Mueller–Hinton broth. The turbidity of bacterial suspension was adjusted to 0.5 McFarland standard (which represents 1.5 x 10^8 bacteria/ml) using Densi-La-Meter II (Lachema, Brno, CZ) as a spectrophotometric device for inoculum standardization (McFarland 1907).

5.4 Preparation of resveratrol complex with β-Cyclodextrin (β-CD-RSV)

β-CD-RSV complex was prepared using a coprecipitation method described by Bhandari et al. (1998) with minor modifications. β-CD (500 mg) was dissolved in 15 ml of distilled water at 60 °C. After cooling the β-CD solution to 40 °C, resveratrol in ethanol (1:1, v/v) was slowly added to the solution with continuous agitation, to give a final molar ratio of resveratrol/β-CD of 1 (100.25 mg of resveratrol and 500 mg of β-CD). The vessel was sealed and stirred for 3 h, after which the resulting slurry was refrigerated overnight at 4 °C. The cold precipitate was recovered by vacuum-filtration and washed with 1.5 ml distilled water and 1.5 ml of ethanol and then dried in a vacuum oven at 75 °C for 24 h. The final dry complex powder was stored in an airtight glass desiccator at room temperature. Using this approach, the final inclusion complex of β-CD-RSV was prepared.

5.5 HPLC analysis

To examine the effectiveness of the CD complex formation, the content of resveratrol in the complex was analyzed by high-performance liquid chromatography using the liquid chromatograph LC5000 (INGOS, Prague, CZ) equipped with UV-vis detector and RP-18e (5 μm) column (Merck KGaA, Darmstadt, DE). The column temperature was set to 24 °C, sample volume of 20 μl was injected and 25 % acetonitrile in water (adjusted to pH 3 using orthophosphoric acid) was used as a mobile phase using constant flow of 1.5 ml/min and isocratic elution. The measurement was carried out at the wavelength of 306 nm. The stock solution of resveratrol was prepared in methanol and appropriately diluted to obtain a series of

standard solutions of concentrations (10, 100, 250, 500 µg/ml) to create an external calibration curve to enable the quantitative determination of resveratrol in the complex. Data were collected and processed in Chromulan data station (PiKRON, Prague, CZ) and Excel (Microsoft Corporation, Redmont, USA). The analysis was performed at the Department of Quality of Agriculture Products. Quantification was evaluated using linear calibration curve (Figure 10).

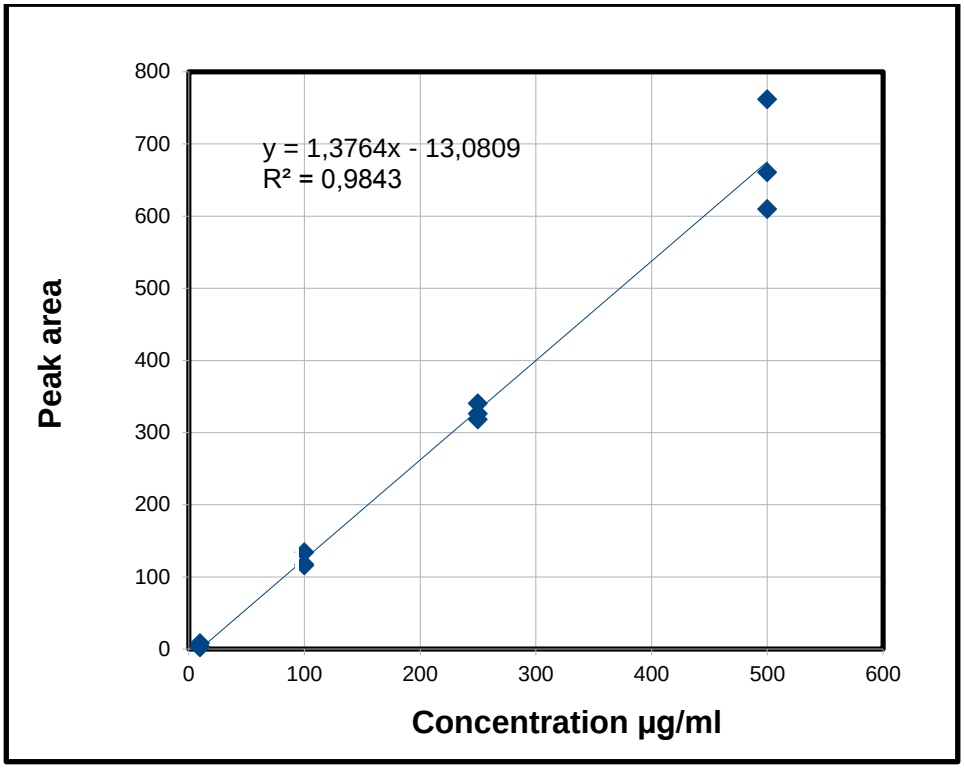


Figure 10 Calibration curve for resveratrol quantification

5.6 Antibacterial assay

The *in vitro* anti-staphylococcal activity of selected stilbenes was determined by the broth microdilution method using 96-well microtiter plates following the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2009), slightly modified according to the recommendations previously proposed for effective assessment of the anti-infective potential of natural products (Cos et al. 2006). Samples were two-fold diluted in a range of 0.5–512 µg/ml and inoculated with bacterial suspension with concentration 5×10^5 CFU/ml, the range of antibiotics concentrations depended on the strain sensitivity to the drug. Microtiter plates were incubated

at 37 °C for 24 h, and bacterial growth was then measured spectrophotometrically as turbidity using a Multimode Reader Cytation 3 (BioTek Instruments, Winooski, USA) at 405 nm. The MICs were expressed as the lowest concentrations which showed at least ≥ 80 % reduction of micro-organisms' growth compared to that of the compound-free growth control. In order to confirm optical density measurement results MTT was added to each well and afterwards the viability of bacteria was checked visually. The results corresponding to both spectrophotometric measurement and MTT assay were used for calculation of MICs values. The assay was performed as three independent experiments each carried out in triplicate and median/ modal values were used for final MICs determination. Oxacillin was used as the positive antibiotic reference control.

