CZECH UNIVERSITY OF LIFE SCIENCES PRAGUE

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Evaluation of *in vitro* synergistic antimicrobial effect of antibiotics with isoflavonoids and their metabolites

Master Thesis

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Abstract

Demethyltexasin (6,7,4'-trihydroxyisoflavone) occurs in soy-based foods, such as doenjang and tempeh but is also metabolized in the liver from isoflavone daidzein that is commonly present in legumes. Demethyltexasin was examined for its antimicrobial and combinatory effect with the oxacillin antibiotic against five strains of *Staphylococcus aureus*, including three resistant to methicillin.

The minimum inhibitory concentrations were determined by the broth microdilution method, whereas the combinatory effect was evaluated according to the sum of fractional inhibitory concentration (ΣFIC) indices. Synergy was obtained for the combination with oxacillin against three out of five strains tested with ΣFIC range from 0.250 to 0.424. These three strains were also methicillin resistant. Demethyltexasin broke this resistance at concentration range of 16 to 64 μg/ml. An additive effect was also observed for four strains.

Our results proved demethyltexasin to be an efficient compound in synergy assays in combination with oxacillin. It is also able to break the resistance in MRSA strains and therefore it is suggested for further research.

Key words: antibiotic, isoflavonoid, isoflavone, metabolite, plant, synergy, demethyltexasin

Abstrakt

Demethyltexasin (6,7,4'-trihydroxyisoflavone) se vyskytuje ve fermentovaných sójových potravinách, jako je například doenjang a tempeh, ale je také metabolizován v játrech z isoflavonu daidzeinu, což je látka přítomná v bobovitých rostlinách. Zkoumali jsme antimikrobiální účinek této látky a také účinek v kombinaci s antibiotikem oxacilinem a to na pěti standartních kmenech *Staphylococcus aureus*, včetně třech meticilin-rezistentních.

Minimální inhibiční koncentrace byly stanoveny pomocí bujónové mikrodiluční metody a účinek kombinací byl vypočítán jako suma jednotlivých frakčních inhibičních koncentrací (ΣFIC). Synergismus byl pozorován pro kombinaci demethyltexasinu s oxacilinem na třech z pěti testovaných kmenech v rozsahu ΣFIC 0,250 až 0,424. Tyto tři kmeny byly meticilin-rezistentní a demethyltexasin dokázal tuto rezistenci prolomit při rozsahu koncentrací 16 až 64 μg/ml. Kromě synergie byl u čtyř kmenů pozorován také aditivní účinek.

Naše výsledky ukázaly, že demethyltexasin je synergisticky účinná látka v kombinaci s oxacilinem, která dokáže překonat i rezistenci methicilin-rezistentních kmenů *S. aureus* a je doporučen k dalšímu kombinačnímu testování a výzkumu.

Klíčová slova: antibiotikum, isoflavonoid, isoflavon, metabolit, rostlina, synergie, demethyltexasin

Declaration

I declare that I have worked on this thesis independently, using only the sources listed in the bibliography.

In Prague, 18. 4. 2014

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Amálie Balaštíková

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Foreword

The discovery of antibiotics caused a revolution both in human and veterinary medicine. Unfortunately, inappropriate and excessive use resulted in the development of resistance among bacteria. One of the most serious human pathogens within this field is the *Staphylococcus aureus* whose strains are resistant mostly to *β*-lactam antibiotics, such as penicillin, methicillin and vancomycin. *S. aureus* causes great problems mainly in hospitals, where it can lead to epidemic outbreaks of multi-resistant strains, causing difficulties in treatment and narrowing the options for possible antimicrobial agents which can be applied. Therefore, it is necessary to find an efficient alternative.

One way of substituting antibiotics are biologically active secondary metabolites of plants. Amongst such metabolites we can find isoflavonoids, a subclass of flavonoids present mainly in leguminous plants, such as soybeans, chickpea and beans. Isoflavonoids are broadly studied for antimicrobial action and many of them proved to be very effective. Furthermore, they produce various beneficial effects on human health, such as anti-cancer, antioxidative and phytoestrogenic activity.

However, the effect of plant compounds, including isoflavonoids, is usually not as pronounced as in conventionally used antibiotics. Recent research reports that compounds present in plants can enhance the antimicrobial activity of some antibiotics. Therefore, a possible strategy on overcoming complications in the treatment of bacterial infections can be to combine common antimicrobial agents and plant compounds. Only a few studies deal with the combination of isoflavonoids and antimicrobial agents but they show interesting results. In addition, the possible synergistic effects of their metabolites remain totally unexplored.

1. Literature review

1.1. *Staphylococcus aureus*

Staphylococcus spp. (from Greek "staphylé", which means a bunch of grapes) are gram-positive cocci belonging to the family *Bacillaceae*. They are nonmotile, non-spore forming and mostly facultative anaerobes. Staphylococci are widespread in nature and are mainly found living on skin, skin glands and mucous membranes of mammals and birds. Nevertheless, they can sometimes also be found in the mouth, blood, mammary glands, intestinal, genitourinary and upper respiratory tract. Staphylococci found in humans and other primates include: *S. aureus, S. epidermidis, S. capitis, S. caprae, S. saccharolyticus, S. warneri, S. pasteuri, S. haemolyticus, S. himinis, S. lugdunensis, S. auricularis, S. saphrophyticus, S. cohnii, S. xylosus* and *S. simulans* (Murray at al., 1999).

S. aureus was first identified in 1880 by a surgeon, Sir Alexander Ogston, as a cause of acute suppuration in humans (Ogston and Witte, 1984). Since then it has been well documented as a human opportunistic pathogen which grows in the temperature range of 15 to 45 °C. The word "aureus" means "golden" in Latin, because most colonies have a characteristic orange-yellow coloring on agar plates and because *S. aureus* is also responsible for golden yellow pus indicating infection (Anonymous, 2014a). The place of residence is mainly the anterior nares (Noble et al., 1964).

1.1.1. Cell structure and function

All bacteria, including *S. aureus*, belong to the prokaryotes, which do not have a nucleus, and their genetic information is contained in a single circular structure of the chromosome (Freeman-Cook et al., 2006). They may also have small extra-chromosomal elements such as plasmids, prophages and transposons. Genes for resistance to antibiotics are found on the chromosome as well as on these extrachromosomal elements and can be transferred between staphylococcal strains, species or even other bacteria (Schaberg and Zervos, 1986).

Approximately 50% of the cell wall of *S. aureus* is made of peptidoglycan. Peptidoglycan consists of alternating polysaccharide subunits of *N*-acetylglucosamine and *N*-acetylmurmaric acid with 1,4-*β* linkages. The peptidoglycan chains are cross-linked by tetrapeptide chains bound to *N*-acetylmuramic acid and by a pentaglycine bridge specific

for *S. aureus*. Peptidoglycan may have endotoxin-like activity, stimulating the release of cytokines by macrophages (Lowy, 1998).

The cell wall also contains teichoic acids (polymers composed of glycerol phosphate or ribitol phosphate). These acids are in part responsible for the overall negative electrical charge of the cell surface and they also bind Ca^{2+} and Mg^{2+} cations for transport into the cell. Certain teichoic acids are covalently bound to membrane lipids, these are called lipoteichoic acids.

Most staphylococci also create capsules on their outer surface. These polysaccharide layers assist them in attaching to the surface components of host tissues and to solid surfaces in nature. In such a way the staphylococci can form a thick layer of cells called the biofilm. Biofilm-associated infections are a significant cause of morbidity due to indwelling medical devices (such as catheters and prosthetic heart valves). Bacteria in biofilms are often resistant to antibiotics because the biofilm offers a protective barrier. Encapsulated bacteria are also more difficult for the phagocytic cells of the immune system to recognize and destroy. This polysaccharide layer also helps against desiccation (Madigan et al., 2010; Tortora et al., 2012). Another element involved in the formation of the biofilm are surface proteins. They facilitate the attachment to host proteins such as fibronectin, laminin, vitronectin and collagen (on epithelial and endothelial surfaces). Protein A also has anti-phagocytic properties (Gyles et al., 2011).

Enzymes

Staphylococci produce various enzymes, such as hyaluronidase, lipase and protease that destroy tissue but also such enzymes as β-lactamase, PBPs and coagulase. The first group of enzymes mentioned may facilitate the spread of infection to adjoining tissues. Hyaluronidase and hyaluronate lyase are enzymes digesting hyaluronic acid. This depolymerization of hyaluronic acid contributes to the infective process by promoting spread through degradation of tissues. Lipase and an enzyme known as FAME (fatty acid-metabolizing enzyme) have a negative effect on immune functions, since they break down fatty acids and other lipids produced in response by an infected host, serving as surfactants that disrupt bacterial membrane. Proteases have been shown to block the action of antibodies by cleaving and inactivation. They probably also facilitate the protection against antimicrobial peptides, such as neutrophil defensins and platelet microbial proteins. Proteases may also destruct tissue proteins and enhance invasiveness. They may also play a

role in obtaining nutrients from the environment. Another important enzyme is β-lactamase, crucial in inactivating penicillin. Important are also penicillin-binding proteins, enzymes located in the cytoplasmic membrane involved in the cell-wall assembly. Coagulase, an extracellular protein that binds prothrombin to form a complex called staphylthrombin, converts fibrinogen into fibrin. This may help the bacterium to protect itself against phagocytic and immune defense by causing clotting (Gyles et al., 2011; Lowy, 1998).

Toxins

Staphylococci produce numerous toxins such as α-toxin, exfoliatin, PTSAGs, enterotoxins and TSS-1. α-Toxin is a protein that lyses cytoplasmic membranes by the formation of pores. The resulting losses of vital molecules lead to cell death. Another toxin, exfoliatin, causes intercellular splitting of the epidermis between the spinous and the granular layer by disrupting intercellular junctions. A group of enzymes called the pyrogenic toxin superantigens are important in interaction with the host defense system. They stimulate T cells and macrophages to release cytokines, particularly tumor necrosis factor-α and interleukin-1. Another activity of these toxins is pyrogenicity. *S. aureus* can also produce enterotoxins, such as protein enterotoxins A, B and C, which stimulate vomiting in humans and animals. These toxins are quite resilient to boiling and digestive enzymes. The toxic shock syndrome toxin-1 has similar properties as enterotoxins. It stimulates cytokines but also has toxic effect on endothelial cells, leading to capillary leakage, hypotension and shock (Ryan and Ray, 2003).

1.1.2. Types of disease

Infections produced by *S. aureus* are typically acute, aggressive and pyogenic. Of nosocomial infections, the ones caused by this bacterium have the highest morbidity and mortality. If left untreated, *S. aureus* can spread to surrounding tissues or other organs. Some of the most common infections caused by *S. aureus* are infections of the skin, such as furuncles and boils, cellulitis, impetigo and postoperative infections of various sites. Some of the more serious infections are bacteremia, pneumonia, osteomyelitis, acute endocarditis, myocarditis, pericarditis, cerebritis, meningitis, scalded skin syndrome and abscesses of the muscles, urogenital tract, CNS and various intra-abdominal organs. Food poisoning and toxic shock syndrome are also attributed to *S. aureus* (Murray et al., 1999).

The most common staphylococcal infections are furuncles and boils that develop in hair follicles or glands. Furunculosis is often a complication of acne. Infection at the base of the eyelash gives rise to the common stye. The infected patient is often a carrier of that particular *Staphylococcus* strain in anterior nares. No surgical or antibiotic treatment is needed and it is resolved by spontaneous drainage of pus. However, infection can spread from furuncle in adjacent subcutaneous tissues, forming abscesses. The lesion is then called carbuncle and it is most often located at the back of the neck. Carbuncles are more serious and may be the cause of bacteremia (bloodstream invasion) (Fluit and Schmitz, 2003; Ryan and Ray, 2003).

