CZECH UNIVERSITY OF LIFE SCIENCES PRAGUE

Faculty of Tropical AgriSciences

Department of Crop Sciences and Agroforestry

Anti-inflammatory and Antimicrobial Activity of Isoflavonoids

DOCTORAL THESIS

Author: Ing. Jana Hummelová

Thesis supervisor: prof. Ing. Ladislav Kokoška, Ph.D.

2018

ACKNOWLEDGEMENT

I would like to thank the Grant Agency of the Czech Republic (project no. GACR 525/09/0994) for the financial support of this work. I express my sincere thanks to Ing. Přemysl Landa, Ph.D. from the Laboratory of Plant Biotechnologies, Institute of Experimental Botany, Academy of Sciences, Czech Republic, for his precious help with the anti-inflammatory experiments, and to Ing. Johana Rondevaldová, Ph.D. from the Laboratory of Ethnobotany and Ethnopharmacology, Faculty of Tropical AgriSciences, Czech University of Life Sciences Prague, for her invaluable help with the antimicrobial experiments.

I want to, above all, sincerely thank my thesis supervisor, prof. Ing. Ladislav Kokoška, Ph.D., for his encouragement and thoughtful suggestions and guidance on the substantive aspects of this work.

CERTIFICATION

I, hereby undersigned Jana Hummelová, declare that this Dissertation thesis, submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy (Ph.D.) in the Faculty of Tropical AgriSciences, Czech University of Life Sciences Prague, is wholly my own work unless otherwise referenced or acknowledged. The document has not been submitted for qualifications at any other academic institute and I agree with its publication.

Prague, October 30, 2018

…….

Ing. Jana Hummelová

ABSTRACT

In this study, 19 structurally related isoflavonoids were tested for their possible anti-inflammatory and antimicrobial properties. The first part of the research was focused on the assessment of anti-inflammatory activity of the isoflavonoids expressed as an inhibition of cyclooxygenase-2 (COX-2) and 5-lipoxygenase (5-LOX) enzymes *in vitro*. None of the tested compounds was active at least half as much as indomethacin, a standard COX inhibitor. The results showed that genistein inhibited the activity of COX-2 enzyme by 23.6 %, 39.9 % and 25.5 % in concentrations 50, 100, and 150 μM, respectively. In contrast, the most of the isoflavonoid structures exhibited 5-LOX inhibitory effect, whereas the most promising results were observed in biochanin A and formononetin, which inhibited the leukotrienes B_4 (LTB_4) production by 88.2 % and 78.9 % at 100 μM. For comparison, zileuton, a generally used inhibitor of 5-LOX enzyme, inhibited the LTB_4 production by 95.4 % at the same concentration. In the second part, the *in vitro* antibacterial activity of isoflavonoids against nine Gram-positive and Gram-negative bacteria was determined using the broth microdilution method, and the results were expressed as minimum inhibitory concentrations (MICs). Demethyltexasin, hydroxydaidzein, biochanin A, demethylretusin and genistein produced significant antibacterial activity (MICs $\geq$ 16 μg ml^{-1}). The most effective compound, demethyltexasin, was subsequently tested for its growth-inhibitory effect against *Staphylococcus aureus*, and it exhibited significant antistaphylococcal effects against various standard strains and clinical isolates, including methicillin and tetracycline resistant ones with the MICs ranging from 16 to 128 μg ml^{-1}. Based on its inhibitory activity against *S. aureus* resistant infections, which are often hard to treat due to increasing resistance, especially to β-lactams, demethyltexasin was tested for its possible antistaphylococcal combinatory effects with antibiotics amoxicillin and oxacillin using checkerboard method. For comparison, common therapeutically used combination of amoxicillin/clavulanic acid was tested. Demethyltexasin showed strong synergistic interactions against most of *S. aureus* strains when combined with amoxicillin [sum of fractional inhibitory concentrations ($\sum$ FIC) 0.257–0.461] and oxacillin ($\sum$ FIC 0.109–0.484). When oxacillin was combined with demethyltexasin, resistance to this antibiotic was overcome in many cases. Moreover, antibiotic/demethyltexasin combinations were effective mainly against methicillin-resistant *S. aureus* (MRSA); whereas the commonly used drug amoxicillin/clavulanic acid was effective only against sensitive strains. The results indicated demethyltexasin as a compound able to act synergistically with β-lactams. In addition, some combinations are effective against MRSA

and decrease staphylococcal resistance. Demethyltexasin seems to be a possible candidate for further research focused on antistaphylococcal drug development, especially against antibiotic-resistant strains. Additionally, biochanin A proved certain dual inhibitory effect against both 5-LOX activity and against some bacteria strains. These activities should be verified by future experiments, focused on its possible dual effect *in vivo*. As biochanin A is widely distributed in plants and food, mainly from Leguminosae family, the dual effect can be used in future as synergistic and/or concomitant treatment of infections and inflammation.

Key words: 5-lipoxygenase, isoflavones, structure-activity relationship, MICs, FICs

ABSTRAKT

V této studii bylo testováno 19 isoflavonoidů podobných svou strukturou z hlediska jejich možných protizánětlivých a antimikrobiálních účinků. První část výzkumu byla zaměřena na hodnocení protizánětlivé aktivity isoflavonoidů vyjádřených jako inhibice enzymů cyklooxygenázy-2 (COX-2) a 5-lipoxygenázy (5-LOX) *in vitro* pomocí leukotrien B_4 (LTB_4) kolorimetrické kompetitivní enzymové imunoeseje. Ani jedna ze zkoumaných látek testovaných v koncentracích 50, 100, a 150 μM nedosahovala ani poloviční účinku standardního inhibitoru COX indometacinu. Výsledky ukázaly, že genistein inhiboval aktivitu enzymu COX-2 na výše uvedených koncentracích o 23,6, 39,9 a 25,5%. Naproti tomu většina testovaných isoflavonoidů vykazovala 5-LOX inhibiční účinek, kde byly nejslibnější výsledky pozorovány u biochaninu A a formononetinu, které inhibovaly produkci LTB_4 o 88,2 % a 78,9 % při koncentraci 100 μM. Zileuton, což je obecně používaný inhibitor enzymu 5-LOX, inhiboval produkci LTB_4 o 95,4 % ve stejné koncentraci. Ve druhé části byla testována *in vitro* antibakteriální účinnost vybraných látek proti devíti grampozitivním a gramnegativním bakteriím za použití bujónové mikrodiluční metody a výsledky byly vyjádřeny jako minimální inhibiční koncentrace (MIK). Demetyltexasin, hydroxydaidzein, biochanin A, demetylretusin a genistein ukázaly významnou antibakteriální aktivitu (MIK ≥ 16 μg ml^{-1}). Nejúčinnější látka demetyltexasin vykázala významné antistafylokokové účinky proti různým standardním kmenům a klinickým izolátům bakterie *Staphylococcus aureus*, včetně těch, které jsou rezistentní na meticilin a tetracyklin, s MIK od 16 do 128 μg ml^{-1}. Na základě inhibiční aktivity demetyltexasinu proti rezistentním infekcím způsobené bakterií *S. aureus*, které jsou často obtížně léčitelné kvůli zvyšující se odolnosti, zejména vůči β-laktamům, byl demetyltexasin testován na možný antistafylokokový kombinační účinek s antibiotiky amoxicilinem a oxacilinem za použití šachovnicové metody. Pro srovnání byla testována běžná terapeuticky používaná kombinace amoxicilinu a kyseliny klavulanové. Demetyltexasin vykazoval silné synergické interakce proti většině kmenů *S. aureus* při kombinaci s amoxicilinem [součet frakčních inhibičních koncentrací (Σ FIK) 0,257-0,461] a oxacilinu (Σ FIK 0,109-0,484). Když byl oxacilin zkombinován s demetyltexasinem, rezistence k tomuto antibiotiku byla v mnoha případech překonána. Kromě toho, kombinace antibiotik a demetyltexasinu byly účinné zejména proti meticilin-rezistentnímu *S. aureus* (MRSA); avšak běžně užívaný lék amoxicilin a kyselina klavulanová byl účinný pouze proti citlivým kmenům. Výsledky ukázaly, že demetyltexasin je sloučenina schopná působit synergicky s β-laktamovými antibiotiky. Kromě toho jsou některé kombinace účinné proti

MRSA a snižují stafylokokovou rezistenci. Zdá se, že demetyltexasin je možným kandidátem pro další výzkum zaměřený na vývoj antistafylokokových léků, zejména proti kmenům odolným vůči antibiotikům. Biochanin A navíc ukázal jistý duální inhibiční účinek jak proti aktivitě 5-LOX, tak proti některým kmenům bakterií. Tyto účinky by měly být ověřeny dalšími experimenty zaměřenými na možný duální účinek *in vivo*. Vzhledem k tomu, že biochanin A je hojně rozšířen v rostlinách a potravinách, především z čeledi bobovitých, duální účinek může být v budoucnu použit jako synergická a/nebo souběžná léčba infekcí a zánětů.

Klíčová slova: 5-lipoxygenáza, isoflavony, biologický účinek na základě struktury látky, MIK, FIK

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

5-LOX	5-lipooxygenase
AA	arachidonic acid
anti-LTs	anti-leukotrienes
CNS	central nervous system
CoA	coenzyme A COX cyclooxygenase
COX-1	cyclooxygenase-1
COX-2	cyclooxygenase-2
CZ	Czech Republic
DE	Germany
DMSO	dimethylsulfoxide
DT	demethyltexasin
EFA	essential fatty acid
FIC	fractional inhibitory concentration
FLAP	Five Lipoxygenase Activating Protein
GI	gastrointestinal
IC_{50}	half maximal inhibitory concentration
IFN-γ	interferon-γ
IL-1β	interleukin-1β
IL-6	interleukin-6
IL-8	interleukin-8
IL-12	interleukin-12
LTs	leukotrienes
LTB_4	leukotriene B4
LTC_4	leukotriene C4
LTD_4	leukotriene D4
LTE_4	leukotriene E4

MIC	minimum inhibitory concentration
mRNA	messenger ribonucleic acid
MRSA	methicillin-resistant *Staphylococcus aureus*
NF-κB	nuclear factor-κB
NSAIDs	nonsteroidal anti-inflammatory drugs
PAL	phenylalanine ammonia lyase
PGs	prostaglandins
PK/PD	pharmacokinetic/pharmacodynamics
PLA_2	phospholipase A_2
rpm	revolutions per minute
TFA	trifluoroacetic acid
TNF-α	tumour necrosis factor-α
TNF-β	tumour necrosis factor- β
tNSAIDs	traditional nonsteroidal anti-inflammatory drugs
TXA_2	thromboxane A_2
USA	United States of America
US FDA	United States Food and Drug Administration

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Acute arthritis, example of human musculoskeletal infection caused by *S. aureus*, is relatively seldom in general population, but the incidence is considerably higher in patients with predisposing conditions, particularly those with rheumatoid arthritis, a chronic inflammatory disorder. From this example, it is obvious that focus on anti-inflammatory and antimicrobial potential is crucial for future development of natural-derived products used for treatment of both, infections and inflammation.

As flavonoids, and particularly isoflavonoids, are natural phenolic compounds, their vast biological activities have been already proved. There are several studies proving antimicrobial and anti-inflammatory activities of isoflavonoids *in vitro*, *in vivo* and also in clinical trials. The potential of isoflavonoids may also be in their low cytotoxicity, as isoflavonoids are compounds daily consumed in food and beverages, therefore they represent novel leads, and future studies may allow the development of pharmacologically acceptable antimicrobial and/or anti-inflammatory agents.

The relationship between the chemical structure of a molecule and its biological activity is a powerful concept in discovery of bioactive agents that allows the selection and optimization of ideal structural candidates. This analysis enables the determination of the chemical group responsible for biological effect and allows modification of the compound potency by changing its chemical structure.

Based on our supposition that the activity of the isoflavonoids should be influenced by the presence of the functional groups at certain positions, this study concentrates on the anti-inflammatory and antimicrobial activity of several methoxy and hydroxyl structure-related isoflavonoids and their metabolites *in vitro*, with special focus on structure-activity relationship, including their potential against methicillin-resistant *S. aureus*. Thus, this study might bring new insights into this problematic, with possible chance to identify compound with dual, anti-inflammatory and antimicrobial, effect.

1 LITERATURE REVIEW

1.1 Inflammation

1.1.1 Causes and mechanisms

Inflammation is the body's natural immune response to harmful stimuli, such as tissue injury and invading pathogens (Ohishi et al. 2016), and includes interactions between the immune and nervous systems (Ren & Dubner 2010). It is considered a protective measure taken by the organism to begin the healing process. It is classified as acute, however, under certain conditions, the pain lingers and becomes chronic even after the injury has healed (Ren & Dubner 2010; Da Silveira E Sá et al. 2014).

In response to the stimuli, resident immune cells (macrophages and mast cells) are activated and blood-borne immune cells (leukocytes) are released to the site of injury and promote repair, through the secretion of inflammatory mediators, such as cytokines (e.g., interleukin-1β (IL-1β), IL-6, IL-12, and the chemokine IL-8), tumour necrosis factors (e.g., TNF-α and TNF-β), interferons (e.g., IFN-γ), eicosanoids (e.g., prostaglandins and leukotrienes) and vasoactive amines (e.g., histamine) (Ren & Dubner 2010; Da Silveira E Sá et al. 2014). The inflammatory mediators and several cell types (including mast cells, eosinophils, neutrophils, macrophages, and lymphocytes) are involved in allergic and non-allergic-mediated inflammation (Wu et al. 2011).

Arachidonic acid (AA) is the most abundant polyunsaturated fatty acid found in the phospholipid cell membranes. Activation of the phospholipase A_2 (PLA_2), in response to various stimuli, releases AA, which can be further metabolized by two major enzymatic pathways: cyclooxygenase and 5-lipoxygenase, leading to pro-inflammatory mediators, prostanoids and leukotrienes (LTs), respectively (Charlier & Michaux 2003). The pathways for transformations of AA adopted from Dahlén (2006) are shown as Figure 1.

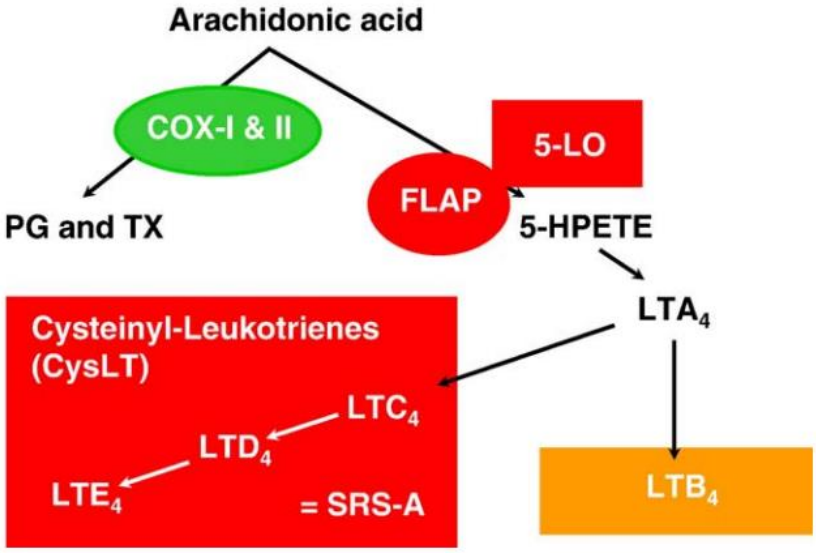


Figure 1. Pathways for transformations of arachidonic acid

Cyclooxygenase (COX, two isoenzymes COX-1 and COX-2) catalyse the formation of prostaglandins (PG) and thromboxane (TX). The biosynthesis of LTs is catalysed by the 5-LOX in co-operation with FLAP. The primary leukotriene intermediate LTA4 is metabolized to LTB4 (in cells equipped with LTA hydrolase such as neutrophils and monocytes) or the cysteinyl-leukotrienes LTC4, LTD4 and LTE4, that made up the biological activity previously known as slow reacting substance of anaphylaxis (Dahlén 2006).

Constitutive cyclooxygenase (COX-1; prostaglandin-endoperoxide synthase) is present in cells and most tissues under physiological conditions and it is considered as a "housekeeping enzyme" due to the constitutive low-levels of expression in most cell types and tissues. High levels of constitutive expression of COX-1 have, instead, been detected in the stomach and platelets whereas COX-2, almost undetectable in healthy man, is induced by some cytokines, mitogens, and bacterial endotoxins and becomes the major contributor to prostanoid synthesis presumably in pathological conditions, such as inflammation (Mitchell et al. 1993; FitzGerald & Patrono 2001; Charlier & Michaux 2003; Perrone et al. 2010). The prostaglandins (PGs) produced by COX-2 play a major role in inflammatory reactions and are responsible for the characteristic inflammatory symptoms (redness, pain, oedema, fever and loss of function) (Charlier & Michaux 2003). Role of COX-1 and COX-2 in arachidonic acid metabolism is shown on Figure 2 adopted from Jachak (2006).

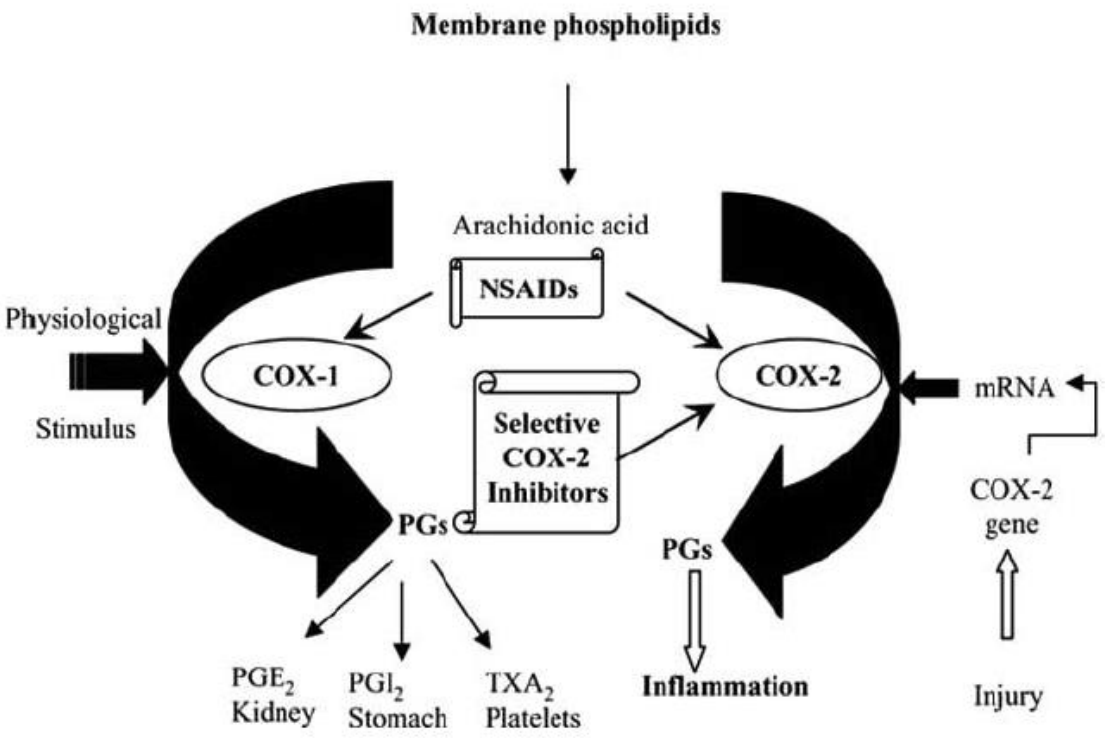


Figure 2. Role of COX-1 and COX-2 in arachidonic acid metabolism

The pivotal lipid mediators of inflammation and allergy are leukotrienes (LTs) with major roles in respiratory and cardiovascular diseases (Pergola & Werz 2010). LTs are one of several substances released by mast cells during an asthma attack and in chronic asthma (Berger 1999). There are two families of LTs - the first group of cysteinyl-leukotrienes (LTC_4, LTD_4, and LTE_4) are molecules of the slow-reacting substance of anaphylaxis. The second group acts primarily in conditions in which inflammation is dependent on neutrophils, they selectively increased the number and percentage of neutrophils and recruit them to areas of tissue damage, e.g. in lung (LTB_4) (Busse 1998; Wu et al. 2011).

For LT biosynthesis, the precursor arachidonic acid (AA) is released from membrane phospholipids by phospholipase A2, and is then metabolized by 5-lipooxygenase (5-LOX) aided by the 5-LOX-activating protein (FLAP) into primary leukotriene intermediate short-lived compound LTA_4, which is metabolized to LTB_4 (in cells equipped with LTA hydrolase such as neutrophils and monocytes) (Dahlén 2006; Baldo & Pham 2016). Thus, 5-LOX is one of the rate-limiting enzymes in the biosynthesis of LTs and targeting 5-LOX represents an attractive strategy for therapeutic intervention (Pergola & Werz 2010). The 5-LOX pathway is shown on Figure 3 adopted from González-Périz & Clària (2007).