6 RESULTS AND DISCUSSION

6.1 Anti-staphylococcal effect of stilbenes

In this study, all plant-derived stilbenes (chemical structures shown in Figure 8) exhibited certain degree of *in vitro* growth-inhibitory activity against at least two out of the eight of tested *S. aureus* strains (Table 3). With the exception of one standard strain and one clinical isolate sensitive to pterostilbene at MIC 64 μg/ml, this compound possessed the strongest anti-staphylococcal effect against all strains with MIC 32 μg/ml. This result corresponds to the findings previously published by Ishak et al. (2016) who described MIC 31.25 μg/ml for two standard *S. aureus* strains. Moreover, in our research, pterostilbene exerted stronger anti-staphylococcal effect than oxacillin indicated by 2-fold lower MICs for two standard strains ATCC 33591 and ATCC 33592. Similarly, piceatannol inhibited growth of all strains at MIC 64 μg/ml except one clinical isolate (MIC 256 μg/ml). Its potency against most drug-resistant standard strains ATCC 33591 and ATCC 33592 was even stronger (64 μg/ml) or equal (64 μg/ml) to the MIC values of test reference antibiotic, respectively. Pinostilbene showed moderate activity with the same MIC value of 128 μg/ml for all tested strains, in addition to these results its MIC against standard strain ATCC 33591 was the same as for oxacillin. MICs of 3′-hydroxypterostilbene, isorhapontigenin and rhapontigenin ranged from 128 to 256 μg/ml. Besides that 3′-hydroxypterostilbene was able to inhibit growth of standard strain ATCC 33591 by same MICs (128 μg/ml) as the test reference antibiotic. Oxyresveratrol was active against all tested strains with MIC 256 μg/ml. Our findings on oxyresveratrol and rhapontigenin are in accordance with previous studies reporting their moderate anti-staphylococcal activity against standard strains (Joung et al. 2016; Kim et al. 2010). Resveratrol exhibited inhibitory effect (MIC 256 μg/ml) only against two standard strains, which is consistent with previous reports showing that resveratrol itself has antibacterial effect at high concentrations (Su et al. 2014). Despite the fact that the anti-staphylococcal activity of pterostilbene, resveratrol, oxyresveratrol, and rhapontigenin has previously been described, our study brings new data on growth-inhibitory effect of 3′-hydroxypterostilbene, isorhapontigenin, piceatannol and pinostilbene against *S. aureus*.

Our findings suggest stilbenes as anti-staphylococcal agents; their side effects are also thought to be less severe than those of traditional antibiotics (Oyama et al. 2016). Nevertheless, as cited in the introduction *in vivo* effectiveness of stilbenes is affected by their limited bioavailability due to rapid metabolism and excretion (Wilson et al. 2008) and their use in practice is determined by their toxicological and technological properties. Regardless there are several studies showing negligible toxicity of stilbenes that are abundant in many commonly consumed foods and beverages such as berries, grapes, red wine and peanuts (Shi et al. 2012; McCormack and McFadden 2012).

Table 3 *In vitro* growth-inhibitory effect of stilbenes against *Staphylococcus aureus*

Compound	Strain tested/ MIC (µg/ml)							
	ATCC 43300	ATCC 25923	ATCC BAA 976	ATCC 29213	ATCC 33591	ATCC 33592	KI1	KI2
3′- hydroxypterostilbene	256	128	256	128	128	128	256	256
Isorhapontigenin	128	256	256	256	256	256	256	256
Oxyresveratrol	256	256	256	256	256	256	256	256
Piceatannol	64	64	64	64	64	64	64	256
Pinostilbene	128	128	128	128	128	128	128	128
Pterostilbene	32	32	32	32	64	32	32	64
Resveratrol	>512	256	>512	>512	>512	256	>512	>512
Rhapontigenin	256	256	256	256	256	128	256	256
Oxacillin*	16	0.125	8	0.125	128	64	1	16

MIC: minimum inhibitory concentration, ATCC: American type culture collection, KI: clinical isolates, * positive (antibiotic) control

6.2 Relationship between structure and anti-staphylococcal effect of stilbenes

As far as the relationship between chemical structure of stilbene compounds and their anti-staphylococcal effects is considered, our results suggest that the position and the number of hydroxyl and methoxy groups in rings A and B (respectively) are important for activity of stilbene compounds.

Among group of hydroxystilbenes (Table 4), piceatannol containing hydroxyl groups at positions B-3′ and -4′, possessed the strongest growth-inhibitory effect against *S. aureus*. In contrast, resveratrol that produced the lowest anti-staphylococcal effect has hydroxyl group on B-4′ only. Piceatannol differs from resveratrol by possessing an additional aromatic hydroxyl group, on that account these results indicate that the more hydroxyl groups stilbenes have, the stronger activity they exhibit. This is in accordance with the evidence that increased hydroxylation of phenolic compounds results in their increased toxicity to microorganisms (Evans and Cowan 2006). In addition, Murias et al. (2005) showed that tetrahydroxy stilbene analogues (e.g. piceatannol) have several thousand-fold higher antiradical activities than trihydroxistilbene resveratrol. These findings suggest that increased number of hydroxyl groups on the ring structure leads to higher biological activity. This phenomena is not corresponding with the study of Plumed-Ferrer et al. (2013) showing that resveratrol is more hydrophilic (has one hydroxyl more) than the more antimicrobial active resveratrol derivate pinosylvine, and this small chemical difference is associated with a clear decrease of the antimicrobial activity. Singh et al. (2019) reported that the increasing number of hydroxyl groups did not result in better antimicrobial activity of resveratrol versus piceatannol or oxyresveratrol. However, both tetrahydroxy stilbenes oxyresveratrol and piceatannol differ significantly in their anti-staphylococcal effects. This data suggests that not only the number of hydroxyl groups of stilbenes but also their position plays a key role in their biological effects. This is in correspondence with Cai et al. (2006), who reported that number and location of the hydroxyl groups influenced stilbenes radical scavenging activity. As all hydroxystilbenes in our study have the same number and position of hydroxyl groups in ring A, we assume that the structure of ring B plays an important role in anti-staphylococcal potential of stilbenes. This is in accordance with Tang et al. (2017) who described that free radical scavenging