Bullous impetigo and scalded-skin syndrome are two expressions denoting the same disease. The former is localized and the latter systemic. Both are produced by exfoliative toxins and both are common among newborns and small children. Considering the scalded skin syndrome, the face, armpits and groin are first affected but the erythema and subsequent desquamation of epithelial sheets can spread to all body parts. With antibiotic treatment the skin heals within two weeks and mortality is lower than 5%. For bullous impetigo fluid-filled vesicles are typical (Crossley et al., 2009; Ryan and Ray, 2003).

The toxic shock syndrome was first described in children but came to public attention during the early 1980s, when many cases were reported in young women using vaginal tampons. The disease is characterized by high fever, vomiting, diarrhea, sore throat and muscle pain. Within 48 hours, it may progress to severe shock with evidence of renal and hepatic damage. A skin rash may develop, followed by desquamation at a deeper level than in the scalded skin syndrome (Ryan and Ray, 2003).

S. aureus is a notorious cause of wound infection, especially after surgery. It can cause a wide variety of deep tissue infections, such as osteomyelitis, arthritis, endocarditis and cerebral, pulmonary, renal and breast abscesses. *S. aureus* can also cause secondary pneumonia (after viral infection). The majority of these infections occur in persons with multiple risk factors for infection, such as diabetes mellitus, leukocyte defects and general reduction of host defense or corticosteroid or cytotoxic therapy. Severe infections are particularly common among intravenous drug abusers (Fluit and Schmitz, 2003).

S. aureus causes also illnesses in animals. In cattle, *S. aureus* is mainly involved in intra-mammary infections, causing considerable economic losses. Unless treatment of acute infection is successful, it becomes chronic. In poultry, *S. aureus* causes arthritis,

bacteremia, dermatitis, osteomyelitis and synovitis and has a high economic impact. *S. aureus* can also affect rabbits. Newborns can suffer from exudative dermatitis, affecting the whole litter and having high mortality. In older young, subcutaneous abscesses, conjunctivitis and purulent rhinitis are typical. Subcutaneous abscesses also occur in broiler rabbits and does. In rabbits of all ages, internal abscesses (e.g. in lungs and liver) as well as arthritis, parodontitis, sinusitis and inflammation of the middle ear due to septicemia may be demonstrated. Mastitis in does may also be acute or chronic (Gyles et al., 2011).

1.1.3. Epidemiology

As far as the acquisition of *S. aureus* is concerned, human population can be divided into three groups: the persistent carriers, persistent non-carriers and intermittent carriers. Approximately 20% of adults almost always carry one type of *S. aureus* strain and can harbor it for a very long period of time. Another 20% of adults almost never carry any *S. aureus* strain. The majority of population are intermittent carriers, who commonly carry staphylococci for a few weeks at a time and then are free from them for some time. These people often carry different phage types in successive periods of carriage. *S. aureus* nasal carriage is also common in monkeys, guinea pigs and dogs (Williams, 1963). The carriage of *S. aureus* seems to be protective against the acquisition of new strains. This effect is reduced after treatment with antibiotics. Younger people (under 20 years of age) are the most frequent nasal carriers, whereas the least frequent are older people (Noble et al., 1964).

Considering methicillin resistant *S. aureus* infection, it can be either health-care associated (HA-MRSA) or community-associated (CA-MRSA). The HA-MRSA (initially known as epidemic-MRSA or EMRSA) emerged in the 1960s and is linked with hospitalization, surgery, hemodialysis, antibiotic treatment and exposure to invasive devices or procedures. On the other hand, Ca-MRSA emerged in 1990s in persons where healthcare associated risk factors were absent, such as in participants in team sports, injection drug users, gay men, people living in dormitories, military barracks etc. The two MRSA strains mentioned above are genetically different. The predominant strains of CA-MRSA are the genetic fingerprint types US 300 and US 400 strains which contain the staphylococcal chromosomal cassette (SCC) *mec* IV. SCC contains the resistance gene against *β*-lactam antibiotics, known as *mec*A. The *mec* of type IV is a smaller genetic

package than are the SCC*mec* I, II, III, and V which are present in the US 100 and 200 strains typical for HA-MRSA. The smaller size of the SCC confers less resistance than the larger SCC and explains why CA-MRSA is susceptible to more classes of antibiotics than HA-MRSA. CA-MRSA is always resistant to the β-lactams and often also to erythromycin but remains susceptible to several other antimicrobial agents. HA-MRSA is resistant to all classes of antibiotics except vancomycin, linezolid, quinoprisitin-dalfopristin, daptomycin, tigecyline, ceftaroline and televancin. Almost all CA-MRSA strains carry the Panton Valentine Leukocidin (PVL), a gene that allows the production of a necrotizing cytotoxin which may be responsible for CA-MRSA's invasiveness and virulence. In contrast, only about five percent of methicillin susceptible strains of *S. aureus* and HA-MRSA carry the PVL gene (Borlaug et al., 2011; David and Daum, 2010; Evans 2008; Monecke et al., 2011). The differences between both types are summarized in Table 1.

Table 1: Differences between HA-MRSA and CA-MRSA (Borlaug et al., 2011; Evans 2008; Weber, 2005)

HA-MRSA	**Conditions**	**CA-MRSA**
Yes	Health care contact	No
Older	Age at infection	Younger
Immunocompromised, hospitalized, dialysis patients, people after surgery	Persons affected	Anyone
35%	Skin and soft tissue infection	75%
Blood, surgical site, site of implant	Area affected	Skin, lungs
Skin-to-skin contact, contaminated equipment or surfaces, poor hand hygiene	Transmission	Skin-to-skin contact, contaminated surfaces, skin openings, crowded living conditions, poor hygiene
many agents	ATB resistance	some agents
Vancomycin, daptomycin, linezolid, tigecycline, trimethoprim, sulfamethoxazole and gentamycin or rifampin for synergy	Treatment	ATB not always required; doxycycline, clindamycin, bactrim
SCCmec types I, II, III, V	Resistance gene	SCCmec type IV
USA 100 and 200	Strain type	USA 300 and 400
Rare (5%)	PVL toxin gene	Frequent (almost 100%)
Common	Toxin production	Rare
Good hand hygiene, careful use of ATBs, post operation surveillance	Prevention	Good hand hygiene, keeping wounds covered, not touching other peoples' cuts and bandages, not sharing personal items

1.1.4. Treatment and antibiotic resistance

In 1928 Sir Alexander Fleming discovered the antibiotic penicillin produced by *Penicillium rubens* (Fleming, 1944; Houbraken et al., 2011; Sarmah et al., 2006). At first this antibiotic was called the "miracle drug" because of its unique and rapid control of infectious bacteria that, before the discovery of penicillin, had been fully expected to kill the patient (Levy, 2002). In 1944 penicillin-resistant strains first emerged (Kirby, 1944). In 1945 Fleming warned that the misuse of penicillin could lead to the selection and propagation of mutant forms of bacteria resistant to the drug. Nevertheless, the drug was available without doctor's prescription until the mid-1950s (Levy, 2002). In early 1960s, methicillin resistant *S. aureus* (resistant to most *β*-lactam antibiotics) emerged as a nosocomial pathogen (Panlilio et al., 1997). It has become a major clinical and epidemiologic problem in hospitals of all sizes since 1980s (Boyce et al., 1997; Murray et al., 1999). The drug of choice to cure MRSA is vancomycin and teicoplanin. However, *S. aureus* develops resistance against these agents as well (Chambers et al., 1997). In 1996 a *S. aureus* with reduced susceptibility to vancomycin was first reported in Japan and in 2002 a vancomycin resistant *S. aureus* (VRSA) was first isolated in the USA (Hiramatsu, 1997; MMWR, 2002). Many strains of MRSA are resistant to multiple classes of antibiotics (such as amikacin, chloramphenicol, clindamycin, erythromycin, gentamicin, kanamycin, neomycin, streptomycin, tetracycline and tobramycin) (Lyon et al., 1984). The antibiotic therapy for MRSA infections is becoming more difficult because of strong biofilm-forming properties (Anonymous, 2014a; Madigan et al., 2010; Tortora et al., 2012).

Infection control

Most boils and superficial staphylococcal abscesses resolve spontaneously without antimicrobial therapy. More serious *S. aureus* infections require aggressive treatment, including incision and drainage of localized lesions, as well as systemic antibiotics. Penicillins and cephalosporins are active against peptidoglycan in *S. aureus* cell wall and vary in their susceptibility to inactivation by staphylococcal *β*-lactamases. Although penicillin G is the treatment of choice for susceptible strains, the penicillinase-resistant penicillins (methicillin, nafcillin and oxacillin) and first-generation cephalosporins are more commonly used because of resistance. For strains resistant to these agents or patients with *β*-lactam allergic reaction, the alternatives are vancomycin, clindamycin,

erythromycin or teicoplanin. However, vancomycin can only be administered intravenously and teicoplanin is only available in Europe and is not approved in the United States (Aldeen and Hiramatsu, 2004; Ryan and Ray, 2003). The incidence of vancomycin resistance has increased steadily, prompting the use of alternative drugs such as quinupristin-dalfopristin, linezolid, and daptomycin (Aldeen and Hiramatsu, 2004). The list of antibiotics generally used against *S. aureus* infections can be seen in Table 2.

Table 2: Antimicrobial agents for treatment of staphylococcal infections (Aldeen and Hiramatsu, 2004)

Antibiotic	**Class**	**Antibiotic**	**Class**
Amoxicillin/ clavulanate	Penicillin combination	Linezolid	Oxazolidononones
Cefalexin	Cephalosporin	Methycillin	Penicillin
Cefazolin	Cephalosporin	Minocycline	Tetracycline
Ciprofloxacin	Fluoroquinolone	Nafcillin	Penicillin
Clindamycin	Lincosamide	Oxacillin	Penicillin
Daptomycin	Lipopeptide	Penicillin G	Penicillin
Dicloxacillin	Penicillin	Penicillin VK	Penicillin
Doxycycline	Tetracycline	Quinupristin-dalfopristin	Streptogramin
Erythromycin	Macrolide	Rifampin	Other
Gentamicin	Aminoglycoside	Teicoplanin	Glycopeptide
Levofloxacin	Fluoroquinolone	Vancomycin	Glycopeptide

Prevention

There is no effective vaccine against *S. aureus*, therefore infection control procedures, such as barrier precautions and disinfection of hands and contaminated surfaces are important in the control of *S. aureus* epidemics (Harvey et al., 2012). Clothes and bedding that may cause infection should be washed at a sufficiently high temperature to destroy staphylococci (70°C or higher) or dry-cleaned. Chlorhexidine or hexachlorophene soaps in showering and washing increase the bactericidal activity of the skin. The outbreak of infection from nasal carriers can be eliminated by the combination of nasal creams containing topical antimicrobials (e.g., mupirocin, neomycin and bacitracin) and oral therapy with antimicrobials that are concentrated within phagocytes and nasal secretions (e.g., rifampin or ciprofloxacin). Attempts to reduce nasal carriage more generally among medical personnel in an institution are usually fruitless and encourage replacement of susceptible strains with multi-resistant ones. Chemoprophylaxis is effective in surgical procedures such as hip joints and cardiac valve replacements. Methicillin,

cephalosporin or vancomycin given during and shortly after surgery may reduce the chance of infection while minimizing the risk for super-infection associated with longer periods of antibiotic administration (Ryan and Ray, 2003). To prevent and treat biofilm caused infections, small molecules and matrix-targeting enzymes inhibiting and disrupting biofilm formation and bactericidal and anti-adhesion coatings seem to be a future possibility (Meng et al., 2013).

Mechanism of resistance

There are two main mechanisms responsible for the resistance of *S. aureus* to *β*-lactam antibiotics. One is the production of *β*-lactamases that destroy the *β*-lactam ring. These *β*-lactamases were responsible for the development of resistance to penicillin and led to the development of the *β*-lactamase-resistant antistaphylococcal penicillins such as methicillin. The other mechanism is an alternation in membrane-bound enzymes called penicillin-binding proteins (PBPs). These proteins perform important functions for cell survival and are the targets of the *β*-lactam antibiotics. All strains of MRSA produce a unique PBP (PBP2a or 2') which catalyzes cell wall construction. Resistance due to the unique PBPs is often called “intrinsic” resistance. PBP 2' is encoded by the *mecA* gene (Chambers et al., 1997 Mulligan et al., 1993). Another mechanism of resistance, for example against tetracycline, is an efflux pump (Hirata et al., 1998).