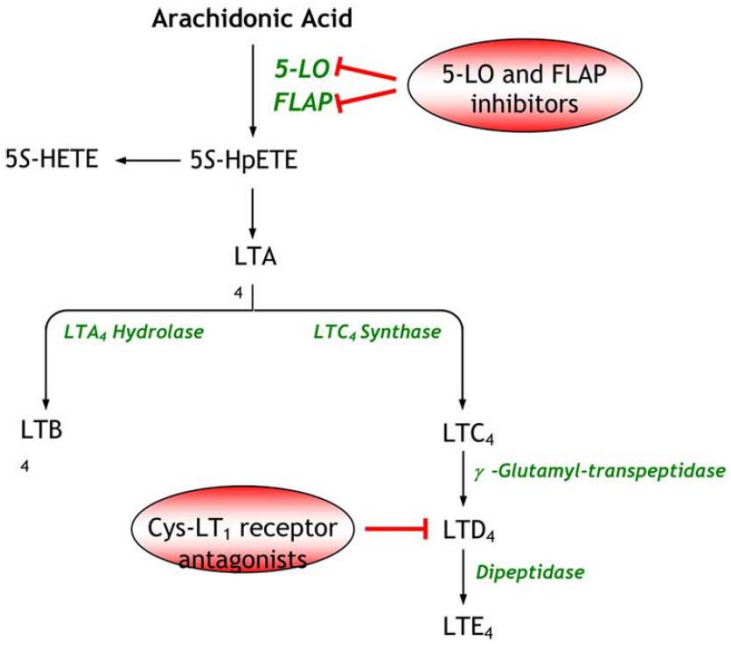


Figure 3. The 5-LOX pathway

5-LOX translocates to the nuclear membrane where it binds its accessory protein (5-LOX-activating protein, FLAP) and transforms AA to 5S-hydroperoxyeicosatetraenoic acid (5S-HpETE), which is reduced to 5S-HETE or transformed to the highly unstable allylic epoxide leukotriene (LT) A_4. LTA_4 is either hydrolysed to LTB_4 by a specific LTA_4 hydrolase or converted into LTC_4 by the addition of the peptide glutathione by a specific LTC_4 synthase. LTC_4 can undergo further metabolism through a series of peptidic cleavage reactions to yield LTD_4 and LTE_4. The clinically relevant LT-modifying drugs include 5-LOX and FLAP inhibitors as well as Cys-LT_1 receptor antagonists (González-Périz & Clària 2007).

1.1.2 Inflammation therapy

1.1.2.1 Inhibitors of COX-1 and -2

To treat arthritis pain, inflammation and other health problems, nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used. This group includes acetylsalicylic acid and the traditional NSAIDs (tNSAIDs), which are derivatives of the propionic acid (ibuprofen, ketoprofen, naproxen), the acetic acid (diclofenac, indomethacin, etodolac, ketorolac), the oxicams (meloxicam, piroxicam), the fenamates (mefenamic acid, tolfenamic acid), and others (nabumetone, nimesulide) (Malm & Borisch 2015), which were developed to mimic the pharmacological effects of aspirin. The tNSAIDs are nonselective, blocking both isoforms of COX, although the relative degrees of COX-1 and COX-2 inhibition vary substantially among these agents. Among them, some were endowed with moderate COX-2 selectivity (e.g., diclofenac). Their therapeutic efficacy, even they are taken in a relatively high dose,

depends on their inhibition of the COX-2-mediated formation of PGE2, which causes inflammation in the joints, and pain and fever in the central nervous system. However, their inhibition of COX-1-mediated PGE2 formation in the gastric mucosa increases the risk of gastrointestinal symptoms, mucosal damage and bleeding (Cairns 2007; Patrignani & Patrono 2015).

By contrast, the coxibs, a group of highly selective inhibitors of the COX-2 isoform of COX, inhibit only the COX-2-mediated pathways, achieving the desired therapeutic goal of reducing inflammation and pain by blocking PGE2 formation in joints and elsewhere. The coxibs spare COX-1-mediated gastric PGE2 production, preserving its gastroprotective actions (Cairns 2007). The first generation of selective COX-2 inhibitors enter clinical trials, with celecoxib and rofecoxib in 1995 (Hawkey 2005). Other selective COX-2 inhibitors are valdecoxib, parecoxib and etoricoxib (FitzGerald & Patrono 2001; Malm & Borisch 2015). The first recognition of a potential difference between selective COX-2 inhibitors and non-selective NSAIDs with regard to possible cardiovascular thrombosis was mentioned in several publications 3 years later, suggesting fewer cardiovascular deaths on rofecoxib than NSAIDs. Ten years later, in 2005, even FDA reviewed cardiovascular safety of selective COX-2 inhibitors, several studies suggested certainly dose dependent cardiovascular problems (Hawkey 2005). For example, an increased incidence of myocardial infarction and sudden cardiac deaths has been observed in patients with high doses of treatment of rofecoxib (Lamprecht et al. 2005). NSAIDs are metabolized in the liver, whereas hepatocytes are responsible for drug metabolism and detoxification. They eliminate potentially toxic compounds but, paradoxically, also result in the generation of toxic or carcinogenic metabolites. Cumulative or overdoses of drugs can induce acute liver failure either directly or indirectly after their biotransformation (González-Ponce et al. 2018). Lastly, both tNSAIDs and coxib may also increase blood pressure and reduce kidney function (Sostres et al. 2010).

1.1.2.2 Inhibitors of 5-LOX

One of the most effective treatments, which inhibits the 5-LOX activity, is zileuton {N-(1-Benzo[*b*]thien-2-ylethyl)-N-hydroxyurea}, which was approved by the US FDA for the prophylaxis and treatment of patients with chronic asthma (D'Urzo & Chapman 2000). This only approved LT synthesis inhibitor, has been, however, later on associated with liver toxicity, which has limited its clinical usefulness (Watkins et al. 2007). Its chemical structure is shown on Figure 4, adopted from Carter et al. (1991).

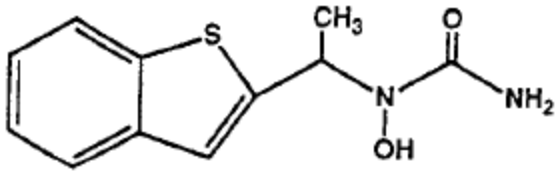


Figure 4. Chemical structure of zileuton

The inflammatory response can be also prevented or controlled by corticosteroids, which act by supressing the formation, release and activity of these endogenous inflammatory cells and mediators (Wu et al. 2011). However, these drugs are usually accompanied with unexpected side effects, and also, they are not considered as a good clinical choice for chronic inflammatory disorders in general (Yu et al. 2016). The first new class of drug introduced into asthma therapy in over 30 years - anti-leukotrienes (anti-LTs) – have attracted considerable attention, particularly as they have been proposed as either an alternative or an addition to inhaled corticosteroids (Barnes 2003). However, several studies suggested relationship between patients with both high-dose corticosteroid therapy and anti-LTs to control their asthma and patients developing Churg–Strauss syndrome (Barnes 2003; Swietlik & Doboszynska 2008; Watelet et al. 2009).

As reviewed by Yu et al. (2016) recent investigations have demonstrated that plant polyphenols, in particular flavonoids, exhibit anti-inflammatory activity both *in vitro* and *in vivo*, mainly through the termination of free-radical reactions, which take part in AA metabolism (Šibul et al. 2016). This not only provides an explanation for the health benefit of vegetarian diets and Chinese medicine, but also identifies potential agents for treating inflammatory disorders, including possible therapy for life-threatening diseases (Yu et al. 2016).

1.1.2.3 Dual inhibitors of COX-2/5-LOX

Considering the pro-inflammatory properties of LTs and prostanoids and the side effects of NSAIDs, coxibs and anti-LTs mentioned in previous two chapters, simple therapeutic approach is insufficient and developing drugs able to inhibit both COX-2 and 5-LOX should not only enhance their individual anti-inflammatory effects, but also reduce the side effects associated with NSAIDs, especially of the gastrointestinal tract, and selective COX-2 inhibitors (Celotti & Laufer 2001; Fiorucci et al. 2001; Charlier & Michaux 2003).

Indeed, it has been shown that COX inhibition by NSAIDs, besides causing a reduction in the synthesis of vasodilatory and gastroprotective PGs, leads to an up-regulation of AA metabolism by the 5-LOX pathway, increasing the formation of LTs and contributing to inflammation and NSAIDs-induced adverse effects. In addition, both COX-2 and 5-LOX enzymes have been involved in the development and progression of numerous types of cancer, so the use of dual inhibitors opens up new perspectives in the prophylactic treatment of this dreadful disease (Charlier & Michaux 2003).

What appears most impressive from the available studies on dual inhibitors is their almost complete lack of gastric toxicity (Celotti & Laufer 2001). Therefore, this new approach focused on dual inhibitors will certainly help to unravel the mechanisms at the root of the undesirable effects of NSAIDs and to develop safer NSAIDs (Fiorucci et al. 2001).

1.2 Infection

1.2.1 Epidemiology & Classification

Infection starts as the invasion and colonization of a host organism's tissues by disease-causing organisms, their multiplication, and the reaction of host tissues to these organisms and the toxins they produce. Infections are caused by microorganisms such as viruses, prions, bacteria, and viroids, and larger organisms like parasites and fungi. Hosts can fight infections using their immune system (Signore & Glaudemans 2011; Signore 2013).

According to Nelson (2006) there are several approaches in classification of infections. Clinicians tend to classify infectious diseases according to their most common clinical manifestation or by the organ systems that are primarily affected (diarrheal, respiratory, CNS, cardiovascular, sepsis). Microbiologists tend to classify infectious diseases according to the causative organisms (bacterial, viral, fungal, parasitic, prion). Epidemiologists usually classify infectious diseases according to the means of transmission (contact, food-borne, water-borne, airborne, vector-borne, perinatal) and the reservoir of the organism (human, animal, soil, water).

For purpose of this thesis, we will focus on most common human bacterial infections, mainly food-borne diseases and musculoskeletal diseases, as they are considered as the most common cause of illness and death in developing countries, associated with bacterial contamination especially with members of Gram-negative bacteria like *Salmonella typhi*, *Escherichia coli* and *Pseudomonas aeruginosa*. Other Gram-positive bacteria including *S. aureus* and *Bacillus cereus* have been also identified as the causal agents of food borne diseases or food spoilage (Mostafa et al. 2018). Extra focus should be paid on the infections

caused by *S. aureus*, a major human pathogen that causes a wide range of clinical infections (Tong et al. 2015), especially on its resistant forms, because 60% of *S. aureus* infections in European region are reported to be methicillin-resistant (MRSA), meaning that treatment with standard antibiotics does not work (WHO 2014). Musculoskeletal infections caused by *S. aureus* are among the most difficult-to-treat infections. For example, osteomyelitis, an inflammation of bone, is usually caused by bacterial infections and it is associated with 6 % mortality and tremendous disease burden through potential for long-term relapses and neurological deficits (Idelevich et al. 2016).

1.2.2 Treatment of infection

The use of antimicrobial agents provides an effective method for the control and treatment of infectious diseases caused by bacteria and certain other microorganisms. The ability to treat bacterial infections with chemotherapeutic agents is one of the most important medical achievements. Antiinfective therapy began with the industrial production of benzylpenicillin in 1941 and was followed by the discovery and development of streptomycin in 1944, chloramphenicol in 1947, chlortetracycline in 1948, semi-synthetic penicillins and cephalosporins from 1958 onwards, fluoroquinolones in the 1980s and oxazolidinones and cationic peptides in the 1990s (Selzer 2000).

Thus, treating and preventing bacterial infections in humans with the use of synthetic antibiotic drugs has been a long time practice in medicine, but their increasing and indiscriminate use causes a selective pressure on the pathogenic bacteria that eventually show antibiotic resistance (Babu et al. 2017). For example, around 90–95% of *S. aureus* strains worldwide are resistant to penicillin and in most of the Asian countries 70–80% of the same strains are methicillin resistant (Hemaiswarya et al. 2008). Among various approaches used for finding agents effective in overcoming the bacterial resistance, naturally occurring anti-infective plant products seem to be one of the most convenient strategies (Cos et al. 2006). Other strategy employed to overcome these resistance mechanisms is the use of combination of drugs, such as β-lactams together with β-lactamase inhibitors. Several plant extracts have already exhibited synergistic activity against microorganisms (Hemaiswarya et al. 2008).

Prevention of food spoilage and food poisoning pathogens is usually achieved by use of chemical preservatives, which have negative impacts including human health hazards of the chemical applications, chemical residues in food and feed chains and acquisition of microbial resistance to the used chemicals. Because of such concerns, the necessity to find potentially effective, healthy, safer and natural alternative preservatives is increased, for

example, plant extracts have been used to control food-borne diseases and preserve foodstuff by several authors (Mostafa et al. 2018).

1.3. Anti-inflammatory and antimicrobial natural products

1.3.1 Non-steroidal anti-inflammatory drugs

The first nonsteroidal anti-inflammatory drug (NSAID) was isolated in 1829 when German scientists were able to recover salicylate from willow bark. More than 130 years later, in the 1960s the next NSAID, indomethacin, was developed. Since then, other NSAID compounds have been brought to market. Although NSAIDs are relatively equipotent clinically, their subtle differences influence their selection in patient use. NSAIDs are advantageous because they lack addictive potential and do not result in sedation or respiratory depression. In addition, these drugs have analgesic properties at lower doses and anti-inflammatory effects at higher doses (Green 2001). They are widely used to treat arthritis pain and inflammation, menstrual and musculoskeletal pain, as well as headache and fever. This group includes acetylsalicylic acid (in a relatively high dose), the traditional NSAIDs (tNSAIDs) (eg, naproxen, ibuprofen, indomethacin and diclofenac) and the coxibs, a group of highly selective inhibitors of the COX-2 isoform of COX (Cairns 2007).

However, because of the significant side effect profiles of NSAID medications, there is a greater interest in natural compounds, such as dietary supplement and herbal remedies, which have been used for centuries to reduce pain and inflammation. Many of these natural compounds also work by inhibiting the inflammatory pathways in a similar manner as NSAIDs. In addition to the COX pathway, many natural compounds act to inhibit nuclear factor-κB (NF-κB) inflammatory pathways. Natural medicines derived from plants are drawing more and more interest in the prevention and treatment of diseases because of their unique characteristics, which include having fewer adverse effects and being more suitable for long-term use compared with synthesized chemicals. Nevertheless, there are problems associated with these dietary supplements, and their use requires knowledge of their biological action, clinical studies (both affirmative and negative), and potential interactions with other nutraceutical products and prescription medications (Wang et al. 2008; Maroon et al. 2010).

Some of the examples of natural anti-inflammatory products, reviewed by Maroon et al. (2010) are as follows:

- the fish oil (in the form of cod liver oil), an omega-3 EFA, for the treatment of muscular, skeletal, and discogenic diseases,
- the bark from the white willow tree, an analgesic and antipyretic agent, contains salicin, which is converted to salicylic acid by the liver and is considered to have fewer side effects than aspirin,
- curcumin, a naturally occurring yellow pigment derived from turmeric (*Curcuma longa*), used as a flavouring spice in food and has long been used in both Ayurvedic and Chinese medicines as an anti-inflammatory agent, a treatment for digestive disorders, and to enhance wound healing,
- green tea has long been recognized to have cardiovascular and cancer preventative characteristics due to antioxidant properties of catechins and subsequently it has been used in the treatment of arthritic disease as an anti-inflammatory agent,
- pycnogenol, derived from the bark of the maritime pine tree (*Pinus maritima*), has been considered helpful for wound healing, treating scurvy, healing of ulcers, and reducing vascular inflammation,
- olibanum, gum resin from trees of *Boswellia* species, can inhibit the leukotriene biosynthesis in neutrophilic granulocytes by inhibiting 5-LOX, thus affecting various inflammatory diseases that are perpetuated by leukotrienes,
- resveratrol, a plant-based polyphenol molecule found in various plant sources where acts as a phytoalexin, shows the anti-inflammatory properties as it suppresses COX-2 by blocking NF-κB activation,
- cat's claw, Peruvian herbs derived from woody vines with small claw-like thorns at the base of the leaf, *Uncaria tomentosa* and *Uncaria guianensis*, used to treat arthritis, bursitis, and intestinal disorders,
- capsaicin, isolated from chili pepper (*Capsicum annum*), inhibits NF-κB, thus producing an anti-inflammatory effect, but is rarely used alone, it is generally mixed into other natural anti-arthritic preparations.

The processes used to prepare herb-derived compounds pose complications when it comes to determining the quantity and concentration of the products. The preparation processes are not standardized, and therefore, the extraction process and the type of plant used may affect the true concentration of the product. In addition, there is a lack of uniformity within and between manufacturers. Although dietary supplements are not held to the same rigorous testing and standards as pharmaceutically derived medications, there are many

regulations that still control their manufacture because these are food products. Some manufacturers inflate nutraceutical products' claims and may not cite possible side effects and potential drug interactions. Bleeding complications are associated with white willow bark, ginger, garlic, and others. Therefore, such medicinal preparations are not without risk (Maroon et al. 2010).

1.3.2 Anti-infective natural drugs

Infectious diseases caused by bacteria, fungi, viruses and parasites are still a major threat to public health, despite the tremendous progress in human medicine (Cos et al. 2006). Natural products have been a particularly rich source of antiinfective agents, yielding, for example, the penicillins in 1940, the tetracyclines in 1948, and the glycopeptides in 1955. Naturally occurring products from plants have played an important role in the discovery of new therapeutic agents since ancient times, for example, quinine obtained from *Cinchona* has been successfully used to treat malaria (caused by protozoa *Plasmodium*). Substantial attention has been focused on exploration and utilization of secondary metabolites of plants (phytochemicals) as an alternative to and/or in combination with traditional antibiotics for treating resistant infections. Among the phytochemicals, flavonoids, isoflavonoids, and related compounds seem to be the most potentially useful candidates, because they are widely distributed in edible plants and they possess broad pharmacologic activity (Mukne et al. 2011). For example, turmeric (*Curcuma longa*) is widely popular in in India, China and South East Asia mainly as spice and as a food preservative along with its use in traditional medicines in preventing and treatment of several diseases and health conditions it is also known to provide antimicrobial activity against several microorganisms. Curcumin, one of the polyphenolic compound present in turmeric, is known to be active against a range of food spoilage and pathogenic microorganisms such as *Aspergillus niger*, *Bacillus subtilis*, *E. coli*, *P. aeruginosa*, and *S. aureus* (Babu et al. 2017). Other food preservative is benzoic acid, because it effectively inhibits the growth of molds, yeasts and some bacteria. The simplest aromatic carboxylic acid, which is contained in resins and oleoresins of shrubs and trees of *Styrax* spp., is also used in cosmetic, hygiene, and pharmaceutical products (Del Olmo et al. 2017).

In addition, spices and aromatic plants have an antimicrobial effectiveness that depends on the kind of plant, its composition and concentration of essential oils, often rich in monoterpens and sesquiterpenes. Studies analysing the antimicrobial activity of essential oil of *Allium sphaerocephalon* revealed the accordance with the popular use of plants of the

Allium genus in traditional medicine, indicating the importance of aroma precursors (cysteine sulfoxides) for a potent biologic activity (Savoia 2012). Allicin, a diallyl thiosulfinate in garlic, exhibits antimicrobial effects on a broad range of bacteria, including *Enterobacter, Escherichia, Klebsiella, Proteus, Pseudomonas, Salmonella, Serratia,* and *Shigella*. Thyme (*Thymus vulgaris*) and oregano (*Origanum vulgare*) have an inhibitory effect on the growth of Gram-negative *Vibrio parahaemolyticus* (Babu et al. 2017). Essential oils are also used for food preservation to avoid the food-borne infections. Hernández et al. (2017) focused on the application of plant essential oils during the meat drying process using oregano essential oil. The application was effective in inhibiting of *Salmonella enteritidis* and *E. coli* growth, without affecting of meat sensory quality. Nedorostova et al. (2011) proved antimicrobial properties of essential oils from *Armoracia rusticana, Origanum syriacum, Allium sativum, Satureja hortensis, S. montana, Thymus vulgaris,* and *T. serpyllum* against different strains of *S. aureus,* including MRSA and resistant clinical isolates.

Mostafa et al. (2018) proved *Punica granatum* and *Syzygium aromaticum* ethanolic extracts to be very effective against the highly susceptible strains of food borne pathogenic bacteria (*S. aureus* and *P. aeruginosa*), suggesting these plant extracts as natural alternative preventives to control food poisoning diseases and preserve food stuff avoiding health hazards of chemically antimicrobial agent applications. Mostafa et al. (2018) also reviewed and summarized the antibacterial effect of avocado, cinnamon, garlic, ginger, guava, lemon, myrtle, and thyme, plants, which are commonly used in food conservation and in traditional medicine.

Miswak (*Salvadora persica*), also known as the toothbrush tree that is used as a chewing stick for oral hygiene since ancient times, contains more than one type of antimicrobial agent that inhibits the growth of both Gram-positive and negative bacteria (*E. coli, S. aureus, Lactobacillus acidophilus, Streptococcus mutans* and *P. aeruginosa*). The biological activity of *S. persica*'s phytochemicals, such as benzyl isothiocyanate and β-sitosterol, inhibit the cariogenic and genotoxic compounds accumulated on the surface of the teeth (Abhary & Al-Hazmi 2016). An *in vitro* study showed inhibitory effect on the growth of *Candida albicans* and proved that *Enterococcus faecalis* is the most sensitive microorganism affected by the use of miswak (Halawany 2012). A strong antibacterial activity against oral pathogens like *S. mutans*, *Bacteroides* sp., *Provotella* sp., *Actinomyces* sp., and *C. albicans*) produced by essential oils from *Mentha* × *piperita*, *Thymus vulgaris*, *Eucalyptus* sp., and *Gaultheria procumbens*, which mix served as a surgery disinfectant and later as agent against cariogenic bacteria in oral care, was reviewed by Vlachojannis et al. (2013). Many plants used

in traditional medicine to treat oral bacterial diseases, like *Punica granatum*, *Syzygium aromaticum*, *Cinnamomun zeylanicum*, and others, showed an antibacterial effect *in vitro*, and justified their use in traditional medicine (Rosas-Piñón et al. 2012). The essential oil from *Melaleuca alternifolia* (so called "tea tree oil") has broad-spectrum antimicrobial activity *in vitro* and it is used in the treatment of a range of superficial dermatoses such as cuts, insect bites, boils, dermatophytosis and for the treatment of acne vulgaris showing the its efficacy against *Propionibacterium acnes* (Enshaieh et al. 2007).