activity of resveratrol analogues mainly depends on the hydroxyl group at ring B-4′ rather than position at the ring A. The role of hydroxyl group at the position B-4′ is considered to be important in stilbenes antibacterial activity (Singh et al. 2019). Experiments utilizing analogs with altered number, position or nature of the phenolic hydroxyl groups demonstrated that the hydroxyl group in the B-4′ position is required for the antioxidant activity, but acts synergistically with the A-3 and -5 hydroxyl groups (Stivala et al. 2001). This evidence is in conformity with our observation. The glycosylation of the hydroxyl group markedly reduced the activity of the stilbenes. As it can be seen from our results, compounds with only one hydroxyl group in ring B is less effective than compounds with two groups. Resveratrol with hydroxyl group at position B-4′, exhibited no or negligible anti-staphylococcal activity. On the other hand, its anti-oxidative activity is well documented, whereas Antus et al. (2014) have proved that this effect was attributed mainly to the presence of hydroxyl groups. Hydroxylation of resveratrol to piceatannol increased the antioxidant and growth-inhibitory effects of the molecule against human promyelocytic leukemia cells (Murias et al. 2005). Correspondingly, piceatannol with hydroxyl groups at ortho-position (B-3′, -4′) was more active than oxyresveratrol with hydroxyl groups at meta-position (B-2′, -4′). In ortho-substitution, two substituents occupy positions next to each other, whereas in meta-substitution the substituents occupy positions 1 and 3 (Figure 11).

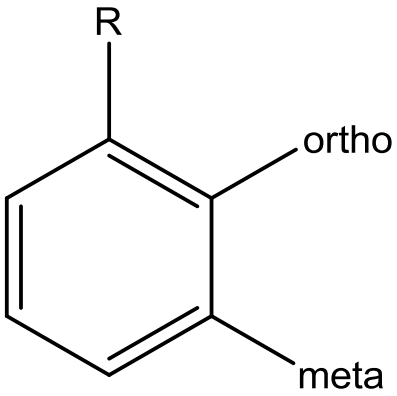
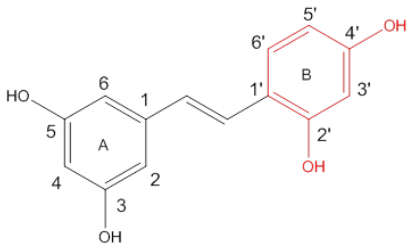
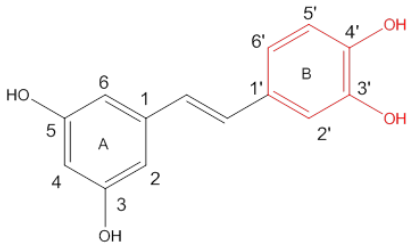
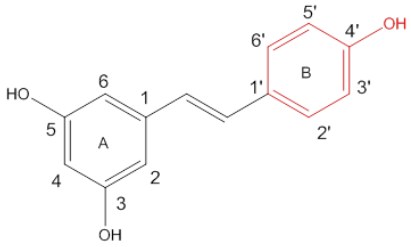


Figure 11 Ortho and meta substitution

These results indicate that ortho-dihydroxy group in stilbene structure seems to be crucial for anti-staphylococcal effect. This observation is in correspondence with previously published reports describing ortho-dihydroxy groups as the most important structural feature of high biological activity for phenolic compounds (e.g. stilbenes

scavenging radicals) (Cai et al. 2006; Murias et al. 2005). In addition, the increasing effect of antibacterial activity due to the presence of ortho-dihydroxy groups in structure of selected various classes of polyphenols such as isoflavones has also previously been proposed (Hummelova et al. 2015).

Table 4 Chemical structures of hydroxystilbenes tested and their growth-inhibitory effect against *S. aureus* strains

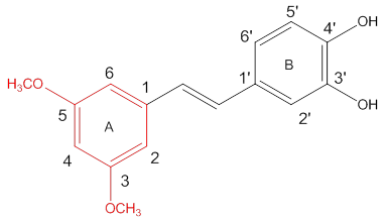
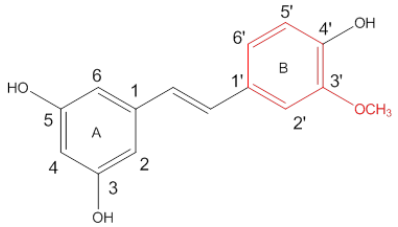
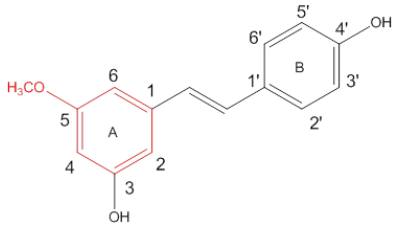
Chemical formula	Chemical Name	IUPAC Name	Anti-staphylococcal activity
	Oxyresveratrol	4-[(E)-2-(3,5-dihydroxyphenyl)ethenyl]benzene-1,3-diol	Moderate
	Piceatannol	4-[(E)-2-(3,5-dihydroxyphenyl)ethenyl]benzene-1,2-diol	Strong
	Resveratrol	5-[(E)-2-(4-hydroxyphenyl)ethenyl]benzene-1,3-diol	Weak