The vancomycin resistant *S. aureus* contains *vanA* vancomycin-resistance gene. These genes have been reported in vancomycin-resistant enterococci. Evidence exists that this vancomycin resistance can be obtained from conjugative transfer from enterococci to *S. aureus* (Chang et al., 2003; Noble et al., 1992).

1.2. Synergistic antimicrobial effect

One of the strategies to overcome resistance in bacteria is to combine various drugs. For example, inhibitors of *β*-lactamases, such as clavulanic acid, sulbactam and tazobactam are administered together with antibiotics as co-drugs (Hemaiswaryaa et al., 2008). Considering staphylococci and synergism, the synergy between cell-wall-active antibiotics and the antimicrobial agents known as aminoglycosides is present when the staphylococcus is sensitive to both of these substances. Such combinations are often used in severe systemic infections when effective and rapid bactericidal action is needed, particularly in compromised hosts (e.g. combination of oxacillin with gentamicin) (Josef et

al., 2010; Ryan and Ray, 2003).

Several approaches, such as the checkerboard, the time-kill assays and the E-test are currently used for the study of synergistic antimicrobial effects. These methods can be based on macro- or micro-dilution techniques in culture broth or agar media. The checkerboard technique is the most common, and as its name suggests, the concentrations of one substance are arranged horizontally and the other vertically in a microtiter plate. The dilutions that are tested are based on the MIC of the substances, usually ranging from a few steps below the expected MIC, to concentrations twice the expected MIC. Measurements are usually made at one time point and therefore do not yield a dynamic view of the antimicrobial interactions. The time-kill assay is a less used and more labor intensive method which involves measuring the number of viable bacteria present in a liquid medium in the presence of a particular combination of antibacterials at different time points. Although time-kill curves are not widely used to study antibacterial interactions, they can be considered a clinically relevant model if the concentrations used represent those achieved at the site of an infection (Chayapa et al., 2014; Langeveld et al., 2014).

E-test consists of two plastic strips coated on one side with a continuous gradient of the individual compounds. For the evaluation of synergy, one compound strip is placed onto an agar plate for one hour and then removed. The second compound strip is placed on top of the gradient left behind by the first strip. The MIC of the combination is taken as the value at which the two inhibition zones intersect (Hemaiswaryaa et al., 2008).

To define synergistic, additive and antagonistic effects of antimicrobial combinations, the FICI (fractional inhibitory concentration index) values are typically calculated (Chayapa et al., 2014). First, the FIC (fractional inhibitory concentration) of each drug is calculated. To do so, we measure the MIC value of each of the compounds in the combination that completely inhibits the growth of the microorganism. Then we divide this value by the MIC of the applicable compound that inhibits the growth of the microorganism-ism by itself. The FIC of the combination is then the sum of these two individual FIC values (Hemaiswaryaa et al., 2008). In short:

$$\Sigma FIC = \frac{MICA(\text{combined with B})}{MICA(\text{alone})} + \frac{MICB(\text{combined with A})}{MICB(\text{alone})}$$ (Verma, 2007).

The result can then be evaluated in several ways. The first is proposed by the European Committee for Antimicrobial Susceptibility Testing: synergistic effect if $\Sigma FIC \leq 0.5$; additive if $\Sigma FIC > 0.5$ to < 1; indifferent if $\Sigma FIC \geq 1$ to < 2, and antagonistic if $\Sigma FIC \geq$

2 (EUCAST, 2000). On the other hand, according to Odds (2003), when the FIC index of the combination is equal to or less than 0.5, the combination is termed as synergistic; when FIC index falls between 0.5 and 4.0, it indicates 'no interaction' between the agents, and a value above 4.0 indicates antagonism between the two compounds. A convenient graphical way of representing the results of combination studies is by the use of an isobologram (Hemaiswaryaa et al., 2008).

Another option is to combine approved antibiotics with compounds derived from plants. For example, it is possible to potentiate the activity of penicillin against *S. aureus* by combining it either with clavulanic acid or the natural compound epigallocatechingallate (EGCg) (Wagner and Ulrich-Merzenich, 2011). Further examples of natural products, such as catechins and flavones, and antibiotics possessing synergistic effect can be seen in Table 3.

Table 3: Synergism between natural products and antibiotics against *S. aureus* strains (Hemaiswaryaa et al., 2008)

Natural product	**Antibiotic**	**Microorganism**	**Mechanism of action**
EGCg	Penicillin	Penicillinase prod. *S. aureus*	Inhibits penicillinase
	β-Lactam	MSSA, MRSA	Binds to the peptidoglycan and inhibits cell wall
	Tetracycline	*S. aureus* with MDR pump	Blocks MDR efflux pumps
Tea catechin	Oxacillin	MRSA	-
Totatrol	Methicillin	MSSA, MRSA	Inhibits PBP 2a production
α-Mangostin	Vancomycin	MRSA	-
Corilagin	Oxacillin	MRSA	Inhibits PBP 2a production or activity
Baicalin	*β*-Lactams	MRSA	Inhibits *β*-lactamase
Pomegranate extract	Chloramphenicol, gentamicin, ampicillin, tetracycline, oxacillin	MRSA	Blocks Nor (A) pump
Sophora-flavanone G	Vancomycin hydrochloride, fosfomycin, methicillin, cefzonam, gentamicin, mino-cycline, levofloxacin	MRSA	-

1.3. Isoflavonoids

Flavonoids are compounds based on a fifteen-carbon skeleton which consists of two phenyl rings (A and B) connected by a three-carbon bridge (C-ring). Flavonoids are synthetized almost solely by plants (with some exceptions, such as marine coral, *Echinophora lamellosa*, and fungi, *Aspergillus candidus* and *Phallus impudicus*). There are about 5000 different kinds of naturally-occurring flavonoids which can be divided into several classes: anthocyanins, aurones, chalcones, flavanones, flavones, isoflavonoids, etc. (Iwashina, 2000; Sharma and Ramawat, 2013).

Isoflavonoids are a biologically active subclass of flavonoid phenolic compounds with a 15-carbon (C6-C3-C6) backbone arranged as a 1,2-diphenylpropane skeleton (Figure 1). In contrast to the parent class of flavonoids, the distribution of the isoflavonoid class in the plant kingdom is relatively limited, probably owing to the sporadic occurrence of isoflavone synthase (Botta et al., 2009). There are more than 1600 structures of isoflavonoids occurring in nature and they can be encountered either in the form of aglycones or glycosides (with glucose, rhamnose or apiose as a sugar component). Glycosides are much less common than aglycones. According to their structure, isoflavonoids can be divided into the following subclasses: isoflavones, isoflavanones, rotenoids, pterocarpans, isoflavans, isoflav-3-enes, coumestans, coumaronochromones, 3-arylcoumarins and 2-arylbenzofurans (Figure 2) (Reynaud et al., 2005; Veitch, 2007).

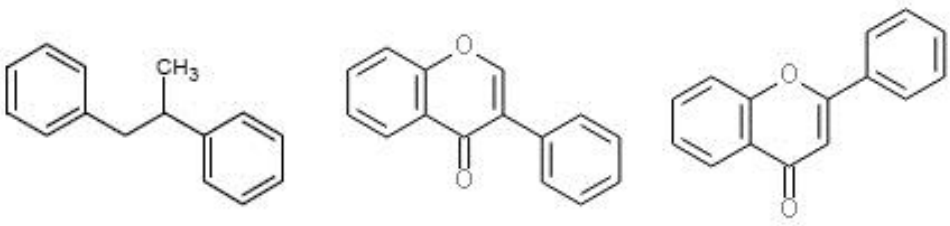


Figure 1: 1, 2-diphenylpropane, flavone (2-phenylchromen-4-one), isoflavone (3-phenylchro-men-4-one)

Isoflavones

Isoflavones differ from corresponding flavones by aromatic ring migration from position C-2 to C-3. Major structural diversity arises from hydroxylation, methoxylation or methylenedioxy substitution (O-substitution), prenylation and glycosidation (Preedy, 2012, Veitch, 2007). Isoflavones are the most frequently reported of all the isoflavonoid subclasses (Veitch, 2013) and can be divided into four categories: (1) aglycones (without

attached glucose) - biochanin A, daidzein, formononetin, genistein and glycitein; (2) glucosides or glucones - daidzin, genistin, glycitin, ononin and sissotrin; (3) acetylglucosides or acetylglucones - 6"-acetyldaidzin, 6"-acetylgenistin, and 6"-acetylglycitin and (4) malonylglucosides or malonylglucones - 6"-malonyldaidzin, 6"-malonylgenistin, and 6"-malonylglycitin (Sharma and Ramawat, 2013). Many isoflavones possess interesting antimicrobial properties (Sato et al., 2006; Mahabusarakam et al., 2004; Kuete et al. 2008; Fukai et al., 2002a; Fukai et al., 2002b).

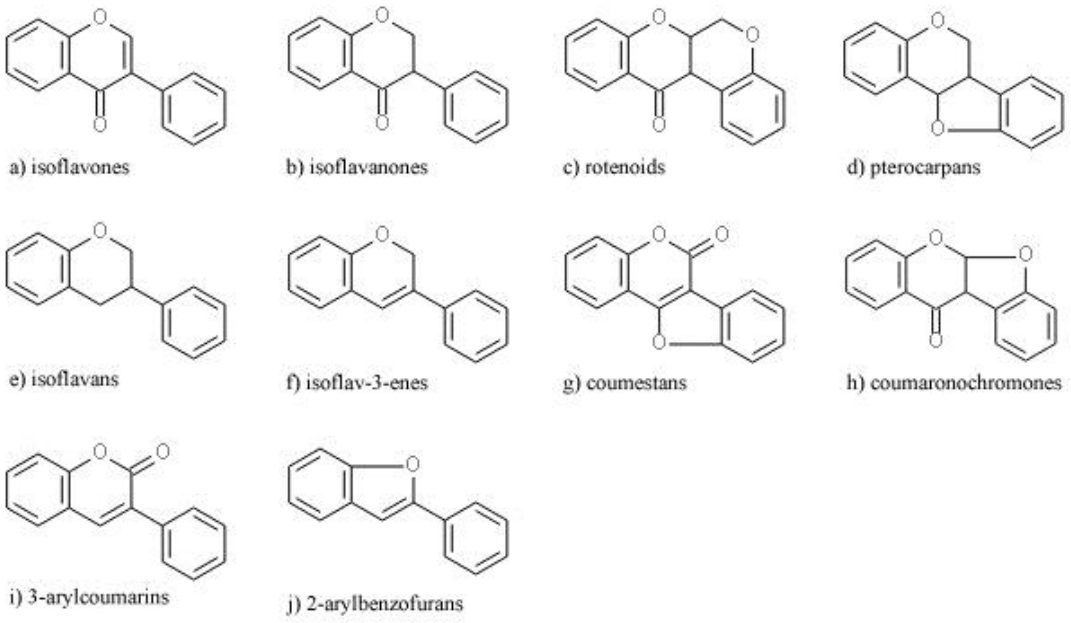


Fig. 2: Structures of basic groups of isoflavonoids (Ingham, 1983; Veitch, 2007)

Rotenoids

Another class of isoflavonoids are rotenoids, which are characterized by the inclusion of an extra carbon atom into a heterocyclic ring. They can be divided into three groups according to their level of oxidation: rotenoids, 12a-hydroxyrotenoids and dehydrorotenoids. Rotenoids are known for their antiviral, insecticidal and piscicidal properties (Boland and Donelly, 1998).