Other plants with potent antimicrobial activity are species of *Pistacia* genus, which resin, a mastic gum, has been evaluated by several authors (Tassou & Nychas 1995; Koutsoudaki et al. 2005; Kalalinia et al. 2008). Their ethnomedicinal uses were reviewed by Rauf et al. (2017). The main components of mastic gum, α-pinene, camphene, β-pinene, β-myrcene, limonene, linalool, and β-caryophyllene, α-thujene, α, β-thujene (Papageorgiou et al. 1991; Koutsoudaki et al. 2005; Kalalinia et al. 2008) exhibited strong inhibitory effects against Gram positive bacteria (*S. aureus*, *B. subtilis*) and *C. albicans*. Moreover it was indicated that β-pinene had the most inhibitory effects against Gram positive bacteria and *C. albicans* while α, β-thujene was more active against Gram negative bacteria (*E. coli, P. aeruginosa*, *Klebsiela pneumonia*) (Kalalinia et al. 2008). As the sensitivity to above mentioned compounds was different for different bacteria tested, it was suggested that the antibacterial effect of mastic oil is due to a number of its components working synergistically (Koutsoudaki et al. 2005). Marone et al. (2001) evaluated antibacterial activity of mastic from *Pistacia lentiscus* against clinical isolates of *Helicobacter pylori* by a microdilution assay, when mastic gum killed 50% and 90% of the strains tested at a concentration of 125 μg ml^{-1} and 500 μg ml^{-1} respectively. The fact that mastic gum has bactericidal activity on *H. pylori* was later confirmed by Dabos et al. (2010) *in vivo*.

It was shown that plant-derived compounds are active against Gram-positive bacteria, successively numerous phytochemicals were shown to act as potential efflux pump inhibitors (EPIs) with antimicrobials for Gram-positive bacteria, while Gram-negative bacteria have innate multidrug resistance to many antimicrobial compounds owing to the presence of efflux pumps. However, the chemical diversity between plants and microorganisms represents an ecological possibility to identify EPIs from natural sources. The literature concerning bacterial resistance modulators from natural plants, described the plant alkaloid reserpine, berberine and methoxylated flavones and isoflavones, which revealed putative interfering activity on efflux (Savoia 2012).

The underuse, overuse, and misuse of antibiotics by humans are the selective pressure, which eventually lead to the development of antibiotic resistance in microbes. Globally, emerging MDR pathogens also called as 'ESKAPE' organisms (*Enterococcus* spp., *S. aureus*, *Klebsiella* spp., *A. baumannii, P. aeruginosa* and *Enterobacter* spp.) are a serious threat to public health. MDR microorganisms can survive the treatment with antimicrobial drugs, thereby standard treatments become ineffective and infections persist, increasing the risk of spread to others. In general, MDR microbes are resistant to three or more antibiotics, however, strains of *Mycobacterium tuberculosis* are extremely drug-resistant that are virtually resistant to all classes of antimicrobials (Subramani et al. 2017).

The rich source of potential drug development is in traditional medicine. Several studies have been dealing with *in vitro* experiments focused on plants used in traditional medicine suggesting them for potential future pharmacology studies (Kloucek et al. 2007; Bussmann et al. 2010; Lulekal et al. 2014; Ulloa-Urizar et al. 2015; Mickymaray et al. 2016; Stanković et al. 2016; Sharma et al. 2017, and others). Due to the emergence of widespread drug resistance, research on new antimicrobial substances must continue, while using of ethnopharmacological knowledge is one attractive way to enhance the probability of success in new drug-finding efforts (Cos et al. 2006).

1.3.3 Natural products with dual effect

Although inflammation is a vital part of the normal response to infection or injury, when it is excessive or prolonged it can prevent healing or even cause further damage (Molan 2001). Therefore, it is necessary to know products, which can act in both, anti-inflammatory and antimicrobial actions. A non-steroidal anti-inflammatory drug, which has been used for medicinal purposes since the ancient Egyptian period is salicylic acid (or sodium salicylate). The acetylation of salicylate to aspirin extended its usage in medicines, it is often used to reduce fever, to relieve pain and as an anti-inflammatory drug, it can be used in low doses to prevent heart attacks. Salicylic acid and salicylate are also key ingredients used in cosmetics in many skincare products and shampoos, as salicylate induces morphological and physiological alterations in bacteria, for instance in *S. aureus* and *E. coli* (Hartog et al. 2010). Damman (2013) described uses of salicylates and their role in gastrointestinal and dermatological diseases, for example, salicylates were used historically with benzoic acid in Whitfield ointment to treat tinea infections. Other well-known natural product, used from ancient time, is honey. Its dual effect was explained and summarized by several authors (Molan 2001; Mandal & Mandal 2011). Among others, we can also mention bovine colostrum

(Yadav et al. 2016), artemisinin from *Artemisia annua* (Kim et al. 2015), and ginger (Grzanna et al. 2005; Islam et al. 2014) as natural products with dual effect.

As isoflavonoids belong to the natural compounds coming from the secondary metabolism of plants synthetized *de novo* when the plant is attacked by microorganisms, they might be one of the future prospective candidates for natural compounds with dual effect and as they are widely distributed even in food, their benefits for human might be invaluable.

1.4 Isoflavonoids

1.4.1 Introduction

Isoflavonoids are a subclass of the more ubiquitous flavonoids, which are naturally occurring polyphenols (Albulescu & Popovici 2007). There are many different types of isoflavonoids, but all have got the same basic characteristic structure with substituents $R_1 – R_6$, and they are divided into subclasses depending on the oxidation level of the central pyran ring (Head 1997; Albulescu & Popovici 2007). Black and white drawing of basic chemical structure of isoflavonoid adopted from Klejdus et al. (2001) is shown as Figure 5.

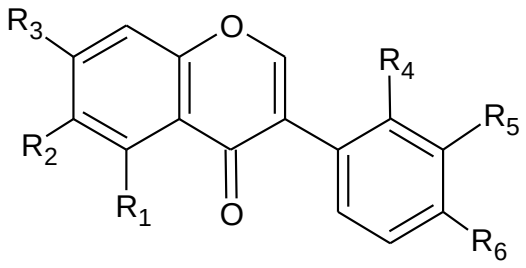


Figure 5. Basic chemical structure of isoflavonoid

Isoflavones are the most abundant of the sub-classes of isoflavonoids (Head 1997), which accumulate predominantly in plants of the Leguminosae family. The main sources of isoflavones are soy (*Glycine max*) and soy-derived products, but isoflavones are also in other species of the Leguminosae family (*Cicer arietinum*, *Medicago sativa*, *Phaseolus* sp., *Pueraria* sp., *Trifolium pratense* and other). Moreover, small amounts of isoflavones are also contained in cereals, fruits, nuts, and vegetables. In plants, isoflavones are usually found in conjugated forms, (glucosides), which are not absorbed intact across the enterocyte of healthy

adults, their bioavailability requires hydrolysis to the corresponding aglycones, by intestinal β-glucosidases (Bustamante-Rangel et al. 2018). Isoflavones act as phytoestrogens and possess antioxidant, anticancer, antimicrobial, and anti-inflammatory activities just like other flavonoids (Yu et al. 2016).

1.4.2 History

The discovery of isoflavonoids as natural products has its origin in the middle of nineteenth century, when Reinsch and Hlasiwetz obtained ononin from the root of *Ononis spinosa* L. Ononin was revealed after eighty years later as the 7-Oglucoside of formononetin (Veitch, 2007). In 1984 it was known 465 structures, in 1989 it was published 630 structures and in 1994 it was published 870 structures (Harborne, 1994). According to Veitch, there was known more than 2000 examples in 2013 (Veitch 2007; Veitch 2009; Veitch 2013). More than 840 new examples were reported in the period from 1997 to 2011, suggesting that interest in this area remains at a high level, leading to the advances in structural elucidation and biosynthesis (Veitch 2013).

1.4.3 Biosynthesis

Isoflavonoids form a large quite distinct subclass of flavonoids, being structural variants, in which the shikimate-derived aromatic ring has migrated to the adjacent carbon of the heterocycles. For the biosynthesis of isoflavonoids is responsible combination of the shikimate pathway and the acetate pathway (Velíšek & Cejpek 2008).

The shikimate pathway is named after its central intermediate, shikimic acid, which was first isolated from fruits of aniseed (*Illicium anisatum*) in 1885 and was named after the Japanese name of the plant *shikimi-no-ki*. In turn, the shikimic acid (shikimate) has given rise to the common name of the pathway. The seven enzymatic steps of the shikimate pathway starting with the condensation of the two metabolites, phosphoenolpyruvate (from glycolysis) and erythrose 4-phosphate (from pentose phosphate pathway), and their cyclization lead to the formation of shikimic acid and end with the synthesis of chorismic acid (chorismate). Active forms of shikimic acid and chorismic acid with coenzyme A (CoA) can access the main classes of phenolic compounds, quoting some transformations to acids of the benzoic acid series (gallic, protocatechuic, etc.) by β-oxidation. Gallic acid itself after the addition of a molecule of phosphoenolpyruvate and additional series of intermediate stages, followed by amination, gives rise the three aromatic amino acids phenylalanine and tyrosine and to the heterocyclic amino acid tryptophan, which are precursors of many biologically active

secondary metabolites and mainly they are the starting point of the phenylpropanoid pathway. (Velíšek & Cejpek 2008; Tzin et al. 2012; Kougan et al. 2013).

The phenylpropanoid pathway is the precursor for several phenylpropanoids such as cinnamic acid, caffeic acid, ferulic acid, sinapic acid, and others (Velíšek & Cejpek 2008; Kougan et al. 2013). The phenylpropanoid pathway starts with the condensation of amimo acids phenylalanine and tyrosine, as C_6-C_3 building blocks, via the first key enzyme phenylalanine ammonia lyase (PAL), catalyzing the deamination of L-phenylalanine into cinnamate. Cinnamate 4-hydroxylase (C4H) is the second enzyme converting cinnamate to *p*-coumarate. 4-Coumarate CoA-ligase (4CL) catalyzes the final step of the phenylpropanoid pathway, converting *p*-coumarate to *p*-coumaroyl-CoA. Then chalcone synthase (CHS) catalyzes the first committed step towards flavonoid biosynthesis, the condensation of one *p*-coumaroyl-CoA molecule and three malonyl-CoA molecules to form a chalcone scaffold. Chalcone reductase (CHR), only identified in legumes, acts on an intermediate of the CHS reaction, yielding deoxychalcone from the coupled catalytic action with CHS. Chalcone isomerase (CHI) catalyzes cyclization of chalcones or deoxychalcone to form the flavonoid core flavanone (Wang 2011).

The biodiversity of phenylpropanoids is the result of the modification and amplification of several basic structures, derived from the shikimate pathway, and it is also emphasized through the contribution of a set of enzymes such as oxygenases, ligases, oxidoreductases, and transferases (Kuete 2013).

Flavanones (e.g., naringenin) act as the core precursors that lead to the production of anthocyanins, condensed tannins, flavones, and various other compounds in all plant species. In legumes, a branch-point enzyme 2-hydroxyisoflavanone synthase (2-HIS, also named as isoflavone synthase, IFS) introduces the isoflavonoid biosynthetic pathway. 2-HIS converts flavanone to 2-hydroxyisoflavanone by performing a novel aryl-ring migration to transfer the aromatic B-ring from position C-2 to C-3 and hydroxylation in position C-2. Dehydration of 2-hydroxyisoflavanones occurs either spontaneously or through catalysis by 2-hydroxyisoflavanone dehydratase to generate isoflavones, e.g., daidzein and genistein (Wang 2011). This rearrangement is generally depicted as a two-step process with two isoflavanones (liquiritigenin to daidzein) as intermediates in the following scheme on Figure 6 adopted from Veitch (2007).

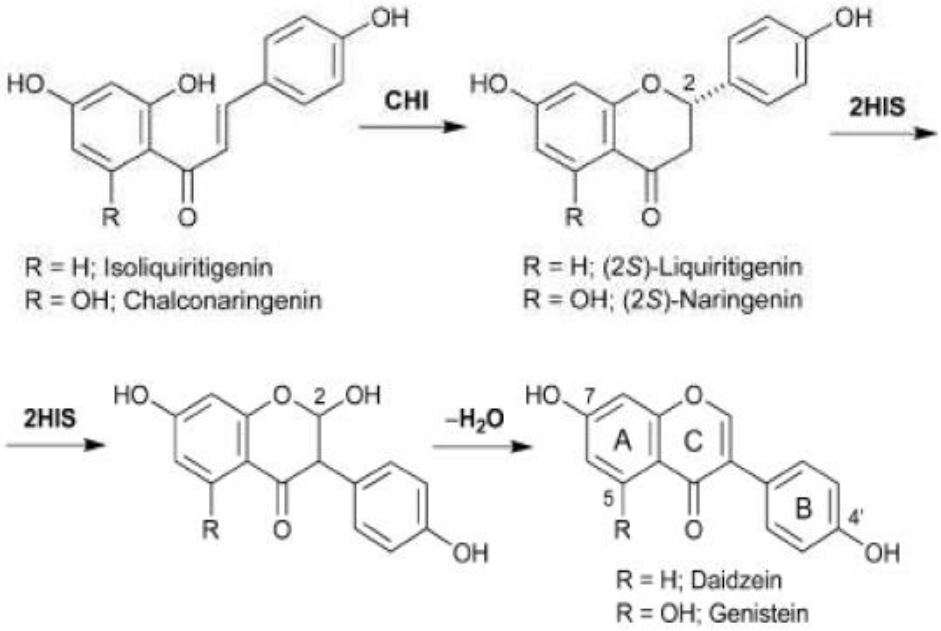


Figure 6. Rearrangement process of isoflavonoids

It has been established that acetate gives rise to ring A and that phenylalanine, cinnamate and cinnamate derivatives are incorporated into ring B and C-2, -3, and -4 of the heterocyclic ring (Friedli G-L 1997). Since chalcones and flavanones are efficient precursors of isoflavonoids, the required aryl migration of ring B from the former 2 or beta position to the 3 or alpha position of the phenylpropanoid precursor must take place after formation of the basic C_{15} skeleton.

1.4.4 Categorization of isoflavonoids

Isoflavonoids are divided into several subclasses to aid their systematic classification. According to the number of substituens on the basic 3-phenylchroman skeleton and mainly according to the different oxidation level of the central pyran ring (3-phenylchroman skeleton) isoflavonoids are divided into classes – isoflavones, isoflavanones, pterocarpans, isoflavans, isoflav-3-enes, 3-arylcoumarins, coumestans and rotenoids (Harborne 1994; Tahara & Ibrahim 1995; Harborne et al. 1999). These major isoflavonoid classes and their biosynthetic relationships adopted from Tahara and Ibrahim (1995) are shown in Figure 7.

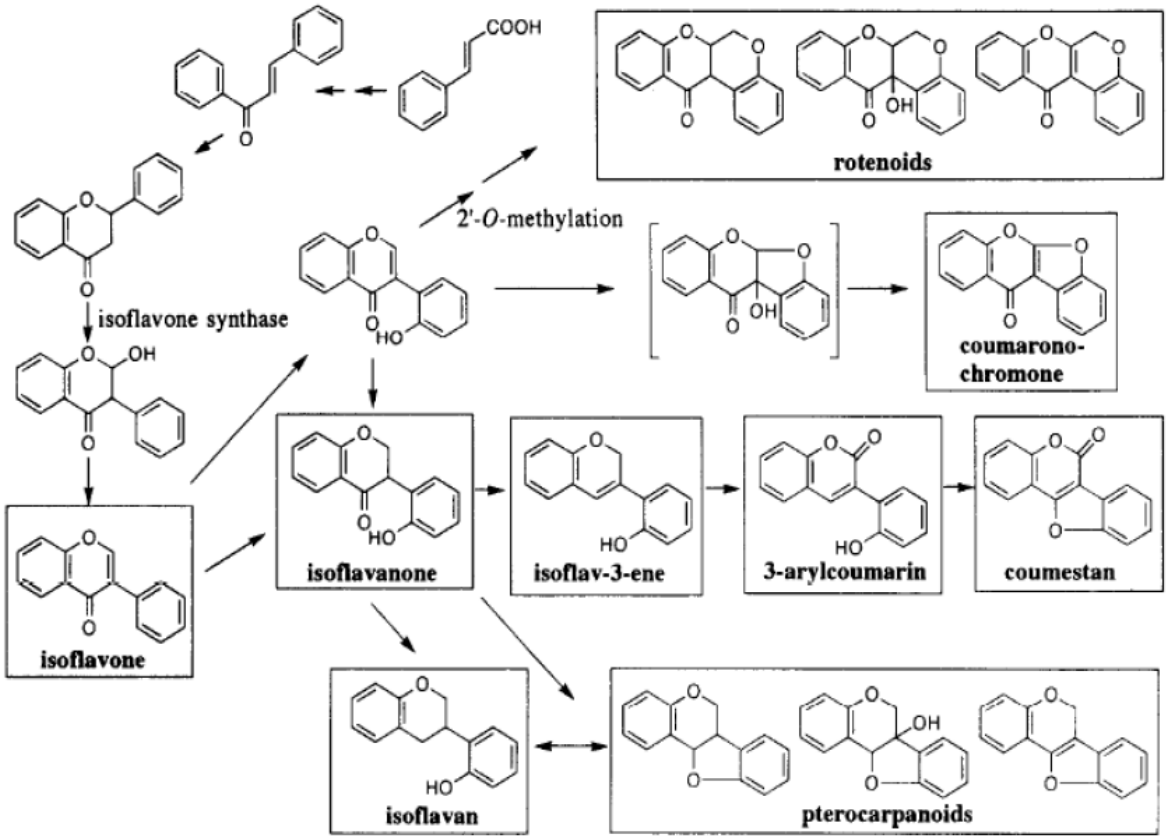


Figure 7. Major isoflavonoid classes

1.4.5 Biological properties of isoflavonoids

Plants produce a lot of organic matters, which do not affect directly growing and progression, so called secondary metabolites. The primary metabolites are basic element for the biosynthetic production of secondary metabolites, but exact border between primary and secondary metabolites does not exist. Some of these metabolites are often exactly divided into several taxonomic groups inside the plant kingdom. Many of their functions are still unknown.

Natural plant secondary metabolites can be divided into three main groups: terpenoids, alkaloids and phenylpropanoids with their relative phenolic compounds. Individual groups of phenolic matters have many conjunctive signs, which are outgoing from their biochemical pathways. One of the most significant groups of phenolic compounds are flavonoids. This gathering consists of many groups of plant metabolites, where chalcons, aurons, flavonons, isoflavonoids, flavonols, catechins and anthocyanidins belong to.

Content of isoflavonoids in plant is affected by many biotic and abiotic factors. Isoflavonoids occur as constitution matters or they can appear by incidence of stress. Isoflavonoids play important role in some function in defensive system of a plant – natural barrier against infection during germination of seeds, attack by insect or damage by pests. These compounds can sustain for certain time theirs biological activity and can influence microbiological proportions in soil or can be used by people – e.g. antifungal properties (Harborne et al. 1999; Klejdus 2004). In family Leguminosae isoflavonoids play their main role in the initiation of a rhizobial symbiosis and interation with the constitutively expressed nodD gene products of the microsymbiont to form a proteinphenolic compound. Different plants release different compounts, e.g. daidzein is an inducer in the *Phaseolus vulgaris/Rhizobium leguminosarum* symbiosis (Goodman 2004).

The pharmacological properties of isoflavonoids are seldom known. In animal models, a soya bean-based diet decreases mammary and prostrate carcinogenesis. Pure genistein is also an anticarcinogen (mammary tumours of the female rat) (Bruneton 1999).

Leguminosae also products isoflavonoids, which are harmful to some animals – rotenoids are used as insecticides or piscidals (rotenone inhibits the electron transport pathway in mitochondria and is therefore toxic to all forms of life) (Harborne et al. 1999).

Isoflavonoids belong to the group of phytoestrogens. Phytoestrogens are phenolic matters imitating a structure of natural mammalian oestrogens and making weak estrogenic activity. The isoflavones and their intestinal metabolites (equol, demethylangolensin) bind to estrogen receptors, and most often, they have a weak estrogenic activity, that's why they are sometimes called "phytoestrogens" (*phyto* = plant, *estrus* = period of fertility for female mammals, gen = to generate) (Bruneton 1999). Food is main source of isoflavonoids for human population. The existence of phytoestrogens was depictured for the first time by Bennetts in 1946 in connection with the infertility of sheep, which were grazing on pastures with large scale of *Trifolium subterraneum* for the long time. In 1954, Bradbury and White isolated from that genistein and formononetin, isoflavonoids with estrogenic effect. Infertility was established at sheep for the first time, because cattle are less sensitive to the effect of phytoestrogens (Bradbury & White 1954).

The occurrence of isoflavones in food raises the question of their potential impact on human health. In the soya bean, the concentration of daidzein, genistein and their glycosyl derivates can reach 3 g kg^{-1}. The same compounds are also found in all of the derived products (soya bean powder, milk, fermented products), at concentrations that vary depending on the industrial manufacturing process.

Isoflavonoids are also tyrosine-kinase inhibitors, which may have a role in the transformation and cell proliferation phenomena (Bruneton 1999).

Epidemiologic studies in human strongly suggest the low incidence of hormonedependent diseases that is observed in Asian and vegetarian populations, correlated with the high consumption of soya bean which is common in those cultures (analysis of phytoestrogens by gas chromatography-mass spectrometry). The soya bean isoflavones, and maybe other constituents as well, seem to have a preventive effect on breast and prostate cancer, as well as colorectal cancer.

Several recent studies suggest that isoflavones and soya bean decrease the symptoms of menopause (hot flashes and others) and reduce the risk of osteoporosis (Bruneton 1999). In order to prevent these menopause-related complications, considerable number of women uses oestrogen substitutes as a hormone-replacing therapy (HRT).

In traditional Chinese medicine is used daidzein, genistein, formononnetin and biochanin A as a medicine for managing alcoholism, fever, cold and flu (Keung 1993; Goodman 2004). On the other hand, isoflavonoids were detected in beer (Lapčík et al., 1998).

Although there are many published studies dealing the topic of positive effects of isoflavonoids (Bruneton, 1999; Harborne et al., 1999; Klejdus, 2004 and others), the placebo-controlled clinical trials referring to the work on humans are insufficient up to the present day. Several clinical studies on climacteric problems were made (Wuttke et al., 2007), and a lot of them showed beneficial effect, e.g. study on frequency and severity of hot flushes.