IUPAC: International Union of Pure and Applied Chemistry,

Pterostilbene, a dimethylated analogue of resveratrol with methoxy groups at positions A-3, -5 and hydroxyl group on B-4′, possessed the strongest anti-staphylococcal effect within all tested compounds. In general, a presence of methylated hydroxyphenyl groups in pterostilbene structure is known to increase its biological activity (Chong et al. 2009). In general, the methoxylation of resveratrol analogues significantly decreased DPPH free radical scavenging activity (Traversi et al. 2016). According to our results, compounds with methoxy groups (Table 5) at the ring A (3′-hydroxypterostilbene, pinostilbene and pterostilbene) produced stronger activity against *S. aureus* than their analogues with methoxy groups at the ring B (isorhapontigenin and rhapontigenin), which suggest the important role of ring A methylation in the anti-staphylococcal effect of stilbenes. In the study of Jeong-Keun et al. (2009), the inhibition activity of rhapontigenin was higher in Gram-positive strains than in Gram-negative strains. Rhapontigenin prepared from rhapontin by bioconversion showed higher antimicrobial activity, suggesting the glucoside structure interferes with antimicrobial activity. Several antibacterial inhibitory mechanisms of phenolic compounds are proposed, such as disruption of the cytoplasmic membrane, change in the permeability of the membrane, and inhibition of the membrane respiratory chain. Lipophilic compounds easily bind to cell membranes, changing membrane properties. Membrane damage blocks its proper function, a proposed mechanism of the antimicrobial action of phenolic compounds. Rhapontigenin disrupts cell membranes, possibly because of presence of hydroxyl and lipophilic groups in its molecule. Nevertheless, the presence of methoxy groups in the ring B has also previously been observed to enhance biological activity of resveratrol analogues (Tang et al. 2017). Considering the influence of the A ring methylation on *S. aureus* growth, pterostilbene (two methoxy groups at positions A-3, -5) produced the strongest inhibitory effect, however, 3′-hydroxypterostilbene with two methoxy groups on A-3,-5 was less active than monomethylated structure of pinostilbene with methoxy group on A-5. Based on these results, we hypothesize that the presence of methoxy group at position A-5 may be significant for the anti-staphylococcal effect of stilbenes. Structure activity relationship analysis of Chen et al. (2013) revealed that methoxy substituents at positions -3, -4, and -5 on the A ring and at position -4′ of the B ring promoted antitumor activity.

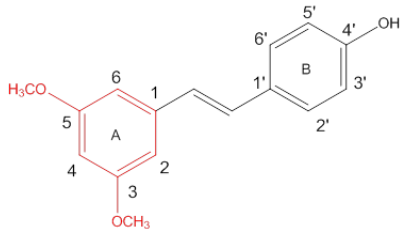
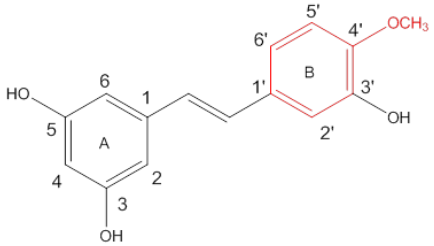
It is very well known that phenolic compounds are produced by plants for their protection against microbial infections (Jeong-Keun et al. 2009), hovewer, the reports on the relationship between the structure of such compounds and their antimicrobial activity are contradictory. Nevertheless, our results suggest that there is clear relation between stilbenes structure and their *in vitro* growth-inhibitory effect against *S. aureus*. This can be supported by several previous studies. For example, Wilson et al. (2008) reported that methoxylated stilbenes are metabolized more slowly, which may have a positive effect on *in vivo* bioactivity. Alternatively, methoxylation may protect stilbenes from metabolic modification and excretion, thereby increasing their biostability and bioavailability. In this regard, methylated structures such as pterostilbene seem to be more promising anti-staphylococcal agents than the hydroxystilbenes. In addition, the results of the structure–activity relationship analysis suggest the important role of the position and the number of hydroxyl and methoxy groups in the resveratrol analogues and denote hydroxyl groups at ortho-position (B-3′, -4′) or two methoxy groups at meta positions A-3, -5 as significant supposition for the anti-staphylococcal effect. An analysis of the structure of resveratrol has found that the substitution of the hydroxyl groups with methoxy groups significantly increases the bioavailability through increased intestinal absorption and enhanced hepatic stability (Traversi et al. 2016). This research also demonstrated that the substitution of hydroxyl groups with methoxy groups may change cellular/molecular targets thus deeply modifying the behavior of the derivatives. In the study of Singh et al. (2019) when all hydroxyl groups were methoxylated antibacterial activity diminished drastically. This shows the importance of the presence of methoxy group along with hydroxyl group in the structure for the antimicrobial activity.

Table 5 Chemical structures of methoxylated stilbenes tested and their growth-inhibitory effect against *S. aureus* strains

Chemical formula	Chemical Name	IUPAC* Name	Anti-staphylococcal activity
	3′-hydroxypterostilbene	4-[(E)-2-(3,5-dimethoxyphenyl)ethenyl]benzene-1,2-diol	Moderate
	Isorhapontigenin	5-[(E)-2-(4-hydroxy-3-methoxyphenyl)ethenyl]benzene-1,3-diol	Moderate
	Pinostilbene	3-[(E)-2-(4-hydroxyphenyl)ethenyl]-5-methoxyphenol	Strong

IUPAC: International Union of Pure and Applied Chemistry

Table 5 Continued

	Pterostilbene	4-[(E)-2-(3,5-dimethoxyphenyl)ethenyl]phenol	Strong
	Rhapontigenin	5-[(E)-2-(3-hydroxy-4-methoxyphenyl)ethenyl]benzene-1,3-diol	Moderate

IUPAC: International Union of Pure and Applied Chemistry,

6.3 Anti-staphylococcal activity of β-CD-RSV complex

With aim to increase anti-staphylococcal effect of a model stilbene resveratrol, we performed susceptibility testing of its complex with β-CD (prepared according to the method described earlier) against standard strains of *S. aureus*. In our tests (Table 6), β-CD-RSV complex showed moderate activity with the MIC value of 256 μg/ml for one standard *S. aureus* strain. Conversely, resveratrol did not exhibit any growth-inhibitory effect against none of standard *S. aureus* strains within the concentration range assayed. According to our best knowledge, this is the first report on anti-staphylococcal effect of resveratrol complex with β-CD.