Pterocarpans

According to Veitch (2013) can pterocarpans be divided into two subgroups: 6a-hydroxypterocarpans and pterocarpenes. Pterocarpans may act as phytoalexines, produced after fungal infection or abiotic stress (Boland and Donelly, 1998).

Isoflavans

This isoflavonoid subclass includes not only isoflavans but also isoflavanquinones and isoflavan-4-ols. Isoflavans, such as vestitol and sativan are of interest as phytoalexins (Veitch, 2007).

1.3.1. Taxonomical distribution of isoflavonoids

The discovery of isoflavonoids as natural products has its origin in 1840s, when Reinsch obtained the first preparations of ononin from the roots of the legume *Ononis spinosa*, which was then recognized as glycoside in 1855. Almost eighty years later the full structure of this compound was determined as the 7-O-glucoside of formononetin (7-hydroxy-4'-methoxyisoflavone). Root preparations of this species are even today used as a herbal drug possessing mild diuretic activity that is attributed to its isoflavone content (Santos-Buelga et al., 2010; Veitch, 2007).

The presence of isoflavonoids is almost entirely restricted to the *Fabaceae* family. The number of reports of isoflavonoid occurrence in the *Caesalpinioideae* and *Mimosoideae* subfamilies remains very small in comparison to the *Papilionoideae* (Santos-Buelga et al., 2010). This emphasizes the importance of isoflavonoids as a diagnostic chemical characteristic of the *Papilionoideae* subfamily (Veitch, 2007). In pre-1982 literature the only sources listed were *Apuleia leiocarpa* (the *Caesalpinioideae* subfamily), *Albizia procera* and *Prosopis juliflora* (both from the *Mimosoideae* subfamily) (Santos-Buelga et al., 2010). Isoflavone C-glycosides were later found in *Cassia javanica* subsp. *nodosa* (Ilyas et al., 1994) and *Senna siamea* (Shafiullah et al., 1996) (both from the *Caesalpinioideae* subfamily). Considering information about aglycones published between 1997 and 2007, the prevalence of different isoflavonoid structures in *Papilionoideae* is as follows: most frequent were isoflavones (34%), followed by pterocarpans (17%) and isoflavanones (15%). Rarest were isoflav-3-enes, 3-arylcoumarins, coumestans and coumaronochromones. (Santos-Buelga et al., 2010). Isoflavonoids are most abundant in soybeans and other legumes, such as *Cicer arietinum* (chickpea), *Pueraria lobata* (kudzu root) and *Trifolium* spp. (see Table 4) (Mazur et al., 1998; Renda et al. 2013). One way to increase the content of isoflavonoids in legumes is sprouting. Sprouting of seeds is known to increase their nutritive value, such as phenolic and flavonoid content and improve the health qualities of foods (Chon, 2013). This effect of increased isoflavonoid amount was

proved in several studies dealing with legumes (Park et al., 2005, Plaza et al., 2003, Sujatha et al., 2009).

Table 4: Isoflavonoid content in different plant species (Mazur et al., 1998; Renda et al. 2013)

Plant name	**Isoflavonoid content µg/100g dry mass**			
	Daidzein	**Genistein**	**Biochanin A**	**Formononetin**
Glycine max[a]	10,500-56,000	26,800-84,100	trace-15	18-121
Cajanus cajan	12-27	190-737	10-219	5-26
Cicer arietinum	11-192	69-214	838-3,080	94-215
Lens culinaris	3-10	7-19	trace-7	8-11
Phaseolus vulgaris	7-40	7-518	trace-12	0-11
Pisum sativum	4-11	0-23	3-6	0-10
Vigna mungo	7-36	trace-60	0-81	0-2
Vigna unguiculata	21-30	12-56	0-8	0-5
Pueraria lobata (root)	185,000	12,600	1,400	7,090
Sophora japonica	319	265	830	322
Trifolium canescens	21,730	125,3181	460,277	100,534
Trifolium pratense	12,200	4,010	20,400	22,300

[a]Species shown in bold contain the greatest amount of the above mentioned isoflavonoids.

Nevertheless, isoflavonoids can also occasionally be found in other plant families, so far in 61 (see Table 5). Evidence of limited distribution of isoflavonoids in non-leguminous plant families began to emerge in the late nineteenth century with the observation of iridin in rhizomes of the *Iris* species and in 1910 with the discovery of prunetin in the bark of a *Prunus* species (Veitch, 2007). Although isoflavonoids can be found in non-leguminous families, their content there is rather small compared to legumes. As Reynaud et al. (2005) state in their article: 'The increasing sensitivity of isolation and identification methods should make it possible to identify new occurrences of isoflavonoids in families where these molecules are currently considered rare or even non-existent.' From dietary perspective are isoflavonoids (specifically daidzein and genistein) found in cereals (*Hordeum* spp. and *Triticum dicoccum*), oilseeds (*Helianthus* spp., *Cuminum cyminum, Juglans nigra, Corylus avellana pontica, Sesamum* spp. and *Papaver* spp.), berries (*Rubus fructicosus, Fragaria x ananassa* and *Rubus ideaus*), fruits (*Pyrus malus*), vegetables (*Brassica oleracea, Brassica oleracea italica, Brassica oleracea botrytis, Allium sativum* and *Daucus sativus*) (Mazur, 1998; Mazur and Adlerkreutz, 1998). Furthermore, isoflavonoids can also be found in alcoholic beverages. All four

isoflavonoids, biochanin A, daidzein, genistein and formononetin were found in beer (Lapčík et al., 1998). In bourbon, *β*-sitosterol and biochanin A were determined by Rosenblum and his colleagues in 1993.

Table 5: Taxonomical distribution of isoflavonoids in plants[a]

Class	Family	No. of structures	Family	No. of structures
Bryopsida	*Bryaceae*	4		
Pinopsida	*Araucariaceae*	2		
	Cupressaceae	8		
	Podocarpaceae	5		
Liliopsida	*Asphodelaceae*	1	*Liliaceae*	4
	Cyperaceae	6	*Poaceae*	10
	Eriocaulaceae	1	*Smilacaceae*	1
	Iridaceae	52	*Stemonaceae*	3
	Juncaceae	1	*Zingiberaceae*	2
Magnoliopsida	*Amaranthaceae*	3	*Myriciaceae*	2
	Apiaceae	4	*Myristicaceae*	13
	Apocynaceae	1	*Myrtaceae*	6
	Asclepiadaceae	12	*Nyctagicaeae*	19
	Asteraceae	21	*Nymphaeceae*	1
	Bombacaceae	1	*Ochnaceae*	17
	Brassicaceae	6	*Papaveraceae*	2
	Cannabaceae	10	*Polygalaceae*	3
	Caryophyllaceae	1	*Polygonaceae*	1
	Celastraceae	1	*Rhamnaceae*	8
	Clusiaceae	3	*Rosaceae*	5
	Connaraceae	1	*Rubiaceae*	4
	Convolvulaceae	20	*Rutaceae*	7
	Corylaceae	1	*Sapotaceae*	2
	Crassulaceae	1	*Scrophulariaceae*	6
	Cucurbitaceae	1	*Solanaceae*	1
	Erythroxylaceae	8	*Sterculiaceae*	3
	Euphorbiaceae	3	*Theaceae*	4
	Chenopodiaceae	19	*Urticaceae*	1
	Magnoliaceae	1	*Verbenaceae*	1
	Malvaceae	2	*Violaceae*	2
	Melastomataceae	1	*Vitaceae*	1
	Menispermaceae	1	*Zygophyllaceae*	4
	Moraceae	18		

[a](Alvez et al. 2010; Benavides et al., 2007; Koblovská et al., 2006; Lapčík, 2007; Macková et al. 2006; Mazur and Adlercreutz, 1998; Reynaud et al. 2005, Guo et al. 2007).

According to Mazur and Adlerkreutz (1998), most of the black and green teas contained small amounts of biochanin A, daidzein, formononetin and genistein, ranging from 5 to 78 μg/100g. A study of Alves et al. (2010) revealed interesting facts about coffee and its isoflavonoid content. Not only did they assess the presence of daidzein, genistein and formononetin in coffee but they also found out that the total isoflavone level was six times higher in robusta coffee than in arabica (mainly due to formononetin). They also learned that during roasting the content of isoflavones decreased, whereas their extractability increased. After comparing different brewing methods they realized that filtered brew presented the lowest concentrations of isoflavones, whereas mocha the highest.

Isoflavonoids are not present only among plants, but are also produced by several fungi e.g. *Arthrobactor*, *Aspergillus saitoi*, *A. oryzae*, *Fusarium* spp., *Micrococcus*, *Monascus pilosus* and *Streptomyces* spp. (Chen et al., 2009; Hong et al., 2013; Huang et al., 2010; Huang et al., 2013; Seo et al., 2013), bacteria e.g. *Micromonospora* spp. and mycobacteria *Mycobacterium phlei* (Gutierrez-Lugo et al., 2005; Hudson and Bentley, 1969).

1.3.2. Metabolism of isoflavonoids

What happens to isoflavonoids after ingestion has been documented for several isoflavones (such as biochanin A, daidzein, genistein and formononetin). Once ingested, free aglycone forms of these isoflavones produced by acid or enzymatic hydrolysis of the conjugated isoflavones (genistin, daidzin) are available for absorption. Biochanin A and formononetin may also be demethylated by gut bacteria to genistein and daidzein respectively (Kelly et al., 1993). These can either be absorbed or further metabolized (Setchell and Cassidy, 1999). Genistein is then converted by gut bacteria to dihydrogenistein, followed by a cleavage of the C-ring to form 6'-hydroxy-*O*-desmethylangolensin, which can be further degraded by the colonic microflora to 4-hydroxyphenyl-2-propionic acid. Decarboxylation can then lead to the putative metabolic end product 4-ethylphenol. Daidzein is transformed by the gut microflora to dihydrodaidzein, which can be further metabolized to both equol and *O*-desmethylangolensin (see Figure 3) (Kulling et al., 2001).

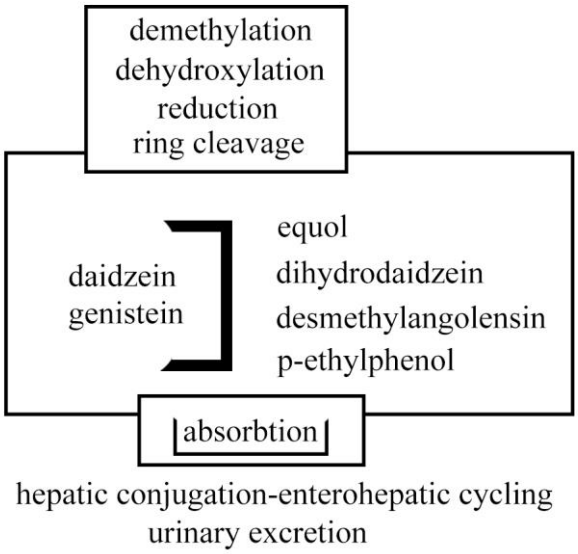


Figure 3: Major biotransformations in the metabolism of isoflavones in humans and animals (Setchell and Cassidy, 1999)

However, not everybody can produce equol. It is reported that it can be produced only by 20-60% of healthy adults (Yokohama and Suzuki, 2008). Whether you can or cannot produce equol depends on the composition and the enzymatic capability of your gut microflora (Heinonen et al., 2002) and also on high-carbohydrate environment, which causes increased intestinal fermentation, resulting in more extensive biotransformation of

phytoestrogens as well as formation of equol (Setchell and Cassidy, 1999). Daidzein is hydroxylated in the liver by the action of the phase II enzyme cytochrome P450 to produce 6,7,4'-trihydroxyisoflavone (demethyltexasin) and 7,3',4'-trihydroxyisoflavone (Seo et al., 2013).