According to Wuttke et al. (2007), the questionable effect originated in following facts:

- in some of double blind placebo-controlled clinical studies soy/red clover/isoflavone preparations were not superior to placebo
- in animal experiments, only high doses of such preparations were able to expressed expected effect (suppress the activity of the gonadotropin releasing hormone pulse generator as indicated by the reduction of serum luteinizing hormone levels)
- it is uncertain as to whether the low estrogenic potency of isoflavones has an estrogenic, antiestrogenic, or at all any effect on the mammary gland
- the estrogenic effects on the uterus may lead to endometrial hyperplasia and – if unopposed by progestins – to endometrial cancer
- animal experiments suggest putative goitrogenic effects of isoflavones
- estradiol and soy isoflavones appear to improve symptoms of osteoarthritis

• isoflavones may have some mild estrogenic lipid lowering effect in some, but by no means all women

1.4.6 Anti-inflammatory activity of isoflavonoids

The overall anti-inflammatory potential of flavonoids is known, most important mechanisms of action for flavonoids is modulation of the activity of pro-inflammatory enzymes such as COX and LOX, which produce potent inflammatory mediators, prostaglandins and LTs (González-Ponce et al. 2018). The anti-inflammatory capacity of flavonoids has long been utilized in Chinese medicine by applying crude plant extracts. Many investigations have shown that a variety of flavonoid molecules exhibit anti-inflammatory activity both, *in vitro* and in various animal models of inflammation. It may be valuable to study the anti-inflammatory activity of flavonoids, not only in order to establish anti-inflammatory mechanisms, but also for developing a new class of safe anti-inflammatory agents (García-Lafuente et al. 2009).

The first publications describing the anti-inflammatory potential of isoflavonoids are from 80s', when Kuhl et al. (1984) described the inhibition activity of 6,7,4' – trihydroxyisoflavan in human and peripheral blood leukocytes. Based on his methodology, Voß et al. (1992) tested effect of synthesized isoflavonoids *in vitro* with focus on structure activity relationship using porcine leukocytes suggested that tested isoflavans are stronger inhibitors of 5-LOX activity than isoflavones tested. They confirmed their suggestions 23 years later by *in vitro* experiments using human blood, and also *in cellulo* docking, concluding 6 isoflavones with IC_{50} lower or equal to baicalein, which was used for comparison, while texasin (6,7 dihydroxy-4'methoxy isoflavone) was the most selective 5-LOX inhibitor. This team subsequently evaluated whether some of the potent and selective 5-LOX inhibitors were also active against mammalian COX, but none of them inhibit COX-1 or COX-2 at IC_{50} concentrations higher than 150 μM, while ibuprofen and aspirin, which were used as positive controls, shown to inhibit COX-2 activity with $IC_{50} < 20$ μM (Mascayano et al. 2015). Jun et al. (2005) reported the inhibitory effect of genistein, daidzein and biochanin A isolated from kudzu (*Pueraria lobata*), which significantly suppressed AA release *in vitro*, while biochanin A was most active compound and isoflavone glucosides, puerarin and daidzin, showed lower inhibitory activities on the release of AA and its metabolites.

Paradkar et al. (2004) demonstrated *in vivo* that soy-isoflavone diet modulate the inflammation in intestine and liver of mice. Droke et al. (2007) concluded that soy isoflavones attenuate the negative effects of chronic inflammation on bone and cardiovascular health

(Droke et al. 2007). In experiments with mice, Khan et al. (2012b) proved that soy isoflavones have inhibitory effect on expression of COX-2, activation of NF-κB and on proinflammatory cytokines. Kim et al. (2009) revealed that the administration of daidzein *in vivo* attenuates myocardial damage via inhibition of NF-κB activation in rats, which may suppress inflammatory cytokine expression. Treatment with daidzein can also reduce the expression of TNF-α in rats (Selzer et al. 2000). Duan el al. (2003) proved anti-inflammatory effects of genistein *in vivo* on a guinea pig model of asthma. García-Lafuente et al. (2009) reviewed the anti-inflammatory potential of genistein, which is the most studied isoflavone, *in vitro* and *in vivo*.

Huang et al. (2005) suggested decreasing effect of soymilk rich in isoflavones on levels of TNF-α in postmenopausal women, when the inhibition rise up to 65.7%. Similarly, findings of Bao et al. (2011) suggested that soy isoflavone as nutritional supplement may provide a novel means for the treatment of airway inflammatory disease and their inhibition activity may be dose-dependent (Hamalainen et al. 2007). Benefit of genistein in asthma patients, supplemented for 4 weeks by this dietary soy isoflavone at physiologically relevant concentrations, supported its potential role in the treatment of asthma (Kalhan et al. 2008).

Beside "simple" anti-inflammatory activity, the pharmacological strategy has focused on dual inhibition of the 5-LOX and the COX-2 pathway in order to establish a more efficient anti-inflammatory therapy with reduced number of side effects. This can be achieved by a single compound either with dual action or by combination of both inhibitors (Pergola & Werz 2010). Despite the assertion that isoflavones are effective cancer prevention agents and have a variety of other health benefits, a significant gap exists between what has been shown *in vitro* and what can be achieved *in vivo*. The low bioavailability is usually not the result of poor absorption but of extensive metabolism of these compounds (Chen et al. 2005). On the other hand, Kim et al. (2006) detected three flavonoids apigenin, luteolin, and cynaroside in *Dystaenia takeshimana,* which showed COX-2/5-LOX dual inhibitory activity, which may suggest that there is a potential that even isoflavonoids can possess dual inhibitory activity and that they need future investigation.

However, the bioavailability of isoflavone can also influence the variability of results - equol as a gut metabolite of daidzein is considered to present higher affinity to estrogen receptor than other subtypes of isoflavones and the ability to produce equol seems to vary from person to person. Therefore, the doses, the form of supplementation and the bioavailability are questions to be investigated (Rosa et al. 2012). On the other hand, the bioavailability of isoflavonoids is highest from all flavonoids (Yao et al. 2004).

1.4.7 Antimicrobial activity of isoflavonoids

Isoflavonoids, naturally occurring in legume-based human diet, belong among a number of possible candidates of antimicrobial compounds, synthesized by plants as protecting agents against pathogenic microorganisms (Wyman & VanEtten 1978; Iinuma et al. 1994). The antimicrobial effect of isoflavones is also attributed to the presence of phenolic hydroxyl groups that have affinity for 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 their lipophilicity and consequently, enhances their antibacterial activity through interaction with cellular membranes (Mukne et al. 2011). Legume extracts rich in prenylated isoflavonoids showed potent antibacterial activity against *Listeria monocytogenes* and methicillin-resistant *S. aureus* suggesting legume seedlings as promising naturally improved preservation antimicrobials of foods (Araya-Cloutier et al. 2017).

The fact, that the "iso" structures of flavonoids are more important for antifungal activity was summarised by Johnson et al. (1976), who compared selected flavonoids and their "iso" forms and suggested substituents at 5, 6, and 7 positions of the A ring as crucial for antifungal activity of isoflavonoids. He also summarised antifungal agents of isoflavonoids origin such as formononetin, biochanin A, and luteone and mentioned the aglycones had very significant activity against certain plant pathogens in comparison with glycosides.

Wyman and VanEtten (1978) described effect of six isoflavonoids on bacteria in the semisolid medium bioassay. Osawa et al. (1992) determined by paper disc assay that four isoflavanones (dihydrobiochanin A, ferreirin, darbergioidin, dihydrocajanin) had potent antibacterial activity against cariogenic bacteria. The effect of seven isoflavonoid compounds against cariogenic oral bacteria was also investigated by Sato el al. (2003) confirming the antibacterial effect of erycristagallin to mutans streptococci was based on a bactericidal action suggesting bacterial cell membrane as one of the operative targets of erycristagallin. Hong et al. (2006) evaluated the direct effect of genistein *in vitro* and, furthermore, suggested the use of genistein in combination with probiotics may augment the effectiveness of antimicrobial therapies currently used in the management of infections. Dastidar et al. (2004) studied eleven isoflavones, while two of them (sophoraisoflavone A, 6,8-diprenylgenistein) showed a significant inhibitory action against bacteria both *in vitro* and *in vivo*. Sophoraisoflavone A and 6,8-diprenylgenistein were most active against *S. aureus*, and then in decreasing order against *Vibrio cholerae*, *Salmonella*, *Shigella, Klebsiella* and *Pseudomonas* spp. Bacteria resistant to antibiotics such as klebsiellae and pseudomonads were also fairly sensitive to

sophoraisoflavone A and 6,8-diprenylgenistein. Both isoflavones showed bacteriostatic activity. They gave significant protection to mice challenged with a virulent *Salmonella* and there were no toxic side effects.

The potential antibacterial activities of genistein was examined by Verdrengh et al. (2004), when exposure to genistein exhibited an inhibitory effect on all staphylococcal strains tested, including methicillin-resistant strains. Furthermore, the growth of *Streptococcus pasteurianus*, *B. cereus*, and *H. pylori* was clearly inhibited by genistein, whereas *E. coli* growth was not suppressed. For comparison, daidzein, which is structurally similar to genistein, was also tested, and inhibited the growth of *S. aureus*, albeit with lower potency than genistein. Their results indicated that genistein exerts potent antibacterial properties *in vitro*, which are possibly mediated by the stabilization of the covalent topoisomerase II-DNA cleavage complex. Flythe and Kagan (2010) described inhibitory effect of biochanin A against *Clostridium sticklandii*. The inhibitory effect of biochanin A was also evaluated by Sklenickova et al. (2010), enhanced by the finding, that this compound is able to inhibit several clostridia, but in the same time does not suppress bifidobacteria, which are important probiotic microorganisms. Khan et al. (2012a) investigate antibacterial potential of *Trifolium alexandrinum* against both, Gram-positive and Gram-negative hospital isolated human pathogenic bacterial strains, confirming the effectiveness of crude leaves extracts against tested human pathogenic bacterial strains causing several tropical diseases. Since Egyptian clover is used as a fodder plant, it could be helpful in controlling various infectious diseases associated with cattle as well and avoid eventual diseases from food chain. The antibacterial effect of isoflavones against some strains of potentially human pathogenic bacteria has also been proven in several previous studies (as reviewed by Mukne et al. 2011). Therefore, it is obvious that isoflavonoids inhibit Gram-positive bacteria more than Gram-negative ones (Gnanamanickam & Smith 1980).

1.4.8 Synergistic antimicrobial effect of isoflavonoids

The discovery of penicillin nearly 90 years ago revolutionized the treatment of bacterial disease. Since that time, numerous other antibiotics have been discovered from bacteria and fungi, or developed by chemical synthesis and have become effective chemotherapeutic options. However, the misuse of antibiotics has lessened the efficacy of many commonly used antibiotics. The emergence of resistant strains of bacteria has seriously limited the ability to treat bacterial illness, and new antibiotics are desperately needed (Cheesman et al. 2017). Two approaches can be followed to address this problem: screening various sources for new leads

for antibiotics or finding ways to disable the resistance mechanisms to existing antibiotics. The spread of multidrug-resistant (MDR) *S. aureus* strains, including methicillin-resistant *S. aureus* (MRSA), has shortened the useful life of antistaphylococcal drugs enormously. Plants are resistant to most microorganisms, but despite extensive efforts to identify metabolites that are responsible for this resistance, no substantial progress has been made. Plants possibly use multiple strategies to deal with microorganisms that evolved over time (Abreu et al. 2017).

Isoflavones isolated from *Lupinus argenteus* were found to potentiate activity of berberine and norfloxacin. The isoflavones increased the uptake of berberine into *S. aureus* cells, indicating that they may be inhibiting a MDR pump (Morel et al. 2003). The ethanolic extract of the aerial parts of the herb *Sophora moorcroftiana* showed significant antibacterial activity against drug-resistant *S. aureus*, however, five flavonoids isolated exhibited antibacterial activity weaker than the extract. In combination with norfloxacin, the antibacterially inactive compound genistein showed significant synergistic activity against drug-resistant *S. aureus*. In an efflux experiment to elucidate a plausible mechanism for the observed synergy, genistein showed marginal inhibition of the NorA efflux protein (Wang et al. 2014). Mbaveng et al. (2015) assessed the antimicrobial activity of selected natural products against Gram-negative MDR bacteria, among which isoflavonoids neobavaisoflavone and daidzein showed inhibitory activity against all tested strains. Isoflavonoids from *Cytisus striatus* were found to potentiate the effect of ciprofloxacin and erythromycin against MRSA strains revealing a clear synergy between isoflavonoids and the tested antibiotics, showing their great potential for applications in the clinical therapy of infections with antibiotic-resistant microorganisms such as MRSA (Abreu et al. 2017).

1.4.9. Structure-activity relationship of isoflavonoids

It is obvious that there exists a relation between the chemical constitution and the biological activity of compounds (Brown & Fraser 1868). This connection is called structure-activity relationship (SAR) and it is an approach, which considers that drugs' biological activities are induced by their molecular structure, and every change in the molecular structure leads to a change of these properties. This phenomenon has wide applications in medical chemistry, one of them being the design of new drugs (Putz et al. 2016).

Likewise, as other secondary plant metabolites, chemical structures of isoflavones are changed after digestion through a human or animal gastrointestinal tract into other structurally related compounds, which can possess biological activities significantly differing from their precursors (Pandey & Rizvi 2009). Additionally, there are several studies dealing with

antitumour (Lee et al. 2011a; Lee et al. 2011b) and antioxidant (Roh et al. 2011; Villares et al. 2011) activities of isoflavones' metabolites proving these compounds to be more biologically active than their precursors (Lee et al. 2011a; Lee et al. 2011b; Roh et al. 2011). Despite the vast biological potential of isoflavones' metabolites, no report has been published on their antibacterial nor anti-inflammatory activity. Moreover, the previous studies of isoflavones showed that hydroxyl groups and their methylation at certain positions often occur during digestion. This significantly affects their biological properties (Cushnie & Lamb 2005; Villares et al. 2011) and plays a key role in their anti-inflammatory and antimicrobial potential.

2 HYPOTHESIS

Musculoskeletal infections caused by *S. aureus* are relatively seldom in general population, but the incidence is considerably higher in patients with predisposing conditions, particularly those with rheumatoid arthritis, a chronic inflammatory disorder. Therefore, it is obvious that focus on anti-inflammatory and antimicrobial potential is crucial for future development of natural-derived products used for treatment of both, infections and inflammation. This "dual" effect has been already proven in several natural compounds, as for example in salicylate from willow bark, which are used for medicinal purposes from ancient times. As soy, rich in isoflavonoid content, is used in traditional Chinese medicine to treat oedema, common cold, skin disease, diarrhoea, leg ulcers and other disorders, and is widely-consumed mainly in Asian countries, where there is generally lower incidence of musculoskeletal diseases, we supposed that in-depth analysis of isoflavonoids and their activities may contribute to medicinal uses of these perspective compounds.

Since isoflavonoids, and especially isoflavones, are directly associated with human dietary ingredients and health, there is need to evaluate relationship between their structure and function. The bioavailability, metabolism, and biological activity of isoflavonoids depend upon the configuration, total number of hydroxyl groups, and substitution of functional groups about their nuclear structure. Based on the supposition that the biological activity of the isoflavonoids should be influenced by the presence of the functional groups at certain positions, the evaluation of anti-inflammatory and antimicrobial activity of selected methoxy and hydroxyl structure-related isoflavonoids and their metabolites *in vitro* can lead to the identification of key functional groups and their positions. This approach can generate knowledge necessary for development of new effective anti-inflammatory and antimicrobial drugs.

3 OBJECTIVES

The main aim of the thesis is evaluation of *in vitro* anti-inflammatory and antimicrobial activity of structure related isoflavonoids and identification of their possible dual anti-inflammatory/antimicrobial effect.

The specific objectives of the study are:

I) determination of inhibitory effect of isoflavonoids on COX-2 and 5-LOX enzymes

II) assessment of growth-inhibitory effect of isoflavonoid compounds against bacterial pathogens involved in musculoskeletal infections

III) evaluation of combinatory effects of most prospective isoflavonoid structures with other agents.

4 MATERIALS AND METHODS

4.1 Chemicals

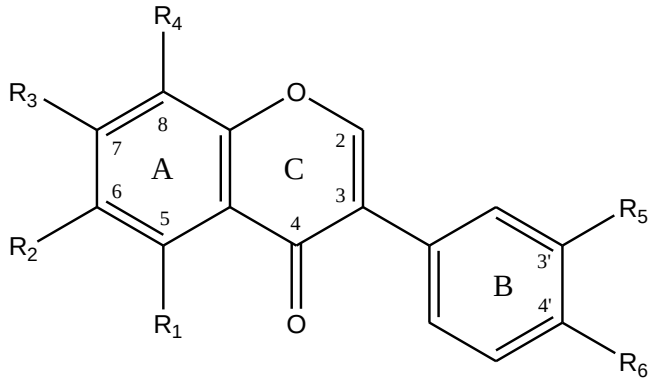
In this study, we tested 19 commercially obtained structure-related isoflavones (see Figure 8) (INDOFINE Chemical Company, Inc., Hillsborough, New Jersey, USA). The purity of the tested compounds varied from 97 % to 99+ %, as shown in the following Table 1. As dissolvent for the tested compounds was used dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Prague, CZ), solutions were stored at -20°C.

For COX-2 assay, human recombinant COX-2, porcine hematin, L-epinephrine, disodium ethylenediamine tetraacetate (Na_2EDTA), ethanol (EtOH), arachidonic acid (AA), and formic acid were purchased from Sigma-Aldrich (Prague, CZ). Tris was purchased from Bio-Rad (Prague, Czech Republic).

For 5-LOX assay, eicosatetraynoic acid (ETYA), calcium Ionophor A23187, indomethacin, trypan blue, and gentian violet, were purchased from Sigma-Aldrich (Prague, CZ). Dextran T-500 was purchased from Roth (Karlsruhe, Germany). Ammonium chloride (NH_4Cl), disodium hydrogen phosphate (Na_2HPO_4), sodium chloride (NaCl), and potassium dihydrogen phosphate (KH_2PO_4) were obtained from Lach-Ner (Neratovice, CZ) and zileuton was donated by Farmak (Olomouc, CZ). Potassium chloride (KCl) and sodium hydroxide (NaOH) were purchased from Lachema (Brno, CZ). Calcium chloride ($CaCl_2 \cdot 2H_2O$) and acetic acid (CH_3COOH) were obtained from Penta (Prague, CZ).

For antimicrobial susceptibility testing, ciprofloxacin, erythromycin, oxacillin, tetracycline, and vancomycin (Sigma-Aldrich, Prague, CZ) were dissolved at an appropriate concentration in deionized water or ethanol (Lach-Ner, Neratovice, CZ) prior to testing.

For combinatory antimicrobial effect, amoxicillin, clavulanic acid (salt potassium clavulanate), as well as acetonitrile, trifluoroacetic acid (TFA), and α-cyano-4-hydroxycinnamic acid (HCCA) were purchased from Sigma-Aldrich (Prague, CZ).



No.	Name of isoflavone	R_1	R_2	R_3	R_4	R_5	R_6
1	6,4'-dimethoxy-7-hydroxyisoflavone	H	OCH_3	OH	H	H	OCH_3
2	6,7,4'-trimethoxyisoflavone	H	OCH_3	OCH_3	H	H	OCH_3
3	7-hydroxyisoflavone	H	H	OH	H	H	H
4	7,4'-dimethoxyisoflavone	H	H	OCH_3	H	H	OCH_3
5	7,8,4'-trimethoxyisoflavone	H	H	OCH_3	OCH_3	H	OCH_3
6	6,7-dimethoxyisoflavone	H	OCH_3	OCH_3	H	H	H
7	5,7,4'-trimethoxyisoflavone	OCH_3	H	H	OCH_3	H	OCH_3
8	7-hydroxy-6methoxyisoflavone	H	OCH_3	OH	H	H	H
9	6,7,4'-trihydroxyisoflavone	H	OH	OH	H	H	OH
10	7,3',4'-trihydroxyisoflavone	H	H	OH	H	OH	OH
11	7,4'-dimethoxy-5-hydroxyisoflavone	OH	H	OCH_3	H	H	OCH_3
12	5,7-dihydroxy-4'-methoxyisoflavone	OH	H	OH	H	H	OCH_3
13	7,8,4'-trihydroxyisoflavone	H	H	OH	OH	H	OH
14	7-methoxyisoflavone	H	H	OCH_3	H	H	H
15	5,4'-dihydroxy-7-methoxyisoflavone	OH	H	OCH_3	H	H	OH
16	7,4'-dihydroxy-6-methoxyisoflavone	H	OCH_3	OH	H	H	OH
17	7,4'-dihydroxyisoflavone	H	H	OH	H	H	OH
18	5,7,4'-trihydroxyisoflavone	OH	H	OH	H	H	OH
19	7-hydroxy-4'-methoxyisoflavone	H	H	OH	H	H	OCH_3

Figure 8. Chemical structures of tested compounds **[*1 – 19*]**

Table 1. Purity of the tested compounds and their synonyms

No.	Chemical name of compound	Synonym	Purity [%]
1	6,4'-dimethoxy-7-hydroxyisolfavone	afrormosin	98
2	6,7,4'-trimethoxyisoflavone		98
3	7-hydroxyisoflavone		99
4	7,4'-dimethoxyisoflavone		99.4
5	7,8,4'-trimethoxyisoflavone		98+
6	6,7-dimethoxyisoflavone		98
7	5,7,4'-trimethoxyisolfavone		98
8	7-hydroxy-6-methoxyisoflavone		98
9	6,7,4'-trihydroxyisoflavone	demethyltexasin	98
10	7,3',4'-trihydroxyisoflavone	hydroxydaidzein	97
11	7,4'-dimethoxy-5-hydroxyisoflavone		98
12	5,7-dihydroxy-4-methoxyisoflavone	biochanin A	99+
13	7,8,4'-trihydroxyisoflavone	demetylretusin	98+
14	7-methoxyisoflavone		99
15	5,4'-dihydroxy-7-methoxyisoflavone	prunetin	98+
16	7,4'-dihydroxy-6-methoxyisoflavone	glycitein	97
17	7,4'-dihydroxyisoflavone	daidzein	99
18	5,7,4'-trihydroxyisoflavone	genistein	99+
19	7-hydroxy-4'-methoxyisoflavone	formononetin	98

4.2 Bacterial strains and growth media

The antimicrobial activity was evaluated against nine bacterial American Typical Culture Collection (ATCC) strains, and they were selected as representatives of both classes of Gram-positive (*B. cereus* ATCC 11778, *E. faecalis* ATCC 51299, *L. monocytogenes* ATCC 7644, *S. aureus* ATCC 29213, *S. epidermidis* ATCC 12228, *Streptococcus pyogenes* ATCC 19615) and Gram-negative (*E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *S. enteritidis* ATCC 13076) bacteria. The standard ATCC strains, including those tested for antistaphylococcal activity of demethyltexasin (*S. aureus* ATCC 25923, ATCC 43300, ATCC

BAA-976 and ATCC 33591), were purchased from Oxoid (Basingstoke, UK) on ready-to-use bacteriological Culti-Loops.