Table 6 *In vitro* growth-inhibitory effect of β-CD-RSV complex against *S. aureus*

Compound	Strain tested/ MIC (μg/ml)		
	ATCC 43300	ATCC 29213	ATCC 33591
Resveratrol	>512	>512	>512
β-CD-RSV complex	>512	>512	256

MIC: minimum inhibitory concentration, ATCC: American type culture collection

According to Lu et al. (2009), the limited water solubility of resveratrol can be overcome by the formation of inclusion complexes with CDs. In the study of Amri et al. (2012), it is described that β-CD does not modify resveratrol pharmacokinetic profile, increases its solubility, improves stability and leads to significant anti-tumor response. On the other hand, Silva et al. (2015) revealed higher MIC values against bacteria *Campylobacter* for the inclusion of pterostilbene with CDs in comparison to the pure compound and they presume that it could be explained by the reduction on the antimicrobial activity of a compound by CDs. In the same study inclusion of pinosylvin with CDs were more active against the bacteria than pure compound.

Although β-CD-RSV complex produced only slight increase of growth-inhibitory effect against one strain of *S. aureus* in comparison with resveratrol, our

findings suggest that stilbene encapsulation with β-CD may increase their anti-staphylococcal potential. We can suppose that the efficacy of CDs depends on stilbene structure and bacteria. However, further studies focused on combination of other stilbene structures, especially methoxylated analogues (e.g. pterostilbene, pinostilbene, 3′-hydroxypterostilbene), with different types of CDs are recommended.

7 CONCLUSION

In this study, eight naturally occurring plant-derived hydroxystilbenes and their methoxylated analogues exhibited significant *in vitro* anti-staphylococcal effect against two clinical isolates and six standard *S. aureus* strains. Pterostilbene, piceatannol and pinostilbene produced the strongest growth-inhibitory activity against all tested *S. aureus* strains. Moreover, in several cases their potency was equal to or even stronger the test reference antibiotic oxacillin. According to the best knowledge, this is first study reporting anti-staphylococcal potential of 3′-hydroxypterostilbene, isorhapontigenin, piceatannol, and pinostilbene.

The results of subsequently performed structure–activity relationship analysis suggest the important role of the position and the number of hydroxyl and methoxy groups in the resveratrol analogues and denote hydroxyl groups at ortho-position (B-3´, -4´) or two methoxy groups at meta-positions A-3, -5 as significant assumption for the anti-staphylococcal effect. This overview is reported here for the first time.

Following an approach of increase the activity of resveratrol, encapsulation with CDs were used. β-CD-RSV complex demonstrated higher antimicrobial effect than pure resveratrol. On that account, it is possible to hypothesize that stilbene encapsulation with β-CD may increase their antibacterial potential. Moreover, this is first report focused on β-CD-RSV complex growth-inhibitory effect against standard *S. aureus* strain.

In summary, the results of our study suggest stilbenes as promising structures for development of novel anti-staphylococcal agents. However, more detailed toxicological and microbiological evaluation should be performed before their practical use can be considered. According to our opinion, the future investigation focused on encapsulation of resveratrol analogues with CDs and growth-inhibitory activity of methoxylated stilbenes against *S. aureus* may bring new promising results.

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9 APPENDICES

Appendix 1 *Curriculum vitae*

PERSONALIA

Name	**Ing. Tereza Žáková**
E-mail	zakovat@ftz.czu.cz
Nationality	Czech

EDUCATION

2012 – 2019	**Doctoral Study**
	Czech University of Life Sciences Prague
	Faculty of Tropical AgriSciences
	Study Programme: Tropical and Subtropical Agriculture
	Thesis: Anti-staphylococcal effect of plant-derived stilbenes
2010 – 2012	**Master's degree**
	Czech University of Life Sciences Prague
	Faculty of Tropical AgriSciences
	Study Programme: Tropical Crop Management and Ecology
	Thesis: Evaluation of grape-derived compounds with antimicrobial properties as alternatives to sulphites in wine
2007 – 2008	**Erasmus Programme**
	Universitat Politècnica de València
	Study Programme: Rural and Agrifood Engineering
	One year's enrolment
2006 – 2010	**Bachelor's degree**
	Czech University of Life Sciences Prague
	Faculty of Tropical AgriSciences
	Study Programme: Agriculture in Tropics and Subtropics
	Thesis: Ethnobotany of the Andes

WORK EXPERIENCE

04/2016 – 12/2017	**Occupational safety and health technician**
	Faculty of Tropical AgriSciences, Czech University of Life Sciences Prague
	Systematic evaluations of the working environment in faculty laboratories, providing information on safety risks in the workplace

ABROAD EXPERIENCES

12/2017 – 06/2018	**Erasmus Mundus Eulalinks Sense**
	University of Veracruz, Mexico
	Fellowship in Laboratory of high technology in Xalapa- LATEX
	Study area: Isolation and purification of secondary metabolites of fungal origin
08/2016	**Student mobility in Vietnam**
	Vietnam National University of Agriculture, Hanoi
	Students coordinator
08/2015	**Student mobility in India**
	Kalinga Institute of Industrial Technology, Bhubaneswar
	Students coordinator
07/2014 – 09/2014	**Erasmus+ traineeship**
	Kaunas University of Technology, Lithuania
	Fellowship in laboratory of Department of Food Science and Technology
	Study area: Antioxidant activity of *Monarda didyma*

PROJECT PARTICIPATION

2018	Chemical composition and biological activity of medicinal and edible tropical plants (IGA 20185019)
2015	Chemical composition and biological activity of extracts and plant derived compounds (IGA 20155012)
2014	Biological activity of extracts and plant derived compounds (IGA 20145021)

2013	Characterization *in vitro* and *in cellulo* testing probiotic bacteria (CIGA 20132023)
2012	Plant quinones as compounds with potential dual anti-inflammatory and antimicrobial activity (CIGA 20125009)

LANGUAGE SKILLS

Czech:	mother tongue
English:	proficient user
Spanish:	proficient user
French:	basic user

Appendix 2 List of author's publications

Publications in scientific journals with IF:

Zakova T., Rondevaldova J., Bernardos A., Landa P., Kokoska L. The relationship between structure and *in vitro* anti-staphylococcal effect of plant-derived stilbenes. Acta Microbiologica et Immunologica Hungarica 2018, 11: 1-10 (IF 1.107).