Both equol and demethyltexasin showed beneficial impact on human health. Equol has antioxidative and estrogenic properties, reduces breast cancer risk and it could also play a role in reducing prostate cancer (Choi and Kim, 2014; Goodman et al., 2009; Lund et al., 2011; Morton et al., 1997; Setchel et al., 2002). Demethyltexasin was found to inhibit proliferation of breast cancer, has antioxidative properties, suppresses adipogenesis and inhibits colon cancer (Lee et al., 2011; Lee and Lee, 2013; Roh et al., 2011; Seo et al., 2013)

Several gut bacteria were identified to facilitate the metabolism of isoflavonoids, such as *Bifidobacterium longum*, *Clostridium* spp., *Eggerthella* spp., *Lactobacillus rhamnosus*, *Lactococcus garviea*, *Slackia* spp., and *Streptococcus* spp. (Sanchez-Calvo et al., 2013). Demethyltexasin was also found in foods such as doenjang, a traditional Korean fermented soybean paste, and tempeh, a fermented soybean food from Indonesia, where it is produced by *Arthrobacter* spp., *Brevibacterium epidermidis* and *Micrococcus* spp. (Klus and Barz, 1995; Klus et al., 1993; Roh et al., 2011).

In the metabolism of isoflavonoids, the importance of the microflora is illustrated by observations that antibiotic administration blocks metabolism and that infants fed soy infant formulas in the first four months of life, when gut microflora is underdeveloped, cannot form significant amounts of equol (Blair et al., 2003; Cruz et al. 1994; Setchell and Cassidy, 1999; Setchell et al. 1998).

1.3.3. Biological effects of isoflavonoids

Isoflavonoids have been studied extensively and have been found to possess numerous biological activities. Soybean isoflavonoids (such as biochanin A, daidzein, genistein, formononetin or a metabolite of daidzein - equol) act as phytoestrogens and may be useful in the treatment of menopausal symptoms (such as hot flashes and osteoporosis) or the prevention of cardiovascular diseases by reduction of blood lipids and blood pressure. Isoflavonoids also possess anti-carcinogenic properties against breast and prostate cancer, their estrogen-like properties, however, rise concerns over possible adverse effects in women with breast cancer or at increased risk of the disease. Many of the health-

promoting benefits of isoflavonoids have been linked to the ability of phenolics to serve as antioxidants (Magee et al., 2010; Morton et al., 1997; Patisaul and Jefferson, 2010; Preedy, 2012; Shetty et al., 2006). Isoflavonoids possess also rhizobial properties, acting as chemoattractants for rhizobia and induce the expression of bacterial nod genes (Cooper et al., 2004; Dakora and Phillips, 1996).

1.3.3.1. Antimicrobial activity

Isoflavonoids can act as phytoalexins and phytoanticipins, possessing antimicrobial activity (Dakora and Phillips, 1996). Phytoalexins are usually defined as: "low molecular weight antimicrobial compounds that are both synthesized by and accumulated in plants after exposure to microorganisms". Excluded from this definition are antibiotic compounds present in plant tissue before microbial infection and those that are produced from preexisting constituents after infection. Such antibiotics are called **phytoanticipins**. Thus, the difference between a phytoalexin and a phytoanticipin is not based on their chemical structure but on how they are produced. It is possible that the same chemical may serve both as phytoalexin and phytoanticipin, even in the same plant (VanEtten et al., 1994). Both phytoalexins and phytoanticipins can also be synthetized after exposure to other stressor (e.g. heavy metals, UV light, herbicides or low nutrient availability). Isoflavonoids can act both as phytoalexins and phytoanticipins (Dakora and Phillips, 1996). The examples of phytoalexins in legumes are shown in Table 6. Isoflavonoid phytoalexins are also involved in symbioses between leguminous plants and soil microorganisms (Reynaud et al., 2005). Isoflavonoid phytoalexins were observed also in *Iris pseudacorus* (*Iridaceae*), producing fungi-toxic stress metabolites (Hanawa et al., 1991). Non-flavonoid phytoalexins are for example stilbenes, benzofurans and furanoacetylenes (Dakora and Phillips, 1996).

Because of the well-known antimicrobial nature of isoflavonoids, many of their representatives, mainly isoflavones, isoflavans and other subgroups such as arylcoumarins and pterocarpans, have been investigated against various human pathogens (El-Seedi and Hesham, 2007; Fukai et al., 2002a; Fukai et al., 2002b; Kuete et al. 2008; Nkengfack et al., 1994; Sato et al., 2006). Unless stated otherwise, the antimicrobial effect in the following text will be evaluated according to standards for *in vitro* antimicrobial effect of natural compounds proposed by Rios and Recio (2005). These standards state that concentrations of compounds higher than 100 μg/ml should be avoided. On the other hand the presence of

activity in concentrations lower than 10 μg/ml is considered very interesting.

Table 6: Representative isoflavonoid phytoalexins in legumes (Dakora and Phillips, 1996)

Pathogen	Inducing microbe	Legume	Phytoalexin
Fungal	*Aschocyta imperfecta*	*Medicago sativa*	coumestrol, daidzein, formononetin
	Phytopthora infestans	*Phaseolus vulgaris*	phaseollin
	Phytophthora vignae	*Vigna unguiculata*	coumestrol, daidzein
	Penicillium expansum		coumestrol
Bacterial	*Pseudomonas glycinea*	*Glycine max*	coumestrol, daidzein
	Pseudomonas spp.	*Phaseolus vulgaris*	coumestrol
Viral	*Tobacco necrosis virus*	*Phaseolus vulgaris*	phaseollin
Nematode	*Pratylenchus scribneri*	*Phaseolus lunatus*	coumestrol

Isoflavones

Among a number of previously tested structures, 8-(*γ*,*γ*-dimethylallyl)-wighteone, erythrinin B, isolupalbigenin, neobavaisoflavone, laburnetin, licoisoflavone B and lupalbigenin showed strong antimicrobial effect against *S. aureus* with MIC (minimum inhibitory concentration) values lower than 10 μg/ml. **Isolupalbigenin** isolated from *Erythrina poeppigiana*, showed MICs against methicillin-resistant *S. aureus* ranges from 1.56 to 3.13 μg/ml, which is comparable to the vancomycin antibiotic used as control (MIC = 0.78-3.13 μg/ml) (Sato et al., 2006).

Lupalbigenin and **derrisisoflavone** isolated from *Derris scandens* showed good antimicrobial activity against *S. aureus* and MRSA with MICs ranging from 2 to16 μg/ml (Mahabusarakam et al., 2004).

Laburnetin isolated from *Ficus chlamydocarpa* possesses MIC of 0.61 μg/ml against *Mycobacterium smegmatis* (control ciprofloxacin has the same MIC). Laburnetin was found to be effective both against the following Gram-positive as well as Gram-negative bacteria: *Bacillus stearothermophilus, Citrobacter freundii, Enterobacter cloacae, Morganella morganii, Proteus mirabilis, Salmonella typhi, S. aureus* and *Streptococcus faecalis* (MICs ranging from 19.53 to 78.12) (Kuete et al. 2008).

Biochanin A is another prospective and widely tested isoflavone. Lechner et al. (2008) found out that although biochanin A showed moderate growth-inhibitory activity (MIC = 256 μg/ml) against *Mycobacterium smegmatis*. Furthermore, biochanin A has a selective antimicrobial activity, inhibiting *Clostridium* spp. (MIC = 64-1024 μg/ml) but not affecting the growth of the beneficial bowel microflora (*Bifidobacteria* spp.) at a

concentration of up to 4096 μg/ml (Sklenickova et al., 2010).

In a study of Lechner et al. (2008) isoflavones called **formononetin** and **genistein** showed the same MICs as biochanin A (MIC = 256 μg/ml) against *M. smegmatis.* In contrast to this study, Kuete et al. (2008) found genistein to be even more effective against *M. smegmatis* and furthermore also against *M. tuberculosis* (both MICs = 19.53 μg/ml). The antimicrobial properties of genistein were proved to be efficient both against Gram-positive and Gram-negative bacterial strains such as *B. brevis, E. cloacae, M. morganii, S. aureus* and *S. epidermidis*, and also fungi *Candida albicans* and *C. glabrata* (MICs ranging from 17.5 to 78.12 μg/ml) (Kuete et al., 2008; Madan et al., 2009; Redko et al., 2007). Another isoflavone that was also found to be effective against *B. brevis* (MIC = 35 μg/ml) was **daidzein** (Redko et al., 2007). All four isoflavones (biochanin A, daidzein, formononetin and genistein) possess also antigiardial properties, with formononetin being the most effective compound followed by daidzein, biochanin A and genistein (Lauwaet et al., 2010).

Alpinumisoflavone, isolated from *Ficus chlamydocarpa,* showed the same antimycobacterial effect against *M. smegmatis* as genistein. It also showed antimicrobial activity against the following Gram-positive and Gram-negative bacteria: *E. cloacae, M. morganii, P. mirabilis, S. aureus, B. stearothermophilus* and fungi *C. albicans* and *C. glabrata* (MICs ranging from 39.06 - 78.12 μg/ml) (Kuete et al., 2008).

5,7,4'-trihydroxy-8,2',5'-tri(3-methylbut-2-enyl) isoflavone isolated from *Flemingia strobilifera* is a potent isoflavone with MICs ranging from 28 to 36 μg/ml for *S. aureus*, MRSA and *S. epidermidis* (Madan et al., 2009).

Several isoflavones possessing a good antimicrobial activity were isolated from *Erythrina* spp. As was mentioned above, **isolupalbigenin** obtained from *E. poeppigiana* showed great activity. Another potent isoflavone from the same plant species is **erythrinin B** that is efficient against methicillin-resistant *S. aureus* (MIC = 6.25-12.5 μg/ml) (Sato et al., 2006). Excellent properties were also shown by **neobavaisoflavone** isolated from *E. eryotricha*, which had great results against *S. aureus* with MIC 2.5 μg/ml. That was even better than streptomycin as control (Nkengfack et al., 1994).

Erysubin isolated from *E. subumbrans* showed good antimicrobial activity (MIC = 50 μg/ml) against *S. aureus*, one strain of vancomycin resistant *S. aureus* and *Streptococcus sorbinus* (Rukachaisirikul et al., 2007). Isoflavones from *E.* × *bidwillii*, **8-*γ,γ*-dimethylallyldaidzein** and **auriculatin,** showed very good antimicrobial activity against

oral microorganism *Fusobacterium nucleatum*, with MIC = 6.25 and 3.2 μg/ml, respectively. On the other hand, it was not potent against other oral microorganisms at tested concentration of 50 μg/ml (including *Lactobacillus casei* and *L. fermentum*) (Iinuma et al., 1994).

Isoflavones obtained from *Gylcyrrhiza* spp. also possess interesting antimicrobial activity. One such compound is **6,8-diisoprenyl-5,7,4'-trihydroxyisoflavone** from *G. uralensis* with MIC = 2 μg/ml against oral Gram-positive bacterium *Streptococcus mutans* (He et al., 2006). Another potent isoflavone isolated from the same plant species is **licoisoflavone B** with MIC = 12.5 μg/ml against methicillin-sensitive and methicillin-resistant *S. aureus* strains and with MIC = 6.25 μg/ml against *Helicobacter pyroli* strains (Fukai et al., 2002a; Fukai et al., 2002b).

Hatano et al. (2000) found out that **8-(*γ*,*γ*-dimethylallyl)-wighteone** (MIC = 8 μg/ml) and **gancaonin, glisoflavone, glycoricone, glycyrrhisoflavone, isoangustone A, isowighteone** and **semilicoisoflavone B** (MIC = 16 - 64 μg/ml) from licorice are effective against MSSA and MRSA strains.