Nine human clinical isolates of staphylococcal strains, methicillin-resistant (MR 12001, MR 12004, CCM 7112, CCM 7115), methicillin-sensitive (MS 12001), tetracycline-resistant (TR 12001, TR 12002), erythromycin-resistant (ER 12001) and epidemic methicillin-resistant *S. aureus* (EMR 15), were obtained from the Motol University Hospital (Prague, CZ). The identification of clinical isolates was performed by Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). With the exception of *S. pyogenes* that was grown in a brain heart infusion (Oxoid, Basingstoke, UK), all bacteria were tested in Mueller-Hinton broth (Oxoid, Basingstoke, UK). For *E. faecalis*, Mueller-Hinton broth was enriched with 1 % of glucose (Sigma-Aldrich, Prague, CZ). The sensitivity of standard strains to ciprofloxacin and the susceptibility of clinical isolates to oxacillin, erythromycin, tetracycline, and vancomycin were checked.

For inoculum preparation, overnight cultures of each bacteria strain were directly suspended in 10 ml of broth (Mueller–Hinton or brain heart infusion). The turbidity of bacterial suspension was adjusted to 0.5 McFarland standard (which represents 1.5 x 108 bacteria ml^{-1}) using Densi-La-Meter II (Lachema, Czech Republic) as a spectrophotometric device for inoculum standardization.

4.3 COX-2 assay

This *in vitro* assay was performed according to the procedure previously described by Reininger and Bauer (2006) with human recombinant COX-2. COX-2 (0.5 unit/reaction) was added to 180 µl of incubation mixture that consisted of 100mM tris buffer (pH 8.0), 5 µM porcine hematin, 18mM L-epinephrine, and 50 µM Na_2EDTA. The test samples were dissolved in DMSO. Compounds were tested in concentrations of 50, 100, and 150 µM for screening. 12% ethanol (in case of blanks for the isoflavonoid samples), or pure DMSO (in case of blanks for purified constituents) was added (10 µl) and the mixture was preincubated for 5 min at room temperature. The addition of 5 µl of 10µM AA started the reaction. After 20 minutes of incubation at 37°C, the reaction was stopped by 10 µl of 10% formic acid. All samples were diluted 1 : 15 in ELISA buffer and the concentration of PGE_2 (prostaglandin E_2) produced by the reaction was determined by a PGE_2 ELISA kit (Enzo Life Sciences, USA) according to the manufacturer's instructions. Absorbance relative to PGE_2 concentration was measured with a microplate reader Tecan Infinite M200 (Tecan Group, Switzerland) at 405

nm. The results were expressed as mean of percentage inhibitions of PGE_2 formation against untreated samples (blanks, control). The percentage of inhibition was measured using the following equation:

$$\% \text{ inhibition} = \frac{(\text{Absorbance of control} - \text{Absorbance of test sample})}{\text{Absorbance of control}} \times 100$$

4.4 LTB_4 inhibition assay

The 5-LOX in vitro assay was performed in a slightly modified version of the standard method described previously by Adams M. et al. (2004) at University of Graz.

Venous human blood (45 ml) obtained from healthy donors was sedimented in 20mL of dextran solution (6% dextran T-500, 1% NaCl) at 4°C. After one hour, the supernatant was collected and centrifuged at 1600 rpm at 4°C for 10 min and then the supernatant was discarded. The obtained pellet was washed with phosphate buffered saline (PBS, 0.02% KCl, 0.024% KH_2PO_4, 0.8% NaCl, 0.288% Na2HPO4·12H2O, pH 7.4) and again centrifuged. The hereby obtained pellet was lysed (0.17% NH4Cl, 0.2% Tris, pH 7.2) for 5 min at room temperature and then centrifuged at 1400 rpm at 4°C for 5 min. The pellet was washed by PBS again and centrifuged at 1400 rpm at 4°C for 15 min. Finally, the pellet was dissolved in 3mL of PBS and the cells were tested for the viability. Cell vitality test: 20 µl of cell suspension and 10 µl of (0.4 %) trypan blue solution were mixed and then 10 µl of this suspension were examined and quantified in a Bürker haemacytometer with a light microscope at 1000-fold magnification. Due to absorption of trypan blue, dead cells appear larger and darker. The vitality of the cells must be over 95%. Determination of cell concentration: 10 µl of granulocyte cell suspension were dyed using 990 ml aqueous TÜRKS solution and quantified in a Bürker haemacytometer as described by the producer. In the end the cells were diluted to the final concentration of 4500 cell μl^{-1}.

The incubation mixture consisted of 225 µl of the cell suspension, 10 µl of 2mM CaCl2, 10 µl of 10µM ETYA, 5 µl of tested sample, 10 µl of 21 µM Calcium Ionophor A23187, and 5 µl of 120µM AA. The reaction was stopped after 10 min incubation at 37°C with 20 µl of 10% formic acid. Isolated neutrophils incubated with tested substances were checked for the viability using trypan blue to verify that inhibition of LTB_4 biosynthesis was not achieved via cytotoxicity of tested compounds. Samples were diluted 40 times in ELISA

buffer and the concentration of LTB_4 was measured using a commercial LTB_4 ELISA kit (Enzo Life Sciences, Farmingdale, New York USA), according to the manufacturer's instructions. Absorbance relative to LTB_4 concentration was measured at 405 nm using a Tecan Infinite M200, supported by Magellan™, the universal reader control and data analysis software (Tecan Group Ltd., Männedorf, Switzerland). The results were expressed as percentage inhibition of LTB_4 formation against untreated samples (blanks). The percentage of inhibition was measured using the same equation as for evaluation of COX-2 assay. Three independent experiments with at least two replicates were used for the calculation of inhibition curves.

4.5 Broth microdilution method

The *in vitro* antimicrobial activity was determined by the broth microdilution method using 96-well microtiter plates according to CLSI guidelines (2009), modified according to the recommendations previously proposed for more effective assessment of the anti-infective potential of natural products (Cos et al. 2006). The samples were 2-fold diluted in Mueller-Hinton broth (100 μl) in a range of 4 - 128 μg ml^{-1}. Bacterial cultures were diluted to contain 2.5-3 × 10^4 CFU ml^{-1} in microtiter plate, each well was subsequently inoculated with the suspension and microplates were incubated at 37°C for 24 h. Growth of microorganisms were estimated visually as turbidity and more accurately determined by measuring the optical density by Multiscan Ascent Microplate Photometer (Thermo Fisher Scientific, Waltham, USA) at 405 nm. Minimum inhibitory concentrations (MICs) were calculated based on the density of the growth control and were expressed as the lowest concentrations, which showed at least 80% reduction of microorganisms' growth, compared to that of the compound-free growth control. All samples were tested as three independent experiments, each was carried out in triplicate and the results are presented as the mean of MICs obtained from these experiments.

4.6 Checkerboard method

The antistaphylococcal combinatory effect of amoxicillin/clavulanic acid, amoxicillin/demethyltexasin and oxacillin/ demethyltexasin were evaluated by the checkerboard method based on fractional inhibitory concentrations (FICs) (White et al. 1996), both performed on 96-well microtiter plates.

In combinations, eight two-fold serial dilutions of antibiotic (amoxicillin or oxacillin) from horizontal rows of the microtiter plate were subsequently crossdiluted vertically by eight two-fold serial dilutions of the test compound (demethyltexasin or clavulanic acid). Microplates so arranged can be used to screen 64 different combinations of concentrations. The initial concentrations used in the combinations for both clavulanic acid and demethyltexasin were 256 μg ml^{-1}, whereas for amoxicillin and oxacillin various starting concentrations were used depending on the staphylococcal strain's susceptibility to the antibiotics tested.

Assay microplate preparation and serial dilution were performed through the automated pipetting platform Freedom EVO 100 equipped with a four-channel liquid handling arm (Tecan, Männedorf, CH). Plates were inoculated by bacterial suspension (final density 5×10^5 cfu ml^{-1}) and incubated at 37°C for 24 hr. The bacterial growth was then measured spectrophotometrically using a Multimode Reader Cytation 3 (BioTek Instruments, Winooski, Vermont, USA) at 405 nm.

The combined effects of the antibiotics with the tested compound (A and B) were then determined based on the value of ∑ FIC, which was calculated according to the following equation:

$$\sum FIC = FIC_A + FIC_B$$

$$FIC_A = MIC_{A\ (in\ the\ presence\ of\ B)} / MIC_{A(alone)}$$

$$FIC_B = MIC_{B(in\ the\ presence\ of\ A)} / MIC_{B(alone)}.$$

In view of the fact that it is a widely accepted norm in MIC testing that variation in a single result places an MIC in a three dilution range (mode ± 1 dilution), the possibilities for reproducibility errors in an MIC checkerboard are considerable. Within the limits of experimental error, ∑ FIC really indicates only ''synergy,'' ''no interaction,'' and ''antagonism'' between agents (Odds 2003).

Therefore, in this study, the antimicrobial combinatory effect was interpreted according to Odds (2003) as follows: a synergistic effect if ∑ FIC ≤ 0.5; no interaction if ∑ FIC > 0.5–4, and antagonistic if ∑ FIC > 4. *S. aureus* 29213 was used as a control strain for antibiotic susceptibility testing and strains were identified as MRSA when oxacillin MIC was ≥ 4 μg ml^{-1} (CLSI 2009). Solvents used as the negative control did not inhibit any strain tested.

4.7 Statistical evaluation

In experiments focused on anti-inflammatory effect, the initial screening of isoflavonoids' effect on COX-2 activity was done in duplicate for each concentration (50, 100, 150 μM). As none of the obtained values was active at least as a half of the inhibition activity of the standard COX-2 inhibitor in any concentrations in this screening test, the results are presented as the mean of inhibition [%] obtained from these experiments. The percentage of inhibition was measured using the following equation:

$$\%\ \text{inhibition} = \frac{(\text{Absorbance of control} - \text{Absorbance of test sample})}{\text{Absorbance of control}} \times 100$$

In experiments focused on 5-LOX inhibitory activity of isoflavonoids, the results were expressed as percentage inhibition of LTB_4 formation against untreated samples (blanks). The percentage of inhibition was measured using the same equation as for evaluation of COX-2 assay. Three independent experiments with at least two replicates were used for the calculation of inhibition curves. The obtained data were evaluated by Dixon's test, Grubbs' test and Z-score test for overall evaluation, and for detection, identification and rejection of outliers. The obtained values were rejected when all three test confirmed them as outliers. The probability value (the *p*-value) was evaluated by two-tailed Student's t-test. In the results, the compound, which is not significantly different from zileuton with the level of significance $p < 0.05$ is marked with star (*).

For assessment of antibacterial action, all samples were tested as three independent experiments, each was carried out in triplicate and the results are presented as the mean of minimum inhibitory concentrations obtained from these experiments.

5 RESULTS AND DISCUSSION

5.1 Inhibitory effect of isoflavonoids on COX-2 activity

The results of COX-2 screening showed that none of the tested compounds within the tested concentration range of 50, 100, 150 μM was active at least half as much as indomethacin, a standard COX inhibitor, which inhibited the COX-2 enzyme by 86.4 % (SD ± 1.5), 94.6 % (SD ± 0.9) and 92.8 % (SD ± 1.4) within the same concentration range. As shown in Figure 9, the highest inhibitory activity from tested compounds showed genistein, followed by 7,4'-dimethoxy-5-hydroxyisoflavone and 7-methoxyisoflavone. Genistein inhibited the activity of COX-2 enzyme by 23.6 % (SD ± 8), 39.9 % (SD ± 17.7) and 25.5 % (SD ± 14.7) in the above mentioned concentration range. The results are presented as the mean of inhibition [%] obtained from the screening.

The anti-inflammatory potential of genistein was already described. For example, Hertrampf et al. (2005) suggested genistein as an interesting substance of a possible hormone replacement therapy, because genistein is a highly potent inhibitor of COX-2 expression in the vena cava, but has only a limited ability to induce proliferation in tissues like the uterus and the mammary gland. Hooshmand et al. (2007) described the ability of genistein to suppress the production of COX-2 at the dose of 100 μM, while it has no effect on COX-1 production, which is advantageous because COX-1 enzyme is involved in cellular house-keeping functions such as the maintenance of gastrointestinal integrity and vascular homeostasis. Swami et al. (2009) suggested genistein at the concentration of 10 μM significantly reduced the secretion of prostaglandin synthesis by human prostate cancer cell lines and primary prostate epithelial cells by decreasing COX-2 mRNA and protein expression. Despite the above mentioned studies suggesting significant anti-inflammatory properties of genistein, our results showed only weak COX-2 inhibitory activity, which may be explained by different mechanisms of its anti-inflammatory action e.g. regulation of key genes expression instead of direct inhibition of PGE_2.

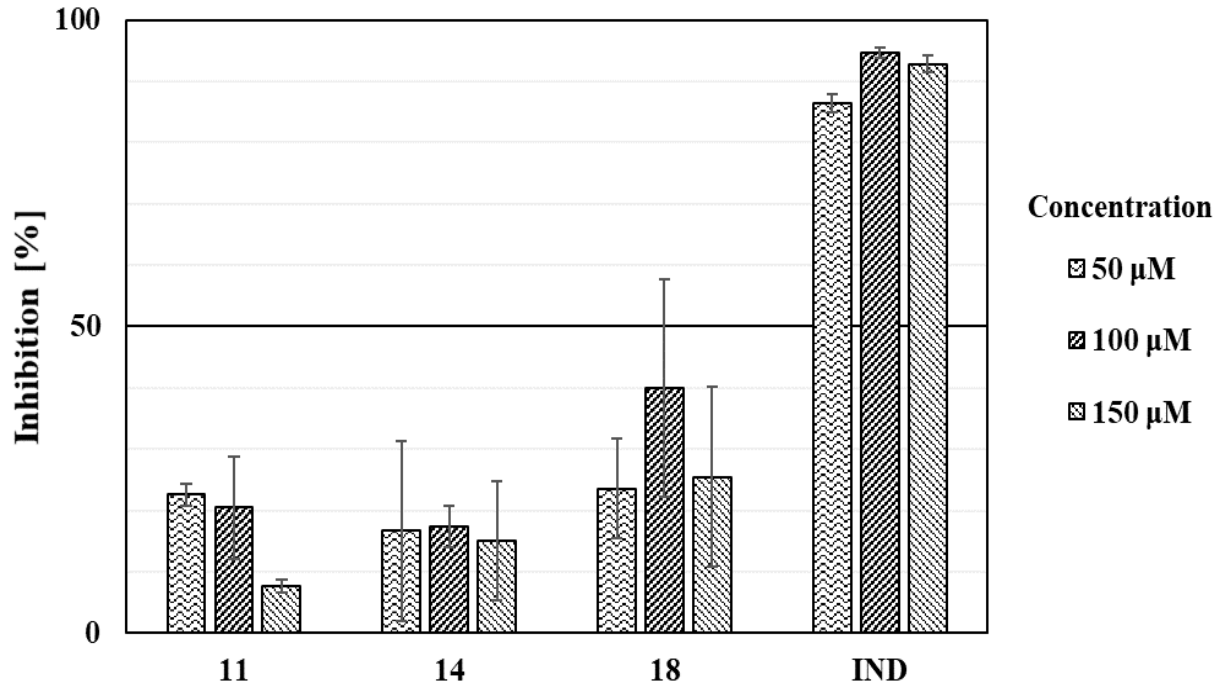


Figure 9. COX-2 inhibition of studied isoflavonoids

11 7,4-dimethoxy-5-hydroxyisoflavone, **14** 7-methoxyisoflavone, **18** genistein, **IND** indomethacin

5.2 Inhibitory effect of isoflavonoids on 5-LOX activity

In contrast to low inhibitory effect of isoflavonoids on COX-2 activity, most of the isoflavonoid structures tested exhibited 5-LOX inhibitory activity, whereas the most promising results were observed in biochanin A, which inhibited the LTB_4 production by 88.2 % (SD ± 5.9) at 100 µM concentration. For the comparison, zileuton, an anti-inflammatory leukotriene pathway inhibitor classified as an inhibitor of the enzyme 5-lipoxygenase, was used, which inhibited the LTB_4 production by 95.4 % (SD ± 3.0) at the same concentration. Based on the statistical evaluation only biochanin A showed the significant activity, with $p < 0.05$. The results are shown in Figure 10. The inhibitory effect of biochanin A was already reported by Jun et al. (2005) together with genistein, daidzein and formononetin isolated from kudzu (*Pueraria lobata*), which significantly suppressed AA release *in vitro*, while biochanin A was most active compound and isoflavone glucosides, puerarin and daidzin, showed lower inhibitory activities on the release of AA and its metabolites. Other perspective compound with good potential to inhibit 5-LOX activity was formononetin, which inhibited the LTB_4

production by 78.9 % (SD ± 7.4), but statistically its activity was not evaluated as significant, with $p < 0.05$. According to Wang et al. (2012), formononetin demonstrated a reduction in some inflammatory mediators such as NF-κB and IL-1β *in vitro*. In addition, this compound was able to decrease the levels of TNF-α and IL-6 (Li et al. 2014).

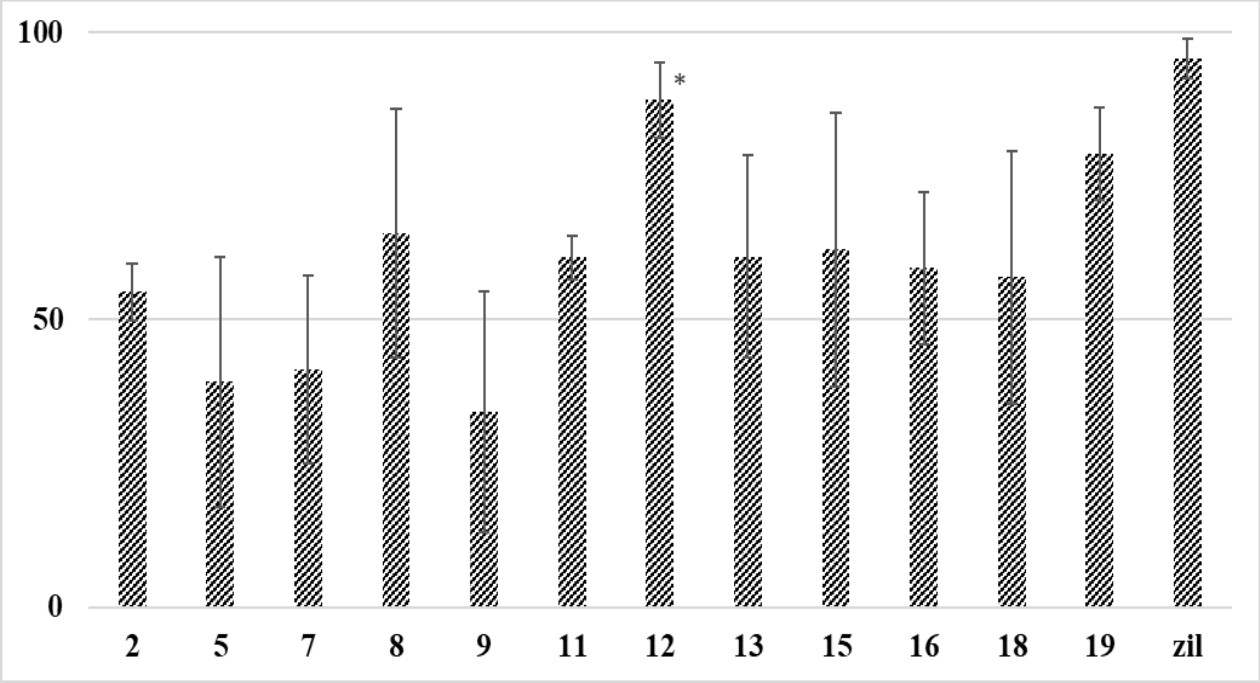


Figure 10. 5-LOX inhibition of studied isoflavonoids (100 μM concentration)

2 6,7,4'-trimethoxyisoflavone, **5** 4',7,8-trimethoxyisoflavone, **7** 5,7,4'-trimethoxyisolfavone, **8** 7-hydroxy-6-methoxyisoflavone, **9** demethyltexasin, **11** 7,4'-dimethoxy-5-hydroxyisoflavone, **12** biochanin A, **13** demetylretusin, **15** prunetin, **16** glycitein, **18** genistein, **19** formononetin, **zil** zileuton

In regards to the structure activity relationship, compounds with low or no inhibitory effect on LTB_4 synthesis were for example 6,4'-dimethoxy-7-hydroxyisoflavone, 6,7-dimethoxyisoflavone, and 7-hydroxyisoflavone, with two methoxy groups in their structure, either in the A-ring or B-ring, or only one hydroxyl group in their structure, suggesting the position of only one methoxy group at the position of C-4' of the ring B and at least one hydroxyl group at the ring A (at C-7 and/or C-5 position) as crucial for the anti-inflammatory activity of isoflavonoids. These suggestions are in correspondence with Jun et al. (2005), who

reported that 5,7-dihydroxyl group in the A-ring of isoflavones could be a key functional group responsible for the strong inhibitory activity of biochanin A.

5.3 Antibacterial effect of isoflavones

Within the 15 isoflavone structurally-related compounds tested by the broth microdilution method, five of them exhibit *in vitro* antimicrobial activity against at least one of the nine bacteria strains tested. Values of MICs determined for these antibacterially active substances (Table 2) showed that the isoflavones' metabolite demethyltexasin possessed the strongest growth inhibitory effect with MIC values of ≥ 16 μg ml^{-1}, followed by biochanin A and genistein with MIC values of ≥ 32 μg ml^{-1} and ≥ 64 μg ml^{-1}, respectively, and finally by two isoflavones' metabolites hydroxydaidzein and demethylretusin, with MIC values of ≥ 128 μg ml^{-1}.