Pastorkova E., **Zakova T**., Landa P., Novakova J., Vadlejch J., Kokoska L. Growth inhibitory effect of grape phenolics against wine spoilage yeasts and acetic acid bacteria. International Journal of Food Microbiology 2013, 161: 209-213 (IF 3.451).

Scientific conference contributions:

Zakova T., Bernardos A., Rondevaldova J., Landa P., Kokoska L. *In vitro* anti-staphyloccocal effect of plant-derived stilbenes. Book of Abstracts (Abstract no. PP 244). 2nd International Conference on Natural Products Utilization: From Plants to Pharmacy Shelf ICNPU 2015, 14-17 October 2015, Plovdiv, Bulgaria.

Pastorkova E., **Zakova T**., Landa P., Novakova J., Kokoska L. Antimicrobial effect of grape derived phenolic compounds against wine spoilage microorganism. Book of Proceedings (Abstract no. P 114) 12th International Nutrition and Diagnostics Conference 27-30 August 2012, Prague, Czech Republic.

Acta Microbiologica et Immunologica Hungarica
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THE RELATIONSHIP BETWEEN STRUCTURE AND *IN VITRO* ANTISTAPHYLOCOCCAL EFFECT OF PLANT-DERIVED STILBENES

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Staphylococcus aureus is a major human pathogen that is responsible for both hospital- and community-acquired infections. Stilbenes are polyphenol compounds of plant origin known to possess a variety of pharmacological properties, such as antibacterial, antiviral, and antifungal effects. This study reports the *in vitro* growth-inhibitory potential of eight naturally occurring stilbenes against six standard strains and two clinical isolates of *S. aureus*, using a broth microdilution method, and expressing the results as minimum inhibitory concentrations (MICs). Pterostilbene (MICs = 32–128 μg/ml), piceatannol (MICs = 64–256 μg/ml), and pinostilbene (MICs = 128 μg/ml) are among the active compounds that possess the strongest activity against all microorganisms tested, followed by 3′-hydroxypterostilbene, isorhapontigenin, oxyresveratrol, and rhapontigenin with MICs 128–256 μg/ml. Resveratrol (MIC = 256 μg/ml) exhibited only weak inhibitory effect. Furthermore, structure–activity relationships were studied. Hydroxyl groups at ortho-position (B-3′ and -4′) played crucial roles for the inhibitory effect of hydroxystilbene piceatannol. Compounds with methoxy groups at ring A (3′-hydroxypterostilbene, pinostilbene, and pterostilbene) produced stronger effect against *S. aureus* than their analogues (isorhapontigenin and rhapontigenin) with methoxy groups at ring B. These findings provide arguments for further investigation of stilbenes as prospective leading structures for development of novel antistaphylococcal agents for topical treatment of skin infections.

Keywords: antimicrobial activity, natural antibacterial agents, minimum inhibitory concentration, *Staphylococcus aureus*, structure–activity relationships

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Introduction

Staphylococcus aureus colonizes the normal human microflora usually found on the skin and mucosa [1], and is a remarkably diverse bacterial pathogen as reflected in its capacity to cause various array of infections (e.g., pneumonia, sepsis, and skin and soft tissue infections) and food poisoning [2–4]. Antibiotics are generally applied as a conventional treatment [5]; however, *S. aureus* has acquired resistance to a majority of clinically used agents [1, 6]. Thus, it is very likely that chemotherapy of *S. aureus* infections will become more difficult in the future [5].

Discovery of new natural antibacterial agents, which include plant-derived compounds, has regained momentum in past years as an important strategy on how to overcome the complications in the anti-infectious therapy [7]. Among these natural substances, plant stilbenes have received considerable interest over the past 20 years due to their pharmacological effects and negligible toxicity verified on various *in vitro* and *in vivo* studies, as well as a few clinical trials [8, 9]. They occur naturally in various plant families, such as the *Cyperaceae*, *Dipterocarpaceae*, *Fabaceae*, *Gnetaceae*, *Moraceae*, *Polygonaceae*, and *Vitaceae*, whereas grapes and related products are considered to be the most important dietary sources of these substances [10]. In previous studies, antimicrobial effect of model stilbene resveratrol and its related structures (e.g., piceatannol, pterostilbene, trans-piceid, and trans-ε-viniferin) has been reported against various food and human pathogenic microorganisms, such as *Acetobacter aceti*, *Acetobacter oeni*, *Bacillus cereus*, *Bacillus subtilis*, *Dekkera bruxellensis*, *Escherichia coli*, *Listeria innocua*, *Listeria monocytogenes*, *Pseudomonas fluorescens*, *Pseudomonas aeruginosa*, *Streptococcus* spp., *Zygosaccharomyces bailii*, and *Zygosaccharomyces rouxii* [11–15]. The *in vitro* growth-inhibitory effect of (E)-3-hydroxy-5-methoxystilbene, oxyresveratrol, pterostilbene, and resveratrol has also previously been described against *S. aureus* and *Staphylococcus epidermidis* [15–18].

It is broadly recognized that the biological activity of a plant-derived compound depends on its chemical structure and that the antioxidant properties of phenolics are closely related to the types of structural terminal groups, as well as to the number and locations of hydroxyls [19]. In the case of resveratrol, it has been reported that the introduction of methoxy substitution in the place of hydroxyl groups improved the compound's antiproliferative effect by apoptosis induction and cell cycle inhibition. The more methoxy groups added, the better the antitumor activity of the compound becomes [20]. Another research showed that structural modifications of the resveratrol increase its bioavailability, while

preserving its beneficial properties in control of atherosclerosis and heart disease [21]. Despite the reports on antistaphylococcal activity of stilbenes, the role of the functional groups at certain positions of resveratrol-related structures in *S. aureus* growth-inhibitory effect has not been reported, to date. For these reasons, the aim of this work is to investigate the relationship between structure and *in vitro* antistaphylococcal effect of various resveratrol-related compounds.