Isoflavans

The most potent isoflavans are **glabridin, licoricidin** and **glyasperin D**, isolated from *G. glabra* and *G. uralensis*, possessing very good antimicrobial properties against methicillin-sensitive and methicillin-resistant *S. aureus* (MICs ranging from 6.25 to 12.5 μg/ml) and also *B. subtilis* (MICs = 3.13-6.25 μg/ml). The best results were showed by licoricidin. These isoflavanes are also active against *H. pyroli* (MICs ranging from 6.25 to 25 μg/ml, licoricidin being the most efficient) (Fukai et al., 2002a; Fukai et al., 2002b). Other very potent isoflavans against *S. aureus* and MRSA are **amorphaquinone** and **pendulone**, isoflavan quinones obtained from *Abrus schimperi* with MICs ranging from 2.5 to 20 μg/ml. These two isoflavans also showed antileishmanial and antiplasmodial activity (Rahman et al., 2011). A different potent antistaphylococcal isoflavan is the **astragalaquinone,** obtained from *Astragalus alexandrinus* and *A. trigonus*, which is effective against *B. subtilis*, *Mycobacterium luteus* and *S. aureus* (MIC = 100, 100 and 10 μg/ml, respectively) (Elsebakhy et al., 1994).

Neovestitol and **vestitol,** two potent isoflavones obtained from Brazilian red propolis (*Dalbergia ecatosphyllum* being its plant source), showed promising antibacterial activity with MICs ranging from less than 6.25 to 100 μg/ml (neovestitol being the more

potent one) against *Actinomyces naeslundii, S. aureus, Streptococcus mutans and S. sorbinus* (Bueno-Silva et al., 2013). Vestitol is also found in licorice and possesses antimicrobial activity against *H. pyroli* (MICs ranging from 12.5-25 μg/ml) (Fukai et al., 2002b).

Isoflavanones

In several studies potent antimicrobial isoflavanones were isolated from *Erythrina* spp. Best antistaphylococcal activity is showed by **orientol F** from *E. variegata* against MRSA (MIC = 3.13-12.5 μg/ml) and **eriotrichin B** isolated from *E. eriotricha* with MIC = 8.3 μg/ml against *S. aureus*. This MIC was almost as good as penicillin control (6 μg/ml) (Tanaka et al., 2002; Nkengfack et al., 1995). **Bidwillon A** isolated from *E.* × *bidwillii* produced good antimicrobial activity against oral bacteria, such as *F. nucleatum, Porphyromonas gingivaris, Prevotella intermedia* and *Actinobacillus actinomycetemcomitans* (MICs ranging from 3.2-50 μg/ml) (Iinuma et al., 1992).

Four isoflavanones (namely 5,7-dihydroxy-2'-methoxy-3',4'-methylenedioxyiso-flavanone, 4',5-dihydroxy-2',3'-dimethoxy-7-(5-hydroxyoxychromen-7yl)-isoflavanone, isoferreirin and parvisoflavanone) isolated from *Uraria picta* possess good antimicrobial activity in the range of MICs from 12.5 to 100 μg/ml against *B. subtilis, Escherichia coli, Proteus vulgaris* and *S. aureus,*. **Isoferreirin** and **parvisoflavanone** were the most potent ones against *S. aureus*. All four isoflavanones mentioned above also showed antifungal activity (Rahman et al., 2007).

Desmodianone A and **B** from *Desmodium canum* possessed inhibitory effect (3-10 μg/ml) against *S. aureus*. They were also active against *B. subtilis, M. smegmatis* and *Streptococcus faecalis* (1-30 μg/ml) (DelleMonache et al., 1996). **Dalversinol A** from *Dalea versicolor* inhibited *S. aureus* with MIC = 31.3 μg/ml (Belofsky et al., 2004). Hatano et al. (2000) learned that **3'-(*γ,γ*-dimethylallyl)-kievitone** (MIC = 8 μg/ml) and **glicoisoflavanone**, **glyasperin F**, **glycyrrhisoflavanone** and **licoisoflavanone** (MIC = 32-64 μg/ml) from licorice are effective against MSSA and MRSA strains.

Pterocarpans

Almost all pterocarpans possessing good antimicrobial activities mentioned in current literature are isolated from *Erythrina* spp. **Erythrabyssin II,** isolated from *E.* × *bidwillii, E. sigmoidea* and *E. subumbrans,* was the most widely tested and one of the most

efficient of all pterocarpans. It is active against *S. aureus* (MIC = 3.1 μg/ml), MRSA (MIC = 0.78-6.25 μg/ml), VRSA (MIC = 3.13-12.5 μg/ml), five *Streptococcus* spp. (MIC = 0.78-3.13 μg/ml) and the following oral bacteria: *F. nucleatum, Porphyromonas gingivaris* and *Prevotella intermedia* with MICs ranging from 6.25 to 50 μg/ml. Other pterocarpans efficient against *S. aureus*, MRSA, VRSA and *Streptococcus* spp. were isolated from *E. subumbrans*. These are: **erybraedin A**, **erycristagallin**, **erystagallin A**, **erythrabissin-1** and **phaseollin** (MICs ranging from 0.78 to 12.5 μg/ml, erycristagallin being the most efficient) (Iinuma et al., 1992; Mitscher et al., 1998; Nkengfack et al., 1994; Rukachaisirikul et al., 2007). Other pterocarpans effective against MRSA are **demethylmedicarpin** and **sandwicensin** (MICs ranging from 6.25 to 50 μg/ml) obtained from *E. poeppigiana,* and 2-(*γ,γ*-dimethylallyl)-6a-hydroxyphaseollidin, cristacarpin, erycristagallin, erystagallin A, eryvarin D, orientanol B, orientanol C, phaseollidin and phaseollin obtained from *E. variegata* (MICs ranging from 3.13 to 100 μg/ml, with **2-(*γ,γ*-dimethylallyl)-6a-hydroxyphaseollidin**, **erycristagallin** and **orientanol B** being the most potent ones). Erycristagallin and orientanol were also found to be effective against vancomycin resistant enterococci (MIC = 6.25 μg/ml). This antibacterial effect may be beneficial while treating MRSA infections because these compounds reduce the risk of developing infections caused by VRE (Sato et al., 2003; Tanaka et al., 2002).

Antistaphylococcal activities were also observed for pterocarpans obtained from *E. mildbraedii* (**erybraedin A**, **B**, **C** and **isoneorautenol**) and *E. eriotricha* (**isoneorautenol, erybraedin A, C, D** and **E**). These are effective against *S. aureus* with MICs ranging from 12.5 to 78.3 μg/ml (Mitscher et al., 1998; Nkengfack et al., 1995). **Glycyrrhizol A** and **B** isolated from *Glycyrrhiza uralensis* showed good antimicrobial activity against oral Gram-positive bacterium *Streptococcus mutans* with respective MICs 1 and 32 μg/ml (He et al., 2006)

Isoflav-3-enes

Glabrene (from *G. glabra* and *G. inflata*) was found to be the most effective compound of this group against MSSA, MRSA and *H. pyroli* (MICs = 12.5 μg/ml). It was also potent against *B. subtilis, Micrococcus luteus, Klebsiella pneumoniae* (6.25-50 μg/ml) (Fukai et al., 2002a; Fukai et al., 2002b). Another good antistaphylococcal agent is **erypoegin A** from *E. poeppigiana*, which was found to inhibit the growth of MRSA (MICs =25-50 μg/ml) (Sato et al., 2003).

Coumestans

Coumestrol, a compound isolated from *E. crista galli,* showed great antimicrobial activity against *B. brevis* with MIC = 4.4 μg/ml (Redko et al., 2007).

Arylcoumarins

Asphodelin A, found in *Asphodelus microcarpus,* exhibited good antibacterial activity against both Gram-positive and Gram-negative bacteria (*S. aureus, E. coli, Pseudomonas aeruginosa*) with MICs ranging from 4 to 16 μg/ml. It also showed antifungal activity (El-Seedi and Hesham, 2007).

Hatano et al. (2000) found **glycycoumarin** and **licoarylcoumarin** from licorice, to be effective against MSSA and MRSA strains (MICs ranging from 16 to 32 μg/ml).

1.3.3.2. Structure-activity relationship and mechanism of action

Differences in core structures between isoflavone, isoflavan, isoflavanone, etc., as well as the presence and position of substituent groups in molecules play an important role in their antibacterial activity. The phenolic hydroxyl groups have an affinity to proteins and therefore act as inhibitors of microbial enzymes as well as their biosynthetic pathways. In addition, substitution of the flavonoid ring system with prenyl groups is thought to increase lipophilicity, which consequently enhances the antibacterial activity through interaction with cellular membranes (Rukachaisirikul, et al. (2007).

The prenyl group

It seems that against Gram-positive bacteria, the presence of a prenyl group at position 6 is crucial (Bojase et al., 2002; Mukne et al., 2011; Sato et al., 2006). Mukne et al. (2011) also state that the removal of the prenyl group or its movement to the C-8 position decrease antimicrobial activity.

On the other hand, Bojase et al. (2002) concluded that the activity is enhanced even when the prenyl group is in position C-8 in A ring and also in position 3' or 5' in B ring. The presence of prenyl group at the C-3' position in the B ring was also confirmed to enhance activity against MRSA. Furthermore, prenyl group at the C-6 position in the A ring also contributes to antimicrobial activity (Sato et al., 2006).

It was also found out that the antimicrobial activity of isoflavones against Gram-

negative bacteria requires a prenyl group at either position 3' or 5' and/or a free hydroxyl group at position 4' and/or 3'. Against fungi a prenyl at position 6 or 8 in A ring and 3' or 5' in the B ring is required. With regard to pterocarpans, the presence of prenyl groups at C-2 or C-4 and C-10 enhances antimicrobial activity (Rukachaisirikul, et al. (2007).

The phenolic hydroxyl groups

According to Mukne et al. (2011), a hydroxyl group should be present at positions C-7 and/or the C-5 position. The hydroxyl group at the C-5 position in the A ring was also confirmed to be essential for anti-MRSA activity (Sato et al., 2006). Also, 2', 4'- or 2', 6'-dihydroxylation of the B ring in the flavanone structure is important for significant antimicrobial activity against MRSA (Tsuchyia et al., 1996). The single presence of a 4'-hydroxylation in the B-ring also enhances activity (Chacha et al., 2005). Removal of the hydroxyl group or its conversion to –CHO abolishes this activity. Methylation of fused pyran ring hydroxyl group decreases antimicrobial activity against *S. aureus* but sharply increases activity against MRSA (Mukne et al., 2011). With regard to pterocarpans, hydroxyl groups at C-3 and C-9 and/or a hydroxyl group at C-6a enhance antimicrobial activity (Rukachaisirikul, et al. (2007). Chacha et al. (2005) also confirmed that for pterocarpans the presence of a hydroxyl group at C-6a is crucial and also that a methoxy group at C-9 was important. On the other hand, in another study the conclusion was different. It seemed that the replacement of the hydroxyl group at C-9 with the methoxy group reduces the antibacterial activity (Rukachaisirikul, et al. (2007). Also, Mukne et al. (2011) concluded that methylation of hydroxyl group together with the absence of prenyl group further decreases antibacterial activity. Antibacterial action of various flavonoids is caused either by (1) inhibiting the nucleic acid synthesis, (2) inhibiting the cytoplasmic membrane function or (3) inhibiting the energy metabolism (Cushnie and Lamb, 2005). Genistein, as one of the most widely investigated isoflavones, inhibits the synthesis of DNA and RNA (genistein inhibits topoisomerase IV that catalyzes the separation of two double-stranded covalently closed circular DNA molecules that are intertwined in a chain) and it also inhibits protein synthesis (Verdrengh et al., 2004; Ulanowska et al., 2006).