Table 2. *In vitro* growth-inhibitory effect of selected isoflavones on potentially pathogenic bacteria

Microorganism tested	**Compound / MIC [μg ml^{-1}]**					
	9	***10***	***12***	***13***	***18***	**CIP**
B. cereus	32		64		128	0.25
E. faecalis		128				0.25
L. monocytogenes	128	128	64			0.5
S. aureus	16					0.5
S. epidermidis	32			128		0.125
S. pyogenes	64		32		64	0.5

MIC minimum inhibitory concentration (value is the mean minimum inhibitory concentrations of three independent experiments each performed in triplicate), ***9*** demethyltexasin, ***10*** hydroxydaidzein, ***12*** biochanin A, ***13*** demethylretusin, ***18*** genistein, **CIP** ciprofloxacin

In contrast to previously described antibacterial effect of biochanin A against some strains of *Clostridia* (Sklenickova et al. 2010), *Chlamydia* (Pohjala et al. 2012), *Mycobacterium* (Lechner et al. 2008) and *S. aureus* (Dastidar et al. 2004) as well as genistein against *Mycobacterium* (Kuete et al. 2008) and *S. aureus* (Dastidar et al. 2004, Verdrengh et al. 2004), according to our best knowledge, there are no reports on the antibacterial effect of demethyltexasin, hydroxydaidzein and demethylretusin. Since these three compounds belong to *ortho*-dihydroxyisoflavones, a class of compounds known as potent anti-inflammatory, anti-allergenic and antioxidant agents contributing to cholesterol-lowering, cardiovascular protection and tumour prevention (Roh et al. 2011), we suggest that isoflavones of this group

may be considered as prospective leading structures for development of new antimicrobial drugs. Especially the activity of demethyltexasin, which showed the most potent antibacterial activity in our experiments and which is found in high levels in some kinds of soy-based fermented food, should be investigated more profoundly. In addition, demethyltexasin, hydroxydaidzein and demethylretusin that represent three major metabolites of hepatic metabolism of daidzein, were more active than their precursor, which showed no or negligible antibacterial activity. This observation is in correspondence with previously published reports describing stronger biological activity of isoflavones' metabolites than their precursors. For example, equol, one of the most biologically active metabolites of daidzein, exerts an antitumour effect due to the inhibition of cell transformation, whereas its precursor is inactive (Lee et al. 2011b). Another example of this phenomenon is hydroxydaidzein, as major metabolite of daidzein, which effectively inhibits the ultraviolet B-induced cyclooxygenase 2 (COX-2) expression, while daidzein has no effect on COX-2 expression levels (Lee et al. 2011a).

Any of the tested compounds did not affect the growth of tested Gram-negative bacterial strains (*E. coli*, *P. aeruginosa*, *S. enteritidis*). This is in correspondence with previous studies describing low antibacterial effect of isoflavones against Gram-negative bacteria (Verdrengh et al. 2004, Mukne et al. 2011).

All the results show that, with the exception of one dihydroxy-methoxy-isoflavone (biochanin A), only trihydroxyisoflavones were antibacterially active. Generally, the position of hydroxyl group on fused pyran ring system is very important for antibacterial activity of flavonoids (Cushnie & Lamb 2005), whereas the hydroxyl groups at C-7 and/or -5 are considered to be crucial (Mukne et al. 2011). Furthermore, the presence of C-4'-hydroxylation is supposed to increase the effectiveness of isoflavonoid structure (Chacha et al. 2005). In the case of genistein, our results are corresponding well with these findings. On the other hand, the most active structure, demethyltexasin, has hydroxyl groups at positions C-6, -7 and -4', suggesting that hydroxyl on C-6 plays an important role in the antibacterial effect of isoflavones. In contrast, the tested compounds containing at least one methoxy group at ring A or methoxy group at position C-4' and at the same time only one hydroxyl group at the ring A, failed to inhibit bacterial growth at MIC values of 128 μg ml^{-1}. This decreasing effect of antibacterial activity due to the presence of methoxy groups in structure of isoflavones has been previously proposed (Cushnie & Lamb 2005).

Since the inhibition of nucleic acid synthesis, a mechanism of antibacterial effect previously suggested for genistein (Verdrengh et al. 2004), is not typical action for which *S. aureus* acquire resistance (Pantosti et al. 2007), it is possible to hypothesize that emergence of resistance to another isoflavones, including demethyltexasin, can be formed in a limited manner only.

5.4 Antistaphylococcal effect of demethyltexasin

Since the initial screening of antibacterial activity of selected isoflavones showed the significant growth-inhibitory effect of demethyltexasin against *S. aureus*, this compound was subsequently tested against nine clinical isolates and four ATCC strains of sensitive- and resistant-staphylococci. All strains of *S. aureus* were inhibited by demethyltexasin within range of MICs 16 - 128 μg ml^{-1} (Table 3).

Table 3. *In vitro* antistaphylococcal effect of demethyltexasin

Bacterial strains tested	Compound / MIC [μg ml^{-1}]				
	DT	**ERY**	**OXA**	**TET**	**VAN**
MSSA (ATCC 25923)	128	0.25	0.25	0.25	2
MSSA (MS 12001)	16	0.25	0.25	0.25	1
MRSA (CCM 7112)	16	>512	512	32	1
MRSA (CCM 7115)	64	>512	512	64	2
MRSA (MR 12001)	32	>512	128	0.25	1
MRSA (MR 12004)	16	>512	32	0.5	2
MRSA (ATCC 43300)	128	>512	16	0.13	2
MRSA (ATCC BAA-976)	128	>512	8	0.25	2
MRSA (ATCC 33591)	128	>512	256	64	2
EMRSA (EMR 15)	32	>512	16	0.25	1

MIC minimum inhibitory concentration (value is the mean minimum inhibitory concentrations of three independent experiments each performed in triplicate), **DT** demethyltexasin, **ERY** erythromycin, **OXA** oxacillin, **TET** tetracycline, **VAN** vancomycin, **ATCC** American type culture collection, **CCM** Czech Collection of Microorganisms, **MSSA** methicillin sensitive *S. aureus*, **MRSA** methicillin resistant *S. aureus*, **EMRSA** epidemic methicillin-resistant *S. aureus*

Among all strains of *S. aureus* tested, MRSA (CCM 7112), MRSA (MR 12004), TRSA (TR 12001) and MSSA (MS 12001) were the most sensitive (MIC 16 μg ml^{-1}) ones. All MSSA strains were suppressed by all antibiotics tested at lower concentrations than demethyltexasin. Moreover, demethyltexasin showed significant antibacterial activity against TR (12001 and 12002) and multidrug-resistant (MDR) MRSA clinical isolates (CCM 7112 and CCM 7115) with MIC values ranging from 16 to 64 μg ml^{-1}. As it can be seen from the results, this study demonstrates that demethyltexasin is effective against various *S. aureus* strains that are resistant to other antimicrobials such as erythromycin, oxacillin tetracycline and vancomycin.

According to our best knowledge, this is the first report on the antistaphylococcal activity of isoflavone's metabolite demethyltexasin, despite a number of studies on the antibacterial activity of plant isoflavonoids against MRSA (Xu and Lee 2001; Tanaka et al. 2002; Sato et al. 2004; Verdrengh et al. 2004; Sato et al. 2006; Mukne et al. 2011).

As far as the possible use of compound demethyltexasin as antimicrobial agent is considered, its technological and toxicological properties should be discussed in detail. Since the toxicity of flavonoids is very low, for example the LD_{50} for rats is varying from 2 to 10 grams per animal, to reach similar doses in humans is quite unrealistic (Havsteen 1983). In addition, foods containing isoflavones, including dietary supplements, are recommended in the treatment of hormone-dependent diseases (Villares et al. 2011). Since demethyltexasin, a compound regularly consumed in fermented soy-based foods such as doenjgang and tempeh (Roh et al. 2011), is excreted in urine after metabolization in the liver (Kulling et al. 2001), it is supposed to be nontoxic to humans. Moreover, Lee at al. (2011b) described significant inhibition of human colon cancer cell proliferation of demethyltexasin, whereas no cytotoxicity to human health cell lines has been observed. Until now, there is no study focused on presence of demethyltexasin in serum, but there are several studies proving presence of daidzein and genistein in blood plasma at C_{max} 1.1 μg ml^{-1} after intake of soy milk (Timan et al. 2014), and occurrence of formononetin in rat serum at C_{max} 5.4 μg ml^{-1} after oral intake of *Dalbergia* extract (Liu et al. 2005), which is in correspondence with C_{max} levels of commonly use antistaphylococcal antibiotics.

The hydrophobicity of isoflavones generally provides longer retention times in the human body (Villares et al. 2011); therefore long-time efficacy can also be expected for demethyltexasin. According to their data sheets, the compounds are generally soluble in polar solvents commonly used in antimicrobial pharmacotherapy (e.g. DMSO). The problem of their hydrophobicity could be overcome by using different techniques, eg. Hanski et al.

(2012) described sublingual film formulation, which showed highly improved dissolution rate and solubility compared to the powder. The possible path way for the absorption of this compound into the blood stream from the gastrointestinal tract is through enterohepatic recycling (Rowland et al. 2003).

Our findings suggest potential contribution to the suppression of staphylococcal infections by consumption of demethyltexasin in an adequate quantity e.g. in the fermented soy-based food, but other pharmacokinetic/pharmacodynamic (PK/PD) modeling will be necessary to support this hypothesis.

5.5 Antistaphylococcal synergistic effect of demethyltexasin with amoxicillin and oxacillin

In this study, demethyltexasin, a human body metabolite of soybean isoflavones, was evaluated for its possible antistaphylococcal combinatory effect with amoxicillin and oxacillin. Demethyltexasin demonstrated significant potentiating antistaphylococcal activity of β-lactam antibiotics especially against MRSA. The results showed many synergistic interactions together with no antagonism occurrence, the data are summarized in Table 4 and 6.

For comparison, common therapeutically used combination of amoxicillin/clavulanic acid was tested (Table 5). These results showed many synergistic interactions between demethyltexasin and amoxicillin against all three tested MRSA (Σ FIC 0.257-0.461), however against sensitive strain there was no interaction. The highest amoxicillin MIC decrease (43-fold reduction) was obtained for MRSA strain ATCC 43300 in demethyltexasin concentration 32 μg ml^{-1} (Σ FIC 0.398). On the other hand, the combination of amoxicillin/clavulanic acid possessed synergy against sensitive *S. aureus* (Σ FIC 0.319-0.396), but there was only one synergistic effect against MRSA (SA6) causing only 2-fold amoxicillin MIC reduction (Σ FIC 0.495).

Demethyltexasin showed strong synergistic interactions against most of *S. aureus* strains when combined with amoxicillin (sum of fractional inhibitory concentrations [∑ FIC] 0.257–0.461), as shown in Table 4, and oxacillin (∑ FIC 0.109–0.484), as shown in Table 6. Moreover, all of these ten strains were MRSA (oxacillin MICs values ranging from 16 to 341 μg ml^{-1}) and the resistance to oxacillin was overcome in most of them when demethyltexasin /oxacillin combination was used. The strongest synergy (Σ FIC 0.109) was obtained against clinical isolate SA3 at demethyltexasin concentration 8 μg ml^{-1}, when 64-fold oxacillin MIC

decrease has been achieved (from 64 to 1 μg ml^{-1}). The highest oxacillin MIC reduction was observed for MRSA 33591, where demethyltexasin concentration 32 μg ml^{-1} caused 170-fold oxacillin MIC reduction (from 341.333 to 2 μg ml^{-1}) with Σ FIC 0.381.

Table 4. *In vitro* inhibitory activity of demethyltexasin/amoxicillin combinations against *S. aureus* strains

S. aureus strain	alone MICs (μg ml^{-1})		MICs of AMX in combination with listed DT concentrations (μg ml^{-1})											
	AMX	DT	+DT 64 μg ml^{-1}		+DT 32 μg ml^{-1}		+DT 16 μg ml^{-1}		+DT 8 μg ml^{-1}		+DT 4 μg ml^{-1}		+DT 2 μg ml^{-1}	
			MIC	ΣFIC	MIC	ΣFIC	MIC	ΣFIC	MIC	ΣFIC	MIC	ΣFIC	MIC	ΣFIC
29213 [A]	2	64	0.042	1.021	0.34	0.670	1.055	0.778	1.333	0.792	1.333	0.729	1.444	0.753
33591 [B]	37.333	96	0.25	0.673	1.944	**0.385**	5.75	**0.321**	8.222	**0.304**	28.444	0.804	30.222	0.830
43300 [B]	21.333	85.333	0.25	0.762	0.5	**0.398**	1.917	**0.277**	3.472	***0.257***	11.111	0.568	19.556	0.940
SA6[B]	2.444	426.667	0.722	**0.445**	0.944	***0.461***	1.444	0.628	1.778	0.746	1.778	0.737	1.889	0.778

Table 5. *In vitro* inhibitory activity of clavulanic acid/amoxicillin combinations against *S. aureus* strains

S. aureus strain	alone MICs (μg ml^{-1})		MICs of AMX in combination with listed DT concentrations (μg ml^{-1})											
	AMX	CLA	+CLA 64 μg ml^{-1}		+CLA 32 μg ml^{-1}		+CLA 16 μg ml^{-1}		+CLA 8 μg ml^{-1}		+CLA 4 μg ml^{-1}		+CLA 2 μg ml^{-1}	
			MIC	ΣFIC	MIC	ΣFIC	MIC	ΣFIC	MIC	ΣFIC	MIC	ΣFIC	MIC	ΣFIC
29213 [A]	2.571	298.667	0.366	**0.357**	0.446	***0.281***	0.714	**0.331**	0.75	**0.319**	0.785	**0.319**	1	**0.396**
33591 [B]	42.667	853.333	21.333	0.575	21.333	0.537	26.667	0.644	26.667	0.634	32	0.755	32	0.752
43300 [B]	16	256	5.667	0.604	10.667	0.792	11.556	0.785	16	1.031	16	1.016	16	1.008
SA6[B]	2.444	1.024	1.056	***0.495***	1.444	0.622	1.444	0.606	1.444	0.599	1.444	0.595	1.444	0.593

Bold values: synergy (Σ FIC ≤ 0.5)

[A] sensitive strain (oxacillin MIC < 4 μg ml^{-1})

[B] methicillin-resistant *S. aureus* strain (oxacillin MIC ≥ 4 μg ml^{-1})

AMX amoxicillin, CLA clavulanic acid, DT demethyltexasin, MIC minimum inhibitory concentration (expressed as an average from three independent tests set in triplicate), Σ FIC sum of fractional inhibitory concentrations

Table 6. *In vitro* inhibitory activity of demethyltexasin/oxacillin combinations against *S. aureus* strains

S. aureus strain	alone MICs (μg ml^{-1})		MICs of OXA in combination with listed DT concentrations (μg ml^{-1})											
	OXA	DT	+DT 64 μg ml^{-1}		+DT 32 μg ml^{-1}		+DT 16 μg ml^{-1}		+DT 8 μg ml^{-1}		+DT 4 μg ml^{-1}		+DT 2 μg ml^{-1}	
			MIC	ΣFIC	MIC	ΣFIC	MIC	ΣFIC	MIC	ΣFIC	MIC	ΣFIC	MIC	ΣFIC
25923[A]	0.25	64	0.004	1.016	0.167	1.168	0.25	1.250	0.25	1.125	0.25	1.063	0,25	1,031
29213 [A]	0.5	64	0.005	1.010	0.086	0.672	0.26	0.770	0.42	0.965	0.5	1.063	0,5	1,031
33591 [B]	341.333	85.333	2	0.756	2	**0.381**	22.667	**0.254**	133.33	***0.484***	341.333	1.047	341,333	1,023
33592[B]	64	149.333	1.167	**0.447**	1.333	**0.235**	1.667	**0.133**	27.333	**0.481**	48.667	0.787	58,667	0,930
43300 [B]	42.667	64	0.291	1.007	0.291	0.507	0.583	**0.264**	1.833	**0.168**	12	**0.344**	42,667	1,031
BAA 976[B]	16	106.667	0.292	0.618	0.333	**0.321**	3.167	**0.348**	6	**0.450**	8	0.538	9,333	0,602
SA1[B]	32	128	0.317	0.510	0.667	**0.271**	3	**0.219**	14.667	0.521	21.333	0.698	26,667	0,849
SA2[B]	128	64	2	1.016	2.333	0.518	7	**0.305**	24	**0.313**	74.667	0.646	106,667	0,865
SA3[B]	64	85.333	0.333	0.755	0.417	**0.382**	0.417	**0.194**	1	***0.109***	18.667	**0.339**	53,333	0,857
SA4[B]	298.667	106.667	1.667	0.606	3	**0.310**	23.333	**0.228**	112	**0.450**	176	0.627	266,667	0,912
SA5[A]	3	48	0.125	1.375	0.625	0.875	0.583	0.528	1.167	0.556	1.667	0.639	1,667	0,597
SA6[B]	256	256	4	**0.266**	42.667	**0.292**	74.667	**0.354**	256	1.031	256	1.016	256	1,008
SA7[A]	1.167	85.333	0.008	0.757	0.583	0.875	1.167	1.188	1.167	1.094	1.167	1.047	1,167	1,023
SA8[B]	32	85.333	0.375	0.762	0.417	**0.388**	2.17	**0.255**	16.667	0.615	18.667	0.630	21,333	0,690

Bold values: synergy (ΣFIC ≤ 0.5)

A sensitive strain (oxacillin MIC < 4 μg ml^{-1})

B methicillin-resistant *S. aureus* strain (oxacillin MIC ≥ 4 μg ml^{-1})

DT demethyltexasin, OXA oxacillin, MIC minimum inhibitory concentration (expressed as an average from three independent tests set in triplicate), ΣFIC sum of fractional inhibitory concentrations

The potentiating effect of isoflavonoids to antibiotics has been reported several times in recent years. Most often tested compound is biochanin A that exerted synergy with ciprofloxacin against eleven MRSA clinical isolates (ΣFIC 0.13-0.5) and one standard *S. aureus* ATCC 25923 (ΣFIC 0.5) (Liu et al. 2011). Wang et al. (2014) described antistaphylococcal combinatory effect of genistein with norfloxacin and of diosmetin with ciprofloxacin, norfloxacin, and streptomycin. In our study, also demethyltexasin provided strong synergistic effect against all MRSA strains tested. Moreover, it is able to cause up to 170-fold reduction in oxacillin MIC. Our results can be compared with review of Cushnie and Lamb (2011), where they indicated most potent synergistic flavonoids/antibiotic antistaphylococcal combinations able to cause from 16 to 1024-fold reduction in antibiotic MIC. Therefore, also demethyltexasin causing up to 170-fold MIC reduction can be denoted as a compound with good potential for further pharmacological research focused on the development of new pharmaceuticals able to overcome microbial drug resistance.

Several modes of action have been proposed for the flavonoids antimicrobial, synergistic and antibiotic resistance-modulating activity, however the exact mechanisms of isoflavonoids group are still not clear. It was described, that there is a significant co-relationship between the antistaphylococcal effect and presence of several functional groups at particular positions in isoflavonoids structure. Its hydroxyl groups have obvious affinity to proteins and presence on C7 and/or C5 position is important (Mukne et al. 2011). Demethyltexasin has one of its hydroxyl groups at the C7 position; moreover, it is also present at the C4′position, which is supposed to increase antimicrobial effectiveness (Chacha et al. 2005). In the case of synergy with β-lactams, certain principles can be engaged: reduction of D-alanylation of teichoic acid in bacterial cell wall resulting in deactivation of modified penicillin-binding proteins 2a (PBP2a, responsible for resistance due to the low affinity to β-lactams), intercalation into the cytoplasmic membrane inducing structural changes and delocalization of PBP2a or disruption of its synthesis, inhibition of production of β-lactamases (enzymes able to destroy β-lactam ring in antibiotics), inactivation of efflux pump, destabilization of bacterial cytoplasmic membrane, and topoisomerase inhibition resulting in prevention of DNA replication. However, some of these processes do not fully explain synergistic effect so it is proposed that isoflavonoids can exert that activity via combination of different mechanisms of action (Cushnie & Lamb 2011).

It is well known that the effectiveness of antibiotic therapy can be affected by daily diet. The negative effect is usually of more interest because of the potential to harm patient,

due to the changes in pharmaceutical, pharmacokinetic, or pharmacodynamic properties of drug. The most important food-drug negative interactions are connected with the fruit juices, mostly with grapefruit juice, able to increase drug bioavailability in blood stream and thus possibility of worse adverse effects. Also co-administration of milk products can influence antibiotic activity, because of the prevention of its absorption which predisposes to treatment failure. On the other hand, it is clear, that food can also have positive effect on antibiotic therapy, however there is a lack of such evidence (Bushra et al. 2011; de Boer et al. 2015). As reviewed by Hemaiswarya and Doble (2006), several herbal extracts or fruits and vegetables phenols (e.g. quercetin or resveratrol) enhance the therapeutic effect of anticancer drugs and their consumption is beneficial. High content of fruits, vegetables, nuts and grains in daily diet brings to the organism large proportion of antioxidants, vitamins, flavonoids and phenols which may interact with drugs and thus increase their activity (Rodríguez-Fragoso et al. 2011). Since demethyltexasin is a compound regularly consumed in fermented soy-based foods (Roh et al. 2011), which is metabolized in the liver from daidzein (Kulling et al. 2001), and its ability to act synergistically with β-lactam antibiotics, we supposed that diet rich in daidzein containing food can positively affect antibiotic therapy. However further research is needed to confirm this suggestion.

In summary, demethyltexasin showed many synergistic interactions with amoxicillin and oxacillin antibiotics, together with no antagonism occurrence against various strains of *S. aureus*. Furthermore, when compared with amoxicillin/clavulanic acid combination, which is active mainly against MSSA, our results with amoxicillin/ demethyltexasin showed synergy mostly against MRSA. According to the best knowledge, our research is the first one reporting antimicrobial combinatory effect of isoflavone's human body metabolite with common antibiotics. The activity of both β-lactam antibiotics was significantly increased when combined with demethyltexasin and effective combinations can overcome bacterial resistance to antibiotics. Demethyltexasin seems to be promising substance for further research focused on the development of new synergistically acting antistaphylococcal drugs especially against antibiotic resistant strains. Moreover, as demethyltexasin is present in many soy bean products it seems that the consumption of such food could positively affect the medical effect of antibiotic therapy. However further research is needed prior to its possible use e.g. to explain exact mechanism of synergistic action or detailed toxicological studies.