Materials and Methods

Chemicals

Isorhapontigenin (purity >95%), piceatannol (purity >98%), pinostilbene (purity >97%), pterostilbene (purity >98%), resveratrol (purity >98%), and rhapontigenin (purity >98%) were purchased from TCI EUROPE N.V. (Zwijndrecht, Netherlands); oxyresveratrol (purity ≥97%) was obtained from Sigma-Aldrich (Prague, Czech Republic). 3′-hydroxypterostilbene (purity = 97%) was received as a gift sample from the Sabinsa Corporation (NJ, USA). The dimethyl sulfoxide (Lach-ner, Neratovice, Czech Republic) has been used as solvent for stilbenes, whereas oxacillin (purity ≥81.5%) and thiazolyl blue tetrazolium bromide (MTT) (purity = 98%) (Sigma-Aldrich) were dissolved in deionized water. The potency of the compound was incorporated in the formula for the preparation of stock solutions, according to EUCAST [22].

Bacterial strains and growth media

The antimicrobial activity was evaluated against six American Typical Culture Collection strains of *S. aureus* (25923, 29213, 43300, 33591, 33592, and BAA 976) purchased from Oxoid (Basingstoke, UK). Two clinical isolates of *S. aureus* (KI1 and KI2) were obtained from the Motol University Hospital, Prague, Czech Republic. Mueller–Hinton broth (Oxoid) was used as the cultivation medium. The identification of clinical isolates was performed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry as it is described in Rondevaldova et al. [23].

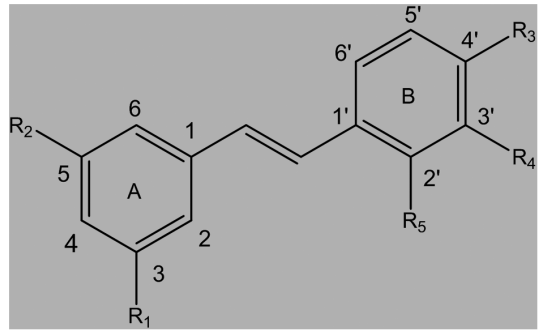
Antimicrobial assay

The *in vitro* antimicrobial activity was determined by the broth microdilution method using 96-well microtiter plates according to CLSI guidelines [24], slightly modified according to the recommendations previously proposed for

effective assessment of the anti-infective potential of natural products [25]. Samples were twofold diluted in a range of 0.5–512 μg/ml and inoculated with bacterial suspension with concentration 5×10^5 CFU/ml. Microtiter plates were incubated at 37 °C for 24 h and bacterial growth was then measured spectrophotometrically as turbidity using a Multimode Reader Cytation 3 (BioTek Instruments, Winooski, VT, USA) at 405 nm. The MICs were expressed as the lowest concentrations, which showed at least ≥80% reduction of microorganisms' growth compared to that of the compound-free growth control. In order to confirm optical density measurement results, MTT was added to each well and afterward the viability of bacteria was checked visually. The results corresponding to both spectrophotometric measurement and MTT assay were used for calculation of MIC values. The assay was performed as three independent experiments each carried out in triplicate and median/modal values, which were used for final MICs determination. Oxacillin was used as the positive antibiotic reference control.

Results

In this study, all plant-derived stilbenes (chemical structures shown in Figure 1) exhibited certain degree of *in vitro* growth-inhibitory activity against



Name	R1	R2	R3	R4	R5
3′-hydroxypterostilbene	OCH_3	OCH_3	OH	OH	H
Isorhapontigenin	OH	OH	OH	OCH_3	H
Oxyresveratrol	OH	OH	OH	H	OH
Piceatannol	OH	OH	OH	OH	H
Pinostilbene	OH	OCH_3	OH	H	H
Pterostilbene	OCH_3	OCH_3	OH	H	H
Resveratrol	OH	OH	OH	H	H
Rhapontigenin	OH	OH	OCH_3	OH	H

Figure 1. Chemical structures of stilbenes tested

Table I. *In vitro* growth-inhibitory effect of stilbenes against *S. aureus*

	Strain tested/MIC (μg/ml)							
Compound	ATCC 43300	ATCC 25923	ATCC BAA 976	ATCC 29213	ATCC 33591	ATCC 33592	KI1	KI2
3′-hydroxypterostilbene	256	128	256	128	128	128	256	256
Isorhapontigenin	128	256	256	256	256	256	256	256
Oxyresveratrol	256	256	256	256	256	256	256	256
Piceatannol	64	64	64	64	64	64	64	256
Pinostilbene	128	128	128	128	128	128	128	128
Pterostilbene	32	32	32	32	64	32	32	64
Resveratrol	>512	256	>512	>512	>512	256	>512	>512
Rhapontigenin	256	256	256	256	256	128	256	256
Oxacillin*	16	0.125	8	0.125	128	64	1	16

Note: MIC: minimum inhibitory concentration; ATCC: American type culture collection; KI: clinical isolates.
*Represents reference control.

at least two out of the eight tested *S. aureus* strains (Table I). With the exception of one standard strain and one clinical isolate sensitive to pterostilbene at MIC 64 μg/ml, this compound possessed the strongest antistaphylococcal effect against all strains with MIC 32 μg/ml. This result corresponds to the findings previously published by Ishak et al. [16] who described MIC 31.25 μg/ml for two standard *S. aureus* strains. Similarly, in our tests, piceatannol inhibited growth of all strains at MIC 64 μg/ml except one clinical isolate (MIC = 256 μg/ml). Pinostilbene showed moderate activity with the same MIC value of 128 μg/ml for all tested strains, followed by 3′-hydroxypterostilbene, isorhapontigenin, and rhapontigenin with MIC ranging from 128 to 256 μg/ml. Oxyresveratrol was active against all tested strains with MIC 256 μg/ml. Our findings on oxyresveratrol and rhapontigenin are in accordance with previous studies reporting their moderate antistaphylococcal activity against standard strains. Resveratrol exhibited inhibitory effect (MIC = 256 μg/ml) only against two standard strains, which is consistent with previous reports showing that resveratrol itself has antibacterial effect at high concentrations [26]. Despite the fact that the antistaphylococcal activity of pterostilbene, resveratrol, oxyresveratrol, and rhapontigenin has previously been described, this study brings new data on growth-inhibitory effect of 3′-hydroxypterostilbene, isorhapontigenin, piceatannol, and pinostilbene against *S. aureus*. As far as the relationship between chemical structure of stilbene compounds and their antistaphylococcal effects is considered, our results suggest that the position and the number of hydroxyl and methoxy groups in rings A and B (respectively) are important for activity of stilbene compounds. Although some results differ in one- or two-dilution range, the MICs presented in this work are the

median/modal values obtained from three independent experiments performed in triplicate and thus their values are significantly different.