1.3.3.3. Synergistic effects of isoflavonoids

Until now, only a few studies focused on the synergistic effect of isoflavonoids and antibiotics were carried out. Biochanin A is the most widely studied compound in relation

to synergy. In combination with ciprofloxacin it showed synergistic effect against *S. aureus* and eleven strains of MRSA (FICI ranged from 0.13 to 0.5). Ciprofloxacin alone had MICs ranging from 1-256 μg/ml and they were reduced to 0.25-32 μg/ml when biochanin was added. Biochanin alone had MICs between 64-512 μg/ml and in combination with ciprofloxacin the MICs were reduced to 0.25-64 μg/ml (Liu et al., 2011). In another study, biochanin A potentiated the activity of berberine (16-fold), norfloxacin (4-fold), *α*-linolenic acid (8-fold) and γ-linolenic acid (3-fold) against *S. aureus* and *B. megaterium*. In this study it was also concluded that potentiation of the antibiotic activity may be caused by the inhibition of a multidrug resistance efflux pump (Morel et al., 2003). A study of Lechner et al. (2008) showed further potentiating activity of biochanin A with ethidium bromide (EtBr) against *M. smegmatis*. Biochanin decreased the MICs of EtBr 4 to 8-fold at the concentration of 10 μg/ml and 16 to 32-fold at the concentration of 32 μg/ml. The FICI of value 0.25 between biochanin A and EtBr proved synergism. It was concluded that the hydroxyl group at C-5 is important for EtBr-modulating and efflux-inhibiting activities of isoflavones.

Genistein also showed some modulating activity when combined with berberine (16-fold), norfloxacin (2-fold), *α*-linolenic acid (4-fold) and *γ*-linolenic acid (2-fold) against *S. aureus* and *B. megaterium* (Morel et al., 2003).

In their study, Sato et al. (2006) combined the isoflavones **laburnetin** and **M-Wi-2** with methicillin and oxacillin and used this combination against MRSA. In all but one strain, the MIC values of methicillin and oxacillin in combination with M-Wi-2 were reduced from ≥100 to 6.25–12.5 μg/ml. Laburnetin also showed some modulating effect in combination with methicillin. The resistance of MRSA to *β*-lactams, including methicillin and oxacillin, is ascribed to the production of penicillin-binding protein 2' (PBP 2'), which is encoded by the *mecA* gene. The production of *β*-lactamase by MRSA strains is also thought to participate in the resistance of MRSA to *β*-lactam antibiotics (Chambers 1997). The ability of M-Wi-2 to intensify the susceptibility of MRSA strains to methicillin and oxacillin is thought to be unrelated to the presence of *mecA* or *β*-lactamase production in MRSA strains. Both M-Wi-2 and laburnetin contain aliphatic hydroxyl groups at the *β*-position of the 6-position substituent. This may be related to their action to intensify methicillin sensitivity (Sato et al., 2006).

Isoflavones, isoflavanones, isoflavans and 3-arylcoumarins [3'-(*γ*,*γ*-dimethylallyl)-kievitone, glabridin, glicoricone, glisoflavone, glycycoumarin, isoangustone A,

isowighteone, licoricidin] from licorice also have modulatory effects on the antibiotic oxacillin against MSSA and MRSA. **Licoricidin** showed the best results and in the presence of 8 μg/ml of this isoflavan MICs of oxacillin decreased to less than 1/128-1/1000 of the values in the absence of this compound. Even the presence of 4 μg/ml of licoricidin was still modulating the antibiotic effect of oxacillin very efficiently. It was concluded that licoricidin does not affect the formation of enzymatic protein 2' (PBP 2') (PBP 2' catalyzes cell wall construction and thus causes the resistance of MRSA against β-lactams). The mechanism for the potentiating effect is unclear but licoricidin may affect the enzymatic function of PBP 2'. However, other possible mechanisms, such as increase in the affinity of oxacillin to this enzymatic protein by licoricidin, are also possible (Hatano et al., 2000).

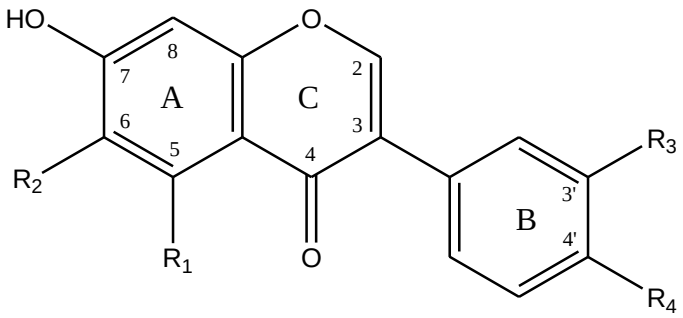
2. Aims of the thesis

Based on the above summarized data showing antimicrobial and synergistic properties of various classes of isoflavonoids, it is possible to suppose that detailed evaluation of selected isoflavone structures and their analogs (e.g. metabolites) could lead to the identification of prospective synergistically acting agents. Therefore, the main aim of this thesis is the evaluation of plant isoflavonoids and their metabolites for their *in vitro* antimicrobial synergistic effect in combination with the conventional antibiotic (oxacillin) against standard strains of *S. aureus*. Another objective is the summarization of data on *S. aureus*, isoflavonoids, antimicrobial activity and synergy.

3. Materials and methods

3.1. Chemicals

Bacteria were grown in Mueller-Hinton broth (MHB; Oxoid, Basingstoke, UK). The final pH 7.6 of the broth has been adjusted by Tris-buffered saline (Sigma–Aldrich, Prague, CZ) and hydrochloric acid 0.1 M (Lach-Ner, Neratovice, CZ). Oxacillin was obtained from Sigma–Aldrich (Prague, CZ) Biochanin A (***1***), demethyltexasin (***2***), genistein (***3***) and hydroxydaidzein (***4***) were purchased from Indofine Chemical Company (Hillsborough, New Jersey, USA). Dimethyl sulfoxide (Penta, Prague, CZ) and deionized water were used as solvents. Structures of tested isoflavones are shown in Figure 4.



No.	Name of compound	R_1	R_2	R_3	R_4
1	5,7-dihydroxy-4'-methoxyisoflavone	OH	H	H	OCH_3
2	6,7,4'-trihydroxyisoflavone	H	OH	H	OH
3	5,7,4'-trihydroxyisoflavone	OH	H	H	OH
4	7,3',4'-trihydroxyisoflavone	H	H	OH	OH

Figure 4: Chemical structures of tested isoflavones

3.2. Bacterial strains

The synergistic effect was evaluated against five *S. aureus* subsp. *aureus* American Typical Culture Collection (ATCC) strains, namely, ATCC® 25923™ (methicillin-sensitive, MSSA), ATCC® 29213™ (methicillin-sensitive, MSSA), ATCC® 33591™ (MRSA), ATCC® 43300™ (MRSA) and ATCC® BAA-976™ (MRSA) which were purchased from Oxoid (Basingstoke, UK).

3.3 Antimicrobial assay

Minimum inhibitory concentrations (MICs) were determined *in vitro* by the broth microdilution method according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2009), modified according to the recommendations proposed for effective assessment of the anti-infective potential of natural products (Cos et al., 2006), using 96-well microtiter plates. Samples of tested isoflavonoids and oxacillin were two-fold diluted in MHB (100 ml) and inoculated with bacterial suspension to reach the final density $5x10^5$ of colony-forming units per milliliter. Microtiter plates were incubated at 37°C for 24 hours. Afterwards, bacterial growth was measured as turbidity by Multiscan Ascent Microplate Photometer (Thermo Fisher Scientific, Waltham, USA) at 405 nm. MICs were expressed as the lowest concentrations that inhibited the growth of the test bacteria by ≥ 80% compared to that of the agent-free growth control. All tests were performed as three independent experiments, each carried out in triplicate. Finally, the mean of the values obtained was calculated. *S. aureus* ATCC 29213 was used as a quality control strain for antibiotic susceptibility testing.

3.4. Synergy evaluation

The fractional inhibitory concentrations (FICs) were evaluated by the checkerboard assays. Two-fold serial dilutions of oxacillin prepared in horizontal rows of the microtiter plate were subsequently cross-diluted vertically by two-fold serial dilutions of demethyltexasin. The combinatory effect was assessed mathematically by determining the fractional inhibitory concentration (FIC), which express mathematically the effect of the combination with antibacterial agents. All tests were performed as three independent experiments, each carried out in triplicate. Subsequently, the mean was assessed. For the combination of compounds A and B, the ΣFIC is calculated according to the following equation:

$$\Sigma FIC = \frac{MICA(\text{combined with B})}{MICA(\text{alone})} + \frac{MICB(\text{combined with A})}{MICB(\text{alone})}$$

(Verma, 2007).

The ΣFICs were then evaluated according to the European Committee on Antimicrobial Susceptibility Testing criteria for synergy as follows: synergistic effect if $\Sigma FIC \leq 0.5$; additive if $\Sigma FIC > 0.5$ to < 1; indifferent if $\Sigma FIC \geq 1$ to < 2, and antagonistic if $\Sigma FIC \geq 2$ (EUCAST, 2000).

4. Results and discussion

MICs determination

Based on the literature data reporting their antimicrobial and synergistic properties (Morel et al., 2003; Lechner et al., 2008; Liu et al., 2011; Sklenickova et al., 2010), we have selected two isoflavones (biochanin A and genistein) for determination of their antimicrobial activity. As representatives of isoflavones' metabolites we have chosen two commercially available compounds (demethyltexasin and hydroxydaidzein). In the broth microdilution assay, demethyltexasin showed promising antibacterial activity against *S. aureus* ATCC 29213 with MIC = 64 μg/ml. The other compounds, however, were not active at the highest tested concentration of 128 μg/ml (Table 7).

Table 7: *In vitro* growth inhibitory effect of isoflavonoids and their metabolites against *Staphylococcus aureus* ATCC 29213

Compound	**MIC[a] (μg/ml)**
Biochanin A	≥ 128
Demethyltexasin	64
Genistein	≥ 128
Hydroxydaidzein	≥ 128
Oxacillin[b]	0.25

[a]MIC - minimum inhibitory concentration, [b]antibiotic positive control

As a next step, we decided to test these compounds at the higher concentration of 1024 μg/ml. However, the solubility of biochanin A, genistein and hydroxydaidzein in growth medium was limited (after being dissolved either in DMSO or ethanol). Therefore, we performed further tests with demethyltexasin (***2***) only. The results of susceptibility testing of *S. aureus* strains to demethyltexasin (MICs = 128 μg/ml) and to the oxacillin (MICs ranging from 0.25 to 256 μg/ml) are shown in Table 8. The resistance to oxacillin was confirmed for three out of the five tested strains [(MIC ≥ 4μg /ml); (CLSI, 2009)].

The antimicrobial effect of isoflavones has been previously confirmed in detail for biochanin A. In the study of Liu et al., (2011) its minimum inhibitory concentrations

against 11 strains of MRSA ranged from 128 to 512 μg/ml and against *S. aureus* ATCC 25923 the MIC was 64 μg/ml. In our test, biochanin A was not effective against the tested *S. aureus* ATCC 29213 at the concentration of 128 μg/ml. However, demethyltexasin was active against this strain (MIC 64 μg/ml).

Table 8: *In vitro* growth inhibitory effect of demethyltexasin against selected strains of *Staphylococcus aureus*

***S. aureus* strain**	**MIC[a] (μg/ml)**	
	Demethyltexasin	**Oxacillin**
ATCC 25923	128	0.25
ATCC 33591 (MRSA)	128	256
ATCC 43300 (MRSA)	128	16
ATCC BAA 976 (MRSA)	128	8

[a]MIC, minimum inhibitory concentration; [b]antibiotic positive control

Evaluation of combinatory effect of demethyltexasin and oxacillin

The individual MICs of demethyltexasin and oxacillin against selected staphylococcal strains as well as the MICs of their combinations and corresponding ΣFICs are summarized in Table 9. For the demethyl-oxacillin combination, the synergistic effect was obtained against three out of five *S. aureus* strains tested. These three strains were also methicillin resistant.

The best result (ΣFIC 0.167) was obtained at the concentration of demethyltexasin of 16 μg/ml against methicillin resistant *S. aureus* ATCC 43300 causing 26-fold reduction in the MIC of oxacillin. Synergy was also achieved at the concentration of demethyltexasin of 32 μg/ml for this bacterium and also for ATCC 33591 and BAA-976 at concentrations of 16 and 32 μg/ml for both bacteria (ΣFIC ranging from 0.250 to 0.424). The oxacillin resistance [(MIC ≥ 4μg /ml); (CLSI, 2009)] was totally broken in the cases of MRSA ATCC 43300 and BAA-976 (demethyltexasin concentration of 16 to 64 μg/ml) and ATCC 33591 (32 to 64 μg/ml).