6 CONCLUSIONS

In this study, 15 isoflavone structurally-related compounds were evaluated for their *in vitro* anti-inflammatory and antimicrobial activity. The results of anti-inflammatory assays showed that none of tested compounds was active at least half as much as indomethacin, a standard COX inhibitor. In contrast, majority of isoflavonoid structures exhibited 5-LOX inhibitory effect, whereas the most promising results were observed in biochanin A and formononetin, which inhibited the LTB_4 production by similar manner as 5-LOX inhibitor zileuton. Assessment of direct antimicrobial effect showed that the isoflavones' metabolite demethyltexasin possessed the strongest growth-inhibitory action, followed by biochanin A and genistein, and finally by two isoflavones' metabolites hydroxydaidzein and demethylretusin. Furthermore, demethyltexasin showed strong synergistic interactions against most of *S. aureus* strains when combined with amoxicillin and oxacillin.

Based on the obtained results, the study of the structure-activity relationship of tested compounds suggests methoxy group at the position C-4' and hydroxyl group at position C-5 and/or -7 as crucial for inhibitory effect of isoflavonoids against 5-LOX activity. The position of hydroxyl group on fused pyran ring system is generally very important for antibacterial activity of flavonoids, the hydroxyl groups at C-7 and/or -5 are considered to be crucial, and the presence of C-4'-hydroxylation is supposed to increase the effectiveness of isoflavonoid structure. Moreover, the most active structure in this study, demethyltexasin, has hydroxyl groups at positions C-6, -7 and -4', suggesting that hydroxyl on C-6 plays an important role in the antibacterial effect of isoflavones, too. In contrast, the tested compounds containing at least one methoxy group at ring A or methoxy group at position C-4' and at the same time only one hydroxyl group at the ring A, failed to inhibit bacterial growth.

These conclusions suggest demethyltexasin as a compound with good potential for further pharmacological research focused on the development of new synergistically acting pharmaceuticals able to overcome microbial drug resistance. In addition, biochanin A seems to be promising and effective agent with dual anti-inflammatory and antimicrobial effect. Nevertheless, this suggestion should further be verified by tests *in vivo*. In general, this work offers new insight into the knowledge of biological activities of compounds of leguminous plants.

7 REFERENCES

Abhary M, Al-Hazmi AA. 2016. Antibacterial activity of Miswak (*Salvadora persica* L.) extracts on oral hygiene. Journal of Taibah University for Science **10**: 513-520.

Abreu AC, Coqueiro A, Sultan AR, Lemmens N, Kim HK, Verpoorte R, van Wamel WJB, Simões M, Choi YH. 2017. Looking to nature for a new concept in antimicrobial treatments: isoflavonoids from *Cytisus striatus* as antibiotic adjuvants against MRSA. Scientific Reports **7**: Article number 3777.

Adams M, Kunert O, Haslinger E, Bauer R. 2004. Inhibition of Leukotriene Biosynthesis by Quinolone Alkaloids from the Fruits of *Evodia rutaecarpa*. Planta Medica **70**: 904-908.

Albulescu M, Popovici M. 2007. Isoflavones – biochemistry, pharmacology and therapeutic use. Revue Roumaine de Chimie **52**: 537-550.

Araya-Cloutier C, den Besten HMW, Aisyah S, Gruppen H, Vincken J-P. 2017. The position of prenylation of isoflavonoids and stilbenoids from legumes (Fabaceae) modulates the antimicrobial activity against Gram positive pathogens. Food Chemistry **226**: 193-201.

Babu D, Kushwaha K, Sehgal S, Juneja VK. 2017. Antimicrobials of Plant Origin. Pages 85-100 in Juneja V, Dwivedi H, Sofos J, editors. Microbial Control and Food Preservation. Food Microbiology and Food Safety. Springer, New York.

Baldo BA, Pham NH. 2016. Mechanisms of Hypersensitivity. Pages 37-90 in Baldo BA and Pham NH, editors. Drug Allergy: Clinical Aspects, Diagnosis, Mechanisms, Structure-Activity Relationships. Springer, New York.

Bao ZS, Hong L, Guan Y, Dong XW, Zheng HS, Tan GL, Xie QM. 2011. Inhibition of airway inflammation, hyperresponsiveness and remodeling by soy isoflavone in a murine model of allergic asthma. International Immunopharmacology **11**: 899-906.

Barnes PJ. 2003. Anti-leukotrienes: Here to stay? Current Opinion in Pharmacology **3**: 257-263.

Berger A. 1999. What are leukotrienes and how do they work in asthma? The BMJ **319**: 90.

Bradbury RB, White DE. 1954. Estrogens and Related Substances in Plants. Vitamins and Hormones **12**: 207-233.

Brown AC, Fraser TR. 1868. On the Connection between Chemical Constitution and Physiological Action; with special reference to the Physiological Action of the Salts of the Ammonium Bases derived from Strychnia, Brucia, Thebaia, Codeia, Morphia, and Nicotia. Journal of anatomy and physiology **2**: 224–242.

Bruneton J. 1999. Pharmacognosy, Phytochemistry, Medicinal Plants. Intercept, Andover.

Bushra R, Aslam N, Khan AY. 2011. Food-Drug Ineractions. Oman Medical Journal **26**: 77-83.

Busse WW. 1998. Leukotrienes and Inflammation. American Journal of Respiratory and Critical Care Medicine **157**: S210-S213.

Bussmann RW, Malca-García G, Glenn A, Sharon D, Chait G, Díaz D, Pourmand K, Jonat B, Somogy S, Guardado G, Aguirre C, Chan R, Meyer K, Kuhlman A, Townesmith A, Effio-Carbajal J, Frías-Fernandez F, Benito M. 2010. Minimum inhibitory concentrations of medicinal plants used in Northern Peru as antibacterial remedies. Journal of Ethnopharmacology **132**: 101-108.

Bustamante-Rangel M, Delgado-Zamarreño MM, Pérez-Martín L, Rodríguez-Gonzalo E, Domínguez-Álvarez J. 2018. Analysis of Isoflavones in Foods. Comprehensive Reviews in Food Science and Food Safety **17**: 391-411.

Cairns JA. 2007. The coxibs and traditional nonsteroidal anti-inflammatory drugs: A current perspective on cardiovascular risks. Canadian Journal of Cardiology **23**: 125-131.

Carter GW, Young PR, Albert DH, Bouska J, Dyer R, Bell RL, Summers JB, Brooks DW. 1991. 5-Lipoxygenase Inhibitory Activity of Zileuton. Journal of Pharmacology and Experimental Therapeutics **256**: 929-937.

Celotti F, Laufer S. 2001. Anti-inflammatory drugs: new multitarget compounds to face an old problem. The dual inhibition concept. Pharmacological Research **43**: 429-436.

Chacha M, Bojase-Moleta G, Majinda RRT. 2005. Antimicrobial and radical scavenging flavonoids from the stem wood of *Erythrina latissima*. Phytochemistry **66**: 99-104.

Charlier C, Michaux C. 2003. Dual inhibition of cyclooxygenase-2 (COX-2) and 5-lipoxygenase (5-LOX) as a new strategy to provide safer non-steroidal anti-inflammatory drugs. European Journal of Medicinal Chemistry **38**: 645-659.

Cheesman MJ, Ilanko A, Blonk B, Cock IE. 2017. Developing New Antimicrobial Therapies: Are Synergistic Combinations of Plant Extracts/Compounds with Conventional Antibiotics the Solution? Pharmacognosy Reviews **11**: 57-72.

Chen J, Lin H, Hu M. 2005. Absorption and metabolism of genistein and its five isoflavone analogs in the human intestinal Caco-2 model. Cancer Chemotherapy and Pharmacology **55**: 159-169.

Clinical and Laboratory Standards Institute (CLSI). 2009. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically 8th ed. Approved Standard M07-A8. CLSI, Wayne, PA.

Cos P, Vlietinck AJ, Berghe DV, Maes L. 2006. Antiinfective potential of natural products: how to develop a stronger *in vitro* 'proof-of-concept'. Journal of Ethnopharmacology **106**: 290–302.

Cushnie TPT, Lamb AJ. 2005. Antimicrobial activity of flavonoids. International Journal of Antimicrobial Agents **26**: 343-356.

Cushnie TPT, Lamb AJ. 2011. Recent advances in understanding the antibacterial properties of flavonoids. International Journal of Antimicrobial Agents **38**: 99-107.

Dabos KJ, Sfika E, Vlatta LJ, Giannikopoulos G. 2010. The effect of mastic gum on *Helicobacter pylori*: A randomized pilot study. Phytomedicine **17**: 296-299.

Dahlén S-E. 2006. Treatment of asthma with antileukotrienes: First line or last resort therapy? European Journal of Pharmacology **533**: 40-56.

Damman CJ. 2013. Salicylates and the microbiota: A new mechanistic understanding of an ancient drug's role in dermatological and gastrointestinal disease. Drug Development Research **74**: 344-352.

Da Silveira E Sá RDC, Andrade LN, De Oliveira RDRB, De Sousa DP. 2014. A Review on Anti-Inflammatory Activity of Phenylpropanoids Found in Essential Oils. Molecules **19**: 1459-1480.

Dastidar SG, Manna A, Kumar KA, Mazumdar K, Dutta NK, Chakrabarty AN, Motohashi N, Shirataki Y. 2004. Studies on the antibacterial potentiality of isoflavones. International Journal of Antimicrobial Agents **23**: 99-102.

De Boer A, van Hunsel F, Bast A. 2015. Adverse food-drug interactions. Regulatory Toxicology and Pharmacology **73**: 859-865.

Del Olmo A, Calzada J, Nuñez M. 2017. Benzoic acid and its derivatives as naturally occurring compounds in foods and as additives: Uses, exposure, and controversy. Critical Reviews in Food Science and Nutrition **57**: 3084-3103.

Droke EA, Hager KA, Lerner MR, Lightfoot SA, Stoecker BJ, Brackett DJ, Smith BJ. 2007. Soy isoflavones avert chronic inflammation-induced bone loss and vascular disease. Journal of Inflammation **4**: Article number 17.

Duan W, Kuo IC, Selvarajan S, Chua KY, Bay BH, Wong W.S.F. 2003. Antiinflammatory effects of genistein, a tyrosine kinase inhibitor, on a guinea pig model of asthma. American Journal of Respiratory and Critical Care Medicine **167**: 185-192.

D'Urzo AD, Chapman KR. 2000. Leukotriene-receptor antagonists: Role in asthma management. Canadian Family Physician **46**: 872-887.

Enshaieh S, Jooya A, Siadat AH, Iraji, F. 2007. The efficacy of 5% topical tea tree oil gel in mild to moderate acne vulgaris: a randomised, double blind placebo-controlled study. Indian Journal of Dermatology, Venereology and Leprology **73**: 22-26.

Fiorucci S, Meli R, Bucci M, Cirino G. 2001. Dual inhibitors of cyclooxygenase and 5-lipoxygenase. A new avenue in anti-inflammatory therapy? Biochemical Pharmacology **62**: 1433-1438.

FitzGerald GA, Patrono C. 2001. The Coxibs, Selective Inhibitors of Cyclooxygenase-2. New England Journal of Medicine **345**: 433-442.

Flythe M, Kagan I. 2010. Antimicrobial Effect of Red Clover (*Trifolium pratense*) Phenolic Extract on the Ruminal Hyper Ammonia-Producing Bacterium, *Clostridium sticklandii*. Current Microbiology **61**: 125-131.

Friedli G-L. 1997. Flavonoids. On-line Tutorials in Phytohemistry. Available from http://www.friedli.com/herbs/phytochem/flavonoids.html#isofla (accessed December 2018).

García-Lafuente A, Guillamón E, Villares A, Rostagno MA, Martínez JA. 2009. Flavonoids as anti-inflammatory agents: Implications in cancer and cardiovascular disease. Inflammation Research **58**: 537-552.

Gnanamanickam SS, Smith DA. 1980. Selective toxicity of isoflavonoid phytoalexins to Gram-positive bacteria. Phytopathology **70**: 894-896.

González-Périz A, Clària J. 2007. New Approaches to the Modulation of the Cyclooxygenase-2 and 5-Lipoxygenase Pathways. Current Topics in Medicinal Chemistry **7**: 297-309.

González-Ponce HA, Rincón-Sánchez AR, Jaramillo-Juárez F, Moshage H. 2018. Natural Dietary Pigments: Potential Mediators against Hepatic Damage Induced by Over-The-Counter Non-Steroidal Anti-Inflammatory and Analgesic Drugs. Nutrients **10**: Article number 117.

Goodman RM. 2004. Encyclopedia of Plant and Crop Science. CRC Press, Boca Raton, Florida.

Green GA. 2001. Understanding NSAIDs: From Aspirin to COX-2. Clinical Cornerstone **3**: 50-58.

Grzanna R, Lindmark L, Frondoza CG. 2005. Ginger - An herbal medicinal product with broad anti-inflammatory actions. Journal of Medicinal Food **8**: 125-132.

Halawany HS. 2012. A review on miswak (*Salvadora persica*) and its effect on various aspects of oral health. The Saudi Dental Journal **24**: 63-69.

Hamalainen M, Nieminen R, Vuorela P, Heinonen M, Moilanen E. 2007. Anti-inflammatory effects of flavonoids: genistein, kaempferol, quercetin, and daidzein inhibit STAT-1 and NF-κB activations, whereas flavone, isorhamnetin, naringenin, and pelargonidin inhibit

only NF-κ B activation along with their inhibitory effect on iNOS expression and NO production in activated macrophages. Mediators of Inflammation (45673) DOI: 10.1155/2007/45673.

Hanski L, Genina N, Skogman M, Parkkisenniemi-Kinnunen U-M, Mäkilä E, Salonen J, Fallarero A, Sandler N, Vuorela PM. 2012. Anti-*C. pneumonia* activity and sublingual formulation of biochanin A. II International Conference on Antimicrobial Research, Lisbon, Portugal: Book of Abstracts: 151.

Harborne JB. 1994. The Flavonoids: Advances in Research Since 1986. CRC Press, Boca Raton, Florida.

Harborne JB, Baxter H, Moss GP. 1999. Phytochemical Dictionary: A Handbook of Bioactive Compounds from plants SE. CRC Press, Boca Raton, Florida.

Hartog E, Menashe O, Kler E, Yaron S. 2010. Salicylate reduces the antimicrobial activity of ciprofloxacin against extracellular *Salmonella enterica* serovar Typhimurium, but not against *Salmonella* in macrophages. Journal of Antimicrobial Chemotherapy **65**: 888-896.

Havsteen B. 1983. Flavonoids, a class of natural products of high pharmacological potency. Biochemical Pharmacology **32**: 1141-1148.

Hawkey CJ. 2005. COX-2 chronology. Gut **54**: 1509-1514.

Head KA. 1997. Isoflavones And Other Soy Constituents In Human Health And Disease. Alternative Medicine Review **2**: 433-450.

Hemaiswarya S, Doble M. 2006. Potential synergism of natural products in the treatment of cancer. Phytotherapy Research **20**: 239-249.

Hemaiswarya S, Kruthiventi AK, Doble M. 2008. Synergism between natural products and antibiotics against infectious diseases. Phytomedicine **15**: 639-652.

Hernández H, Fraňková A, Sýkora T, Klouček P, Kouřimská L, Kučerová I, Banout J. 2017. The effect of oregano essential oil on microbial load and sensory attributes of dried meat. Journal of the Science of Food and Agriculture **97**: 82-87.

Hertrampf T, Schmidt S, Laudenbach-Leschowsky U, Seibel J, Diel P. 2005. Tissue-specific modulation of cyclooxygenase-2 (Cox-2) expression in the uterus and the v. cava by estrogens and phytoestrogens. Molecular and Cellular Endocrinology **243**: 51-57.

Hong H, Landauer MR, Foriska MA, Ledney GD. 2006. Antibacterial activity of the soy isoflavone genistein. Journal of Basic Microbiology **46**: 329-335.

Hooshmand S, Soung do Y, Lucas EA, Madihally SV, Levenson CW, Arjmandi BH. 2007. Genistein reduces the production of proinflammatory molecules in human chondrocytes. Journal of Nutritional Biochemistry **18**: 609-614.

Huang YF, Cao SM, Nagamani M, Anderson KE, Grady JJ, Lu LJW. 2005. Decreased circulating levels of tumor necrosis factor-α in postmenopausal women during consumption of soy-containing isoflavones. Journal of Clinical Endocrinology & Metabolism **90**: 3956-3962.

Iinuma M, Tsuchiya H, Sato M, Yokoyama J, Ohyama M, Ohkawa Y, Tanaka T, Fujiwara S, Fujii T. 1994. Flavanones with potent antibacterial activity against methicillin-resistant *Staphylococcus aureus*. Journal of Pharmacy and Pharmacology **46**: 892 –895.

Idelevich EA, Kreis C, Löffler B, Peters G. 2016. *Staphylococcus aureus*-Associated Musculoskeletal Infections. Pages 229-262id in Bagnoli F, Rappuoli R, Grandi G, editors. *Staphylococcus aureus*. Current Topics in Microbiology and Immunology, vol 409. Springer, Cham.

Islam K, Rowsni AA, Khan MM, Kabir MS. 2014. Antimicrobial activity of ginger (*Zingiber officinale*) extracts against food-borne pathogenic bacteria. International Journal of Science, Environment and Technology **3**: 867-871.

Jachak SM. 2006. Cyclooxygenase Inhibitory Natural Products: Current Status. Current Medicinal Chemistry **13**: 659-678.

Johnson G, Maag DD, Johnson DK, Thomas RD. 1976. The possible role of phytoalexins in the resistance of sugarbeet (*Beta vulgaris*) to *Cercospora beticola*. Physiological Plant Pathology **8**: 225-230.

Jun M, Hong JG, Jeong WS, Ho CT. 2005. Suppression of arachidonic acid metabolism and nitric oxide formation by kudzu isoflavones in murine macrophages. Molecular Nutrition & Food Research **49**: 1154-1159.

Kalalinia F, Behravan J, Ramezani M, Hassanzadeh MK, Asadipour A. 2008. Chemical Composition, Moderate *In Vitro* Antibacterial and Antifungal Activity of the Essential Oil of *Pistacia vera* L. and it's Major Constituents. Journal of Essential Oil-Bearing Plants **11**: 376-383.

Kalhan R, Smith LJ, Nlend MC, Nair A, Hixon JL, Sporn PHS. 2008. A mechanism of benefit of soy genistein in asthma: inhibition of eosinophil p38-dependent leukotriene synthesis. Clinical and Experimental Allergy **38**: 103-112.

Keung WM. 1993. Biochemical studies of a new class of alcohol dehydrogenase inhibitors from *Radix puerariae*. Alcoholism: Clinical and Experimental Research **17**: 1254-1260.

Khan AV, Ahmed QU, Shukla I, Khan AA. 2012a. Antibacterial activity of leaves extracts of *Trifolium alexandrinum* Linn. against pathogenic bacteria causing tropical diseases. Asian Pacific Journal of Tropical Biomedicine **2**: 189-194.

Khan AQ, Khan R, Rehman MU, Lateef A, Tahir M, Ali F, Sultana S. 2012b. Soy isoflavones (daidzein & genistein) inhibit 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced cutaneous inflammation via modulation of COX-2 and NF-κB in Swiss albino mice. Toxicology **302**: 266–274.

Kim JS, Jin CK, Sang HS, Eun JL, Jin W, Bae K, Kun HS, Hyun PK, Sam SK, Hyeun WC. 2006. Chemical Constituents of the Root of *Dystaenia takeshimana* and Their Anti-Inflammatory Activity. Archives of Pharmacal Research **29**: 617-623.

Kim JW, Jin YC, Kim YM, Rhie S, Kim HJ, Seo HG, Chang KC. 2009. Daidzein administration in vivo reduces myocardial injury in a rat ischemia/reperfusion model by inhibiting NF-kB activation. Life Sciences **84**: 227–234.

Kim WS, Choi WJ, Lee S, Kim WJ, Lee DC, Sohn UD, Shin HS, Kim W. 2015. Anti-inflammatory, Antioxidant and Antimicrobial Effects of Artemisinin Extracts from *Artemisia annua* L. The Korean Journal of Physiology & Pharmacology **19**: 21-27.

Klejdus B, Vitamvásová-Štěrbová D, Kubáň V. 2001. Identification of isoflavone conjugates in red clover (*Trifolium pratense*) by liquid chromatography–mass spectrometry after two-dimensional solid-phase extraction. Analytica Chimica Acta **450**: 81-97.

Klejdus B. 2004. Separace a identifikace isoflavonů v rostlinném materiálu [habilitation thesis]. Univerzita Palackého v Olomouci, Olomouc.

Kloucek P, Svobodova B, Polesny Z, Langrova I, Smrcek S, Kokoska L. 2007. Antimicrobial activity of some medicinal barks used in Peruvian Amazon. Journal of Ethnopharmacology **111**: 427-429.

Kougan GB, Tabopda T, Kuete V, Verpoorte R. 2013. Simple Phenols, Phenolic Acids, and Related Esters from the Medicinal Plants of Africa. Pages 225-249 in Kuete V, editor. Medicinal Plant Research in Africa: Pharmacology and Chemistry. Elsevier Inc., New York.

Koutsoudaki C, Krsek M, Rodger A. 2005. Chemical Composition and Antibacterial Activity of the Essential Oil and the Gum of *Pistacia lentiscus* Var. *chia*. Journal of Agricultural and Food Chemistry **53**: 7681-7685.

Kuete V, Ngameni B, Simo CCF, Tankeu RK, Ngadjui BT, Meyer JJM, Lall N, Kuiate JR. 2008. Antimicrobial activity of the crude extracts and compounds from *Ficus chlamydocarpa* and *Ficus cordata* (Moraceae). Journal of Ethnopharmacology **120**: 17-24.