Discussion

Among the group of hydroxystilbenes, piceatannol, which contained hydroxyl groups at position B-3′ and -4′, possessed the strongest growth-inhibitory effect against *S. aureus*. In contrast, resveratrol that produces the lowest antistaphylococcal effect has hydroxyl group on B-4′ only. These results indicate that the more hydroxyl groups the stilbenes have, the stronger activity they exhibit. This is in accordance with the evidence that increased hydroxylation of phenolic compounds results in their increased toxicity to microorganisms [27]. In addition, Murias et al. [28] showed that tetrahydroxy stilbene analogues (e.g., piceatannol) have several 1,000-fold higher antiradical activities than trihydroxystilbene resveratrol. These findings suggest that increased number of hydroxyl groups on the ring structure leads to higher biological activity. However, both tetrahydroxy stilbenes, oxyresveratrol and piceatannol, significantly differ in their antistaphylococcal effects. This data suggests that not only the number of hydroxyl groups of stilbenes plays a key role in their biological effects, but also their position does. This is in correspondence with Cai et al. [29], who reported that number and location of the hydroxyl groups influenced stilbenes radical scavenging activity. As all hydroxystilbenes in this study have the same number and position of hydroxyl groups in ring A, we assume that the structure of ring B plays an important role in antistaphylococcal potential of stilbenes, which is in accordance with Tang et al. [30] who described that free radical scavenging activity of resveratrol analogues mainly depends on the hydroxyl group at ring B-4′ rather than position at the ring A. As it can be observed from the results, compounds with only one hydroxyl group in ring B are less effective than the compounds with two groups. Resveratrol with hydroxyl group at position B-4′ exhibited no or negligible antistaphylococcal activity. Whereas, piceatannol with hydroxyl groups at *ortho*-position (B-3′ and -4′) was more active than oxyresveratrol with hydroxyl groups at *meta*-position (B-2′ and -4′). These results indicate that *ortho*-dihydroxy groups in stilbene structure seem to be crucial for antistaphylococcal effect. This observation is in correspondence with previously published reports describing *ortho*-dihydroxy groups as the most important structural feature of high biological activity for phenolic compounds (e.g., stilbenes scavenging radicals) [29]. In addition, the increasing effect of antibacterial activity due to the presence of *ortho*-dihydroxy groups in structure of selected various classes of polyphenols, such as isoflavones, has also previously been proposed [31].

Pterostilbene, a dimethylated analogue of resveratrol with methoxy groups at positions A-3, -5 and hydroxyl group on B-4′, possessed the strongest antistaphylococcal effect within all tested compounds. In general, a presence of methylated hydroxyphenyl groups in pterostilbene structure is known to increase its biological activity [32]. According to our results, compounds with methoxy groups at the ring A (3′-hydroxypterostilbene, pinostilbene, and pterostilbene) produced stronger activity against *S. aureus* than their analogues with methoxy groups at the ring B (isorhapontigenin and rhapontigenin), which suggest the important role of ring A methylation in the antistaphylococcal effect of stilbenes. Nevertheless, the presence of methoxy groups in the ring B has also previously been observed to enhance biological activity of resveratrol analogues [30]. Considering the influence of the ring A methylation on *S. aureus* growth, pterostilbene (two methoxy groups at positions A-3 and -5) possessed the strongest inhibitory effect; however, 3′-hydroxypterostilbene with two methoxy groups on A-3, -5 was less active than monomethylated structure of pinostilbene with methoxy group on A-5. Based on these results, we hypothesize that the presence of methoxy group at position A-5 may be significant for the antistaphylococcal effect of stilbenes.

Our findings suggest that stilbenes have potential as antistaphylococcal agents; however, their use in practice is determined by their toxicological and technological properties. There are several studies showing negligible toxicity of stilbenes that are abundant in many commonly consumed foods and beverages such as berries, grapes, red wine, and peanuts [33, 34]. Nevertheless, *in vivo* effectiveness of stilbenes is affected by their limited bioavailability due to rapid metabolism and excretion [35]. According to Wilson et al. [35], methoxylated stilbenes are metabolized more slowly, which may have a positive effect on *in vivo* bioactivity. Alternatively, methoxylation may protect stilbenes from metabolic modification and excretion, thereby increasing their biostability and bioavailability. In this regard, methylated structures such as pterostilbene seem to be more promising antistaphylococcal agents than the hydroxystilbenes. In addition, it has been observed that the topical administration facilitates bioavailability of pterostilbene in skin and plasma of hairless mice [36], which suggests this compound as a promising leading structure for the development of novel antistaphylococcal agents, especially for the treatment of staphylococcal skin infections. However, more detailed toxicological and microbiological studies should be determined before their practical use can be considered.

In summary, plant-derived stilbenes exhibited significant *in vitro* antistaphylococcal effect, specifically pterostilbene, piceatannol, and pinostilbene produced the strongest growth-inhibitory activity against all *S. aureus* strains tested. In addition, the results of the structure–activity relationship analysis suggest the

important role of the position and the number of hydroxyl and methoxy groups in the resveratrol analogues and denote hydroxyl groups at *ortho*-position (B-3′ and -4′) or two methoxy groups at positions A-3 and -5 as significant supposition for the antistaphylococcal effect.

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Conflict of Interest

The authors declare no conflict of interest.

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