The biggest reduction of the MIC of oxacillin was observed for the ATCC 43300 strain (171-fold reduction). In addition, our results showed additive antimicrobial effect of demethyltexasin in combination with oxacillin against four out of five *S. aureus* strains tested (all but ATCC 25923) with ΣFIC values ranging from 0.507 to 0.938. The

combination profiles are presented graphically in Figure 5. The isobole curves clearly show the potentiating effect against all but one *S. aureus* strains tested (ATCC 25923) and the synergistic interactions can be read according to the curve indicating the borderline synergy.

The modulating effect of isoflavones has been previously confirmed in detail for biochanin A. In the study of Liu et al., (2011) the synergistic effect was observed in combination with ciprofloxacin against 11 strains of MRSA and for the *S. aureus* ATCC 25923 strain (FICI ranged from 0.13 to 0.5). In our case, demethyltexasin was also synergistically effective against all MRSA strains but did not show synergism against the methicillin sensitive strains ATCC 29213 and 25923, which suggest that the synergistic action of this compound is based on selective inhibition of the specific methicillin resistance.

According to our best knowledge, this is the first report on the synergy and additive effect of demethyltexasin with oxacillin against *S. aureus* strains. These results suggest demethyltexasin as a promising compound acting potentially in combination with the *β*-lactam antibiotic. Oxacillin is a typical anti-staphylococcal agent and its combination with demethyltexasin, therefore, seems to be very perspective.

Table 9: *In vitro* inhibitory activity of demethyltexasin in combination with oxacillin against *S. aureus*

		MIC (µg/ml) of compound alone		OXA with DMT at following concentrations (µg/ml)							
				64		32		16		8	
S. aureus strain	Test	DMT	OXA	MIC	ΣFIC	MIC	ΣFIC	MIC	ΣFIC	MIC	ΣFIC
ATCC 43300 (MRSA)	1	128	32	0.125	0.504	0.125	0.254	1	0.156	16	0.563
	2	128	16	0.125	0.508	0.250	0.266	0.5	0.156	16	1.063
	3	128	16	0.125	0.508	0.125	0.258	1	0.188	16	1.063
	Mean	128	21.333	0.125	0.507	0.167	**0.259**	0.833	**0.167**	16	0.896
ATCC 33591 (MRSA)	1	64	256	2	1.008	2	0.508	32	0.375	256	1.125
	2	64	256	2	1.008	2	0.508	2	0.258	128	0.625
	3	128	256	2	0.508	2	0.258	64	0.375	256	1.063
	Mean	85.333	256	2	0.841	2	**0.424**	32.667	**0.336**	213.333	0.938
ATCC BAA-976 (MRSA)	1	128	16	0.125	0.508	0.250	0.266	2	0.250	16	1.063
	2	128	8	0.125	0.516	0.250	0.281	1	0.250	4	0.563
	3	128	8	0.125	0.516	0.250	0.281	1	0.250	4	0.563
	Mean	128	10.667	0.125	0.513	0.250	**0.276**	1.333	**0.250**	8	0.729
ATCC 29213	1	64	0.125	0.002	1.016	0.063	1	0.250	2.250	0.250	2.125
	2	64	0.250	0.002	1.008	0.002	0.508	0.250	1.250	0.250	1.125
	3	128	0.250	0.031	0.625	0.500	2.250	0.5	2.125	0.5	2.063
	Mean	85.333	0.208	0.012	0.883	0.188	1.253	0.333	1.875	0.333	1.771
ATCC 25923	1	128	0.250	0.063	0.750	0.250	1.125	0.250	0.125	0.250	1.063
	2	64	0.125	0.031	1.250	0.250	2.250	0.250	2.125	0.250	2.063
	3	64	0.250	0.250	2	0.5	2.5	0.250	1.250	0.250	1.063
	Mean	85.333	0.208	0.115	1.333	0.333	1.958	0.250	1.167	0.250	1.396

DMT, demethyltexasin; OXA, oxacillin; ATCC, American Type Culture Collection; MRSA, methicillin resistant *S. aureus*; ΣFIC, sum of fractional inhibitory concentrations: synergistic effect ΣFIC ≤ 0.5 (bold font).

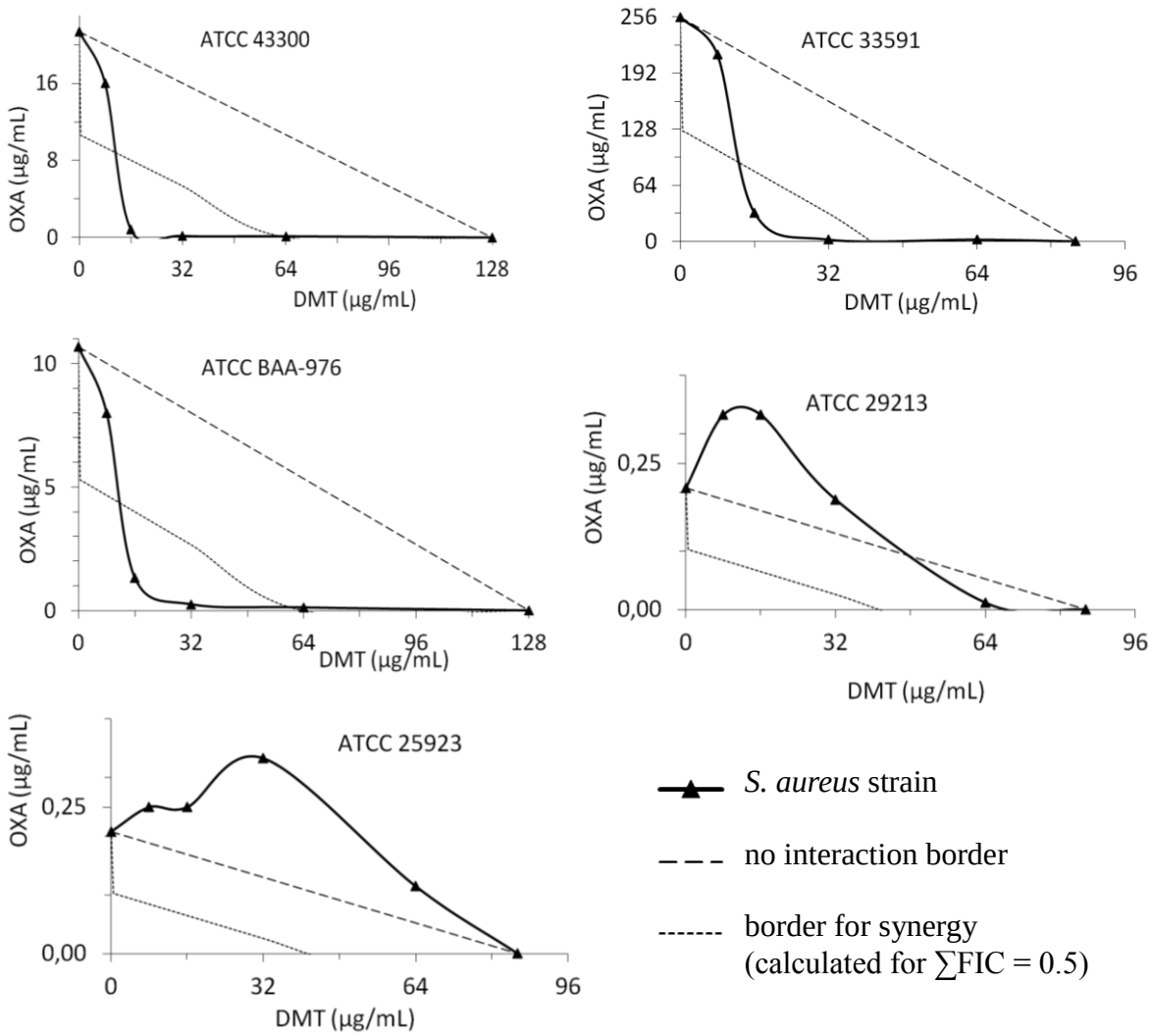


Figure 5: Isobolograms for the interaction of demethyltexasin (DMT) and oxacillin (OXA) against *S. aureus* strains.

Mechanism of action of demethyltexasin

Generally, the phenolic hydroxyl groups have an affinity to proteins and act, therefore, as inhibitors of microbial enzymes as well as their biosynthetic pathways (Rukachaisirikul, et al. (2007). The position of a hydroxyl group at C-7 and/or C-5 is important for antibacterial activity (Mukne et al., 2011). In our case, all the compounds tested had a hydroxyl group at the C-7 position, but only demethyltexasin showed good antimicrobial activity. Also, all the compounds had a hydroxyl group at the C-4' position, which is supposed to increase effectiveness (Chacha et al., 2005). Demethyltexasin, in comparison to the other tested compounds, has a hydroxyl group also at the C-6 position, suggesting that hydroxyl at this position plays an important role in the antibacterial action of isoflavonoids.

Synergism was achieved in methicillin-resistant strains (added concentration of demethyltexasin of 16 and 32 μg/ml), but not in the sensitive ones (ATCC 29213, 25923). As mentioned before, the resistance has been overcome in these MRSA strains. This suggests that demethyltexasin can selectively potentiate the effect of the oxacillin against MRSA. It may affect the function of PBP 2' protein or increase the affinity of oxacillin to this protein. It is also possible that demethyltexasin affects the production of β-lactamases (both mechanisms of resistance are typical for MRSA strains) (Chambers et al., 1997 Mulligan et al., 1993). Further research with demethyltexasin should be performed to assess the exact mechanism of action in synergism.

Toxicity and possible utilization

Generally, the toxicity of flavonoids is very low in animals. In rats, the LD_{50} ranges between 2 to 10 g per animal. When we take into account that a soybean contains approximately 30 mg/100 g of isoflavones (depending on the way of preparation for consumption) and apply it to humans, toxicity in normal diet is quite unrealistic (Bhagwat et al. 2008; Havsteen, 1983).

Furthermore, foods containing isoflavones, including dietary supplements, are recommended in the hormone replacement therapy (Dijsselbloem et al., 2004). In one of such dietary supplements, Menoflavon® forte (extract from red clover), the dosage of isoflavones is 80 mg per day and adverse effects are not known (Anonymous 2011; Anonymous 2014b). Since demethyltexasin occurs in fermented soy-based foods, such as doenjang and tempeh (Klus and Barz, 1995; Roh et al., 2011) and is excreted in urine

(Kulling et al., 2001), it is not considered to be toxic to humans.

Demethyltexasin combined with an antibiotic may be developed into effective antibiotic agents. Since it is present in soy-based foods and is also produced in the human liver as a metabolite of daidzein, which can be found in many leguminous species (Klus and Barz, 1995; Mazur, 1998; Roh et al., 2011; Seo et al., 2013), it seems that the consumption of demethyltexasin and daidzein containing foods could influence antibiotic therapy.

5. Conclusion

Isoflavonoids, compounds occurring mainly in leguminous plants possess various biological properties beneficial for human health such as phytoestrogenic, anticancer, antioxidative and antimicrobial effects. In this study, antistaphylococcal and synergistic properties of selected plant isoflavones (biochanin A and genistein) and two isoflavone metabolites (demethyltexasin and hydroxydaidzein) have been tested for their direct and synergistic antimicrobial effects. As a result, demethyltexasin was found to be a promising compound enhancing the effect of oxacillin. This combination shows synergistic and additive effect against most *S. aureus* strains tested and significantly overcomes staphylococcal resistance to oxacillin. Our findings suggest demethyltexasin as a prospective compound for the development of new synergistically acting anti-staphylococcal agents, effective also against the methicillin resistant strains. However, further research focused on susceptibility of clinical isolates of *S. aureus*, combinations with other antibiotic agents and their delivery techniques will be needed prior to its possible pharmacological application. Our findings could contribute to the development of new preparations designed to overcome the resistance of *S. aureus*.

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