Kuete V. 2013. Phenylpropanoids and Related Compounds from the Medicinal Plants of Africa. Pages 251-260 in Kuete V, editor. Medicinal Plant Research in Africa: Pharmacology and Chemistry. Elsevier Inc., New York.

Kuhl P, Shiloh R, Jha H, Murawski U, Zilliken F. 1984. 6,7,4'-Trihydroxyisoflavan: a potent and selective inhibitor of 5-lipoxygenase in human and porcine peripheral blood leukocytes. Prostaglandins **28**: 783-804.

Kulling SE, Honig DM, Metzler M. 2001. Oxidative metabolism of the soy isoflavones daidzein and genistein in humans *in vitro* and *in vivo*. Journal of Agricultural and Food Chemistry **49**: 3024-3033.

Lamprecht C, Werner U, Werner D, Schaefer S, Renner B, Fromm M, Brune K. 2005. Effect of Selective COX-2 Inhibitors on Heart Rate in Healthy Young Men. The American Journal of Cardiology **95**: 1531-1532.

Lapčík O, Hill M, Hampl R, Wähälä K, Adlercreutz H. 1998. Identification of isoflavonoids in beer. Steroids **63**: 14-20.

Lechner D, Gibbons S, Bucar F. 2008. Plant phenolic compounds as ethidium bromide efflux inhibitors in *Mycobacterium smegmatis*. Journal of Antimicrobial Chemotherapy **62**: 345-348.

Lee DE, Lee KW, Byun S, Jung SK, Song N, Lim SH, Heo Y-S, Kim JE, Kang NJ, Kim BY, Bowden GT, Bode AM, Lee HJ, Dong Z. 2011a. 7,3',4'-trihydroxyisoflavone, a metabolite of the soy isoflavone daidzein, suppresses ultraviolet B-induced skin cancer by targeting Cot and MKK4. Journal of Biological Chemistry **286**: 14246-14256.

Lee DE, Lee KW, Jung SK, Lee EJ, Hwang JA, Lim T-G, Kim BY, Bode A, Lee HJ, Dong ZG. 2011b. 6,7,4'-trihydroxyisoflavone inhibits HCT-116 human colon cancer cell proliferation by targeting CDK1 and CDK2. Carcinogenesis **32**: 629-635.

Li Z, Dong X, Zhang J, Zeng G, Zhao H, Liu Y, Qiu R, Mo L, Ye Y. 2014. Formononetin protects TBI rats against neurological lesions and the underlying mechanism. Journal of the Neurological Sciences **15**: 112-117.

Liu RX, Sun JH, Bi KS, Guo DA. 2005. Identification and determination of major flavonoids in rat serum by HPLC-UV and HPLC-MS methods following oral administration of *Dalbergia odorifera* extract. Journal of Chromatography B Analytical Technologies in the Biomedical and Life Sciences **829**: 35-44.

Liu G, Liang JC, Wang XL, Li ZH, Wang W, Guo N, Wu AP, Shen FG, Xing MX, Liu LH, Li L, Liu MY, Yu L. 2011. *In vitro* synergy of biochanin A and ciprofloxacin against clinical isolates of *Staphylococcus aureus*. Molecules **16**: 6656-6666.

Lulekal E, Rondevaldova J, Bernaskova E, Cepkova J, Asfaw Z, Kelbessa E, Kokoska L, Van Damme P. 2014. Antimicrobial activity of traditional medicinal plants from Ankober District, North Shewa Zone, Amhara Region, Ethiopia. Pharmaceutical Biology **52**: 614-620.

Malm H, Borisch C. 2015. Analgesics, non-steroidal anti-inflammatory drugs (NSAIDs), muscle relaxants, and antigout medications. Pages 27-58 in Schaefer C, Peters P, Miller RK, editors. Drugs During Pregnancy and Lactation: Treatment Options and Risk Assessment: Third Edition. Elsevier Inc., New York.

Mandal MD, Mandal S. 2011. Honey: its medicinal property and antibacterial activity. Asian Pacific Journal of Tropical Biomedicine **1**: 154-160.

Marone P, Bono L, Leone E, Bona S, Carretto E, Perversi L. 2001. Bactericidal Activity of *Pistacia lentiscus* Mastic Gum Against *Helicobacter pylori*. Journal of Chemotherapy **13**: 611-614.

Maroon JC, Bost JW, Maroon A. 2010. Natural anti-inflammatory agents for pain relief. Surgical Neurology International **1**: 80

Mascayano C, Espinosa V, Sepúlveda-Boza S, Hoobler EK, Perry S, Diaz G, Holman TR. 2015. Enzymatic Studies of Isoflavonoids as Selective and Potent Inhibitors of Human Leukocyte 5-Lipo-Oxygenase. Chemical Biology and Drug Design **86**: 894-901.

Mbaveng AT, Sandjo LP, Tankeo SB, Ndifor AR, Pantaleon A, Nagdjui BT, Kuete V. 2015. Antibacterial activity of nineteen selected natural products against multi-drug resistant Gram-negative phenotypes. Springer Plus **4**: Article number 823.

Mickymaray S, Al Aboody MS, Rath PK, Annamalai P, Nooruddin T. 2016. Screening and antibacterial efficacy of selected Indian medicinal plants. Asian Pacific Journal of Tropical Biomedicine **6**:185-19.

Mitchell JA, Akarasereenont P, Thiemermann C, Flower RJ, Vane JR. 1993. Selectivity of nonsteroidal antiinflammatory drugs as inhibitors of constitutive and inducible cyclooxygenase. Proceedings of the National Academy of Sciences of the United States of America **90**, 11693-11697.

Molan P. 2001. Why honey is effective as a medicine: 2. The scientific explanation of its effects. Bee World **82**: 22-40.

Morel C, Stermitz FR, Tegos G, Lewis K. 2003. Isoflavones as Potentiators of Antibacterial Activity. Journal of Agricultural and Food Chemistry **51**: 5677-5679.

Mostafa AA, Al-Askar AA, Almaary KS, Dawoud TM, Sholkamy EN, Bakri MM. 2018. Antimicrobial activity of some plant extracts against bacterial strains causing food poisoning diseases. Saudi Journal of Biological Sciences **25**: 253-258.

Mukne AP, Viswanathan V, Phadatare AG. 2011. Structure pre-requisites for isoflavones as effective antibacterial agents. Pharmacognosy Reviews **5**: 13-18.

Nedorostova L, Kloucek P, Urbanova K, Kokoska L, Smid J, Urban J, Valterova I, Stolcova M. 2011. Antibacterial effect of essential oil vapours against different strains of *Staphylococcus aureus*, including MRSA. Flavour and Fragrance Journal **26**: 403-407.

Nelson KE. 2006. Epidemiology of Infectious Disease: General Principles. Pages 17-49 in Nelson KE, Williams CM, Graham NMH, editors. Infectious Disease Epidemiology: Theory and Practice. Jones and Bartlett Publishers Inc, Maryland.

Odds FC. 2003. Synergy, antagonism, and what the chequerboard puts between them. Journal of Antimicrobial Chemotherapy **52**: 1.

Ohishi T, Goto S, Monira P, Isemura M, Nakamura Y. 2016. Anti-inflammatory Action of Green Tea. Anti-Inflammatory and Anti-Allergy Agents in Medicinal Chemistry **15**: 74-90.

Osawa K, Yasuda H, Maruyama T, Morita H, Takeya K, Itokawa H. 1992. Isoflavanones from the Heartwood of *Swartzia polyphylla* and Their Antibacterial Activity against Cariogenic Bacteria. Chemical and Pharmaceutical Bulletin **40**: 2970-2974.

Pandey KB, Rizvi SI. 2009. Plant polyphenols as dietary antioxidants in human health and disease. Oxidative Medicine and Cellular Longevity **2**, 270-278.

Pantosti A, Sanchini A, Monaco M. 2007. Mechanisms of antibiotic resistance in *Staphylococcus aureus*. Future Microbiology **2**: 323-334.

Papageorgiou VP, Mellidis AS, Argyriadou N. 1991. The Chemical Composition of the Essential Oil of Mastic Gum. Journal of Essential Oil Research 3: 107-110.

Paradkar PN, Blum PS, Berhow MA, Baumann H, Kuo S.-M. 2004. Dietary isoflavones suppress endotoxin-induced inflammatory reaction in liver and intestine. Cancer Letters **215**: 21-28.

Patrignani P, Patrono C. 2015. Cyclooxygenase inhibitors: From pharmacology to clinical read-outs. Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids **1851**: 422-432.

Pergola C, Werz O. 2010. 5-Lipoxygenase inhibitors: a review of recent developments and patents. Expert Opinion on Therapeutic Patents **20**: 355-375.

Perrone MG, Scilimati A, Simone L, Vitale P. 2010. Selective COX-1 Inhibition: A Therapeutic Target to be Reconsidered. Current Medicinal Chemistry **17**: 3769-3805.

Pohjala L, Uvell H, Hakala E, Gylfe A, Elofsson M, Vuorela P. 2012. The isoflavone biochanin A inhibits the growth of the intracellular bacteria *Chlamydia trachomatis* and *Chlamydia pneumonia*. Planta Medica **78**: 1102-1102.

Putz MV, Duda-Seiman C, Duda-Seiman D, Putz AM, Alexandrescu I, Mernea M, Avram S. 2016. Chemical Structure-Biological Activity Models for Pharmacophores' 3D-Interactions. International Journal of Molecular Sciences **17**: Article number 1087.

Rauf A, Patel S, Uddin G, Siddiqui BS, Ahmad B, Muhammad N, Mabkhot YN, Hadda TB. 2017. Phytochemical, ethnomedicinal uses and pharmacological profile of genus *Pistacia*. Biomedicine & Pharmacotherapy **86**: 393-404.

Reininger EA, Bauer R. 2006. Prostaglandin-H-synthase (PGHS)-1 and -2 microtiter assays for the testing of herbal drugs and *in vitro* inhibition of PGHS-isoenzyms by polyunsaturated fatty acids from *Platycodi radix*. Phytomedicine **13**: 164–169.

Ren K, Dubner R. 2010. Interactions between the immune and nervous systems in pain. Nature Medicine **16**: 1267-1276.

Rodríguez-Fragoso L, Martínez-Arismendi JL, Orozco-Bustos D, Reyes-Esparza J, Torres E, Burchiel SW. 2011. Potential Risks Resulting from Fruit/Vegetable–Drug Interactions: Effects on Drug-Metabolizing Enzymes and Drug Transporters. Journal of Food Science **76**: 112-124.

Roh C, Jung U, Jo SK. 2011. 6,7,4 '-trihydroxyisoflavone from Doenjang inhibits lipid accumulation. Food Chemistry **129**: 183-187.

Rosa FT, Zulet MA, Marchini JS, Martinez JA. 2012. Bioactive compounds with effects on inflammation markers in humans. International Journal of Food Sciences and Nutrition **63**: 749-765.

Rosas-Piñón Y, Mejía A, Díaz-Ruiz G, Aguilar MI, Sánchez-Nieto S, Rivero-Cruz JF. 2012. Ethnobotanical survey and antibacterial activity of plants used in the Altiplane region of Mexico for the treatment of oral cavity infections. Journal of Ethnopharmacology **141**: 860-865.

Rowland I, Faughnan M, Hoey L, Wahala K, Williamson G, Cassidy A. 2003. Bioavailability of phyto-oestrogens. British Journal of Nutrition **89**: S45-S58.

Sato M, Tanaka H, Fujiwara S, Hirata M, Yamaguchi R, Etoh H, Tokuda C. 2003. Antibacterial property of isoflavonoids isolated from *Erythrina variegata* against cariogenic oral bacteria. Phytomedicine **10**: 427-433.

Sato M, Tanaka H, Yamaguchi R, Kato K, Etoh H. 2004. Synergistic effects of mupirocin and an isoflavanone isolated from *Erythrina variegata* on growth and recovery of methicillin-resistant *Staphylococcus aureus*. International Journal of Antimicrobial Agents **24**: 241-246.

Sato M, Tanaka H, Tani N, Nagayama M, Yamaguchi R. 2006. Different antibacterial actions of isoflavones isolated from *Erythrina poeppigiana* against methicillin-resistant *Staphylococcus aureus*. Letters in Applied Microbiology **43**: 243-248.

Savoia D. 2012. Plant-Derived Antimicrobial Compounds: Alternatives to Antibiotics. Future Microbiology **7**: 979-990.

Selzer PM, Brutsche S, Wiesner P, Schmid P, Müllner H. 2000. Target-based drug discovery for the development of novel antiinfectives. International Journal of Medical Microbiology **290**: 191-201.

Signore A. 2013. About inflammation and infection. EJNMMI Research **3**: 1-2.

Signore A, Glaudemans AWJM. 2011. The molecular imaging approach to image infections and inflammation by nuclear medicine techniques. Annals of Nuclear Medicine **25**: 681-700.

Sharma A, Flores-Vallejo RDC, Cardoso-Taketa A, Villarreal ML. 2017. Antibacterial activities of medicinal plants used in Mexican traditional medicine. Journal of Ethnopharmacology **208**: 264-329.

Sklenickova O, Flesar J, Kokoska L, Vlkova E, Halamova K, Malik J. 2010. Selective Growth Inhibitory Effect of Biochanin A against Intestinal Tract Colonizing Bacteria. Molecules **15**: 1270-1279.

Sostres C, Gargallo CJ, Arroyo MT, Lanas A. 2010. Adverse effects of non-steroidal anti-inflammatory drugs (NSAIDs, aspirin and coxibs) on upper gastrointestinal tract. Best Practice & Research Clinical Gastroenterology **24**: 121–132.

Stanković N, Mihajilov-Krstev T, Zlatković B, Stankov-Jovanović V, Mitić V, Jović J, Čomić L, Kocić B, Bernstein N. 2016. Antibacterial and Antioxidant Activity of Traditional Medicinal Plants from the Balkan Peninsula. NJAS - Wageningen Journal of Life Sciences **78**: 21-28.

Subramani R, Narayanasamy M, Feussner K-D. 2017. Plant-derived antimicrobials to fight against multi-drug-resistant human pathogens. 3 Biotech **7**: Article number 172.

Swami S, Krishnan AV, Moreno J, Bhattacharyya RS, Gardner C, Brooks JD, Peehl DM, Feldman D. 2009. Inhibition of prostaglandin synthesis and actions by genistein in human prostate cancer cells and by soy isoflavones in prostate cancer patients. International Journal of Cancer **124**: 2050-2059.

Swietlik E, Doboszynska A. 2008. Treatment of Churg-Strauss syndrome with an inhaled corticosteroid after oral steroids discontinuation due to the side effects. Journal of Physiology and Pharmacology **59**: 689-695.

Šibul F, Orčić D, Vasić M, Anačkov G, Nadpal J, Savić A, Mimica-Dukić N. 2016. Phenolic profile, antioxidant and anti-inflammatory potential of herb and root extracts of seven selected legumes. Industrial Crops and Products **83:** 641-653.

Tahara S, Ibrahim R. 1995. Prenylated isoflavonoids - An update. Phytochemistry **38**: 1073-1094

Tanaka H, Sato M, Fujiwara S, Hirata M, Etoh H, Takeuchi H. 2002. Antibacterial activity of isoflavonoids isolated from *Erythrina variegata* against methicillin-resistant *Staphylococcus aureus*. Letters in Applied Microbiology **35**: 494-498.

Tassou CC, Nychas GJE. 1995. Antimicrobial activity of the essential oil of mastic gum (*Pistacia lentiscus* var. *chia*) on Gram positive and Gram negative bacteria in broth and in Model Food System. International Biodeterioration and Biodegradation **36**: 411-420.

Timan P, Rojanasthien N, Manorot M, Sangdee C, Teekachunhatean S. 2014. Efect of synbiotic fermented milk on oral bioavailability of isoflavones in postmenopausal women. International Journal of Food Sciences and Nutrition **65**: 761-767.

Tong SYC, Davis JS, Eichenberger E, Holland TL, Fowler VG Jr. 2015. *Staphylococcus aureus* Infections: Epidemiology, Pathophysiology, Clinical Manifestations, and Management. Clinical Microbiology Reviews **28**: 603-661.

Tzin V., Galili G, Aharoni A. 2012. Shikimate Pathway and Aromatic Amino Acid Biosynthesis. eLS John Wiley & Sons. DOI: 10.1002/9780470015902.a0001315.pub2.

Ulloa-Urizar G, Aguilar-Luis MA, De Lama-Odría MDC, Camarena-Lizarzaburu J, del Valle Mendoza J. 2015. Antibacterial activity of five Peruvian medicinal plants against *Pseudomonas aeruginosa*. Asian Pacific Journal of Tropical Biomedicine **5**: 928-931.

Veitch NC. 2007. Isoflavonoids of the Leguminosae. Natural Product Reports **24**: 417-464.

Veitch NC. 2009. Isoflavonoids of the Leguminosae. Natural Product Reports **26**: 776-802.

Veitch NC. 2013. Isoflavonoids of the Leguminosae. Natural Product Reports **30**: 988-1027.

Velíšek J, Cejpek K. 2008. Biosynthesis of food components. OSSIS, Tábor.

Verdrengh M, Collins LV, Bergin P, Tarkowski A. 2004. Phytoestrogen genistein as an anti-staphylococcal agent. Microbes and Infection **6**: 86-92.

Villares A, Rostagno MA, García-Lafuente A, Guillamón E, Martínez JA. 2011. Content and profile of isoflavones in soy-based foods as a function of the production process. Food and Bioprocess Technology **4**: 27-38.

Vlachojannis C, Winsauer H, Chrubasik S. 2013. Effectiveness and Safety of a Mouthwash Containing Essential Oil Ingredients. Phytotherapy Research **27**: 685-691.

Voß C, Sepulveda-Boza S, Zilliken FW. 1992. New isoflavonoids as inhibitors of porcine 5-lipoxygenase. Biochemical Pharmacology **44**: 157-162.

Wang J, Zhang Q, Jin S, He D, Zhao S, Liu S. 2008. Genistein modulate immune responses in collagen-induced rheumatoid arthritis model. Maturitas **59**: 405-412.

Wang Y, Zhu Y, Gao L, Yin H, Xie Z, Wang D, Zhu Z, Han X. 2012. Formononetin attenuates IL-1β-induced apoptosis and NF-κB activation in INS-1 cells. Molecules **17**: 10052-10064.

Wang SY, Sun ZL, Gibbons S, Zhang WJ, Qing M. 2014. Flavonoids from *Sophora moorcroftiana* and their synergistic antibacterial effects on MRSA. Phytotherapy Research **28**: 1071-1076.

Watelet J-B, Gillard M, Benedetti MS, Lelièvre B, Diquet B. 2009. Therapeutic management of allergic diseases. Drug Metabolism Reviews **41**: 301-343.

Watkins PB, Dube LM, Walton-Bowen K, Cameron CM, Kasten LE. 2007. Clinical pattern of zileuton-associated liver injury: Results of a 12-month study in patients with chronic asthma. Drug Safety **30**: 805-815.

Wang X. 2011. Structure, function, and engineering of enzymes in isoflavonoids biosynthesis. Functional and Integrative Genomics **11**: 13-22.

WHO. 2014. Antimicrobial resistance: global report on surveillance. World Health Organization, Geneva.

White RL, Burgess DS, Manduru M, Bosso JA. 1996. Comparison of three different in vitro methods of detecting synergy: Time-kill, Checkerboard, and E test. Antimicrobial Agents and Chemotherapy **40**: 1914–1918.

Wu X, Adedoyin OO, Mansour HM. 2011. Pulmonary and Nasal Anti-Inflammatory and Anti-Allergy Inhalation Aerosol Delivery Systems. Anti-Inflammatory and Anti-Allergy Agents in Medicinal Chemistry **10**: 215-229.

Wuttke W, Jarry H, Seidlova-Wuttke D. 2007. Isoflavones - Safe food additives or dangerous drugs? Ageing Research Reviews **6**: 150-188.

Wyman JG, VanEtten HD. 1978. Antibacterial Activity of Selected Isoflavonoids. Phytopathology **68**: 583-589.

Xu HX, Lee SF. 2001. Activity of plant flavonoids against antibiotic-resistant bacteria. Phytotherapy Research **15**: 39-43.

Yadav R, Angolkar T, Kaur G, Buttar HS. 2016. Antibacterial and Antiinflammatory Properties of Bovine Colostrum. Recent Patents on Inflammation & Allergy Drug Discovery **10**: 49-53.

Yao LH, Jiang YM, Shi J, Tomás-Barberán FA, Datta N, Singanusong R, Chen SS. 2004. Flavonoids in food and their health benefits. Plant Foods for Human Nutrition **59**: 113-122.

Yu J, Bi X, Yu B, Chen D. 2016. Isoflavones: Anti-Inflammatory Benefit and Possible Caveats. Nutrients **8**: Article number 361.

8 APPENDIX: LIST OF AUTHOR'S PUBLICATIONS

8.1 Publications in scientific journals

Rondevaldova J, **Hummelova J**, Tauchen J, Kokoska L. 2018. *In Vitro* Antistaphylococcal Synergistic Effect of Isoflavone Metabolite Demethyltexasin with Amoxicillin and Oxacillin. Microbial Drug Resistance **24**: 24-29 (IF 2.344).

Hummelova J, Rondevaldova J, Balastikova A, Lapcik O, Kokoska L. 2014. The relationship between structure and *in vitro* antibacterial activity of selected isoflavones and their metabolites with special focus on antistaphylococcal effect of demethyltexasin. Letters in Applied Microbiology **60**: 242-247 (IF 1.471).

Leuner O, Havlik J, Budesinsky M, Vrkoslav V, Chu J, Bradshaw TD, **Hummelova J**, Miksatkova P, Lapcik O, Valterova I, Kokoska L. 2013. Cytotoxic constituents of *Pachyrhizus tuberosus* from Peruvian Amazon. Natural Product Communications **8**: 1423-1426 (IF 0.809).

Leuner O, Havlik J, **Hummelova J**, Prokudina E, Novy P, Kokoska L. 2013. Distribution of isoflavones and coumestrol in neglected tropical and subtropical legumes. Journal of the Science of Food and Agriculture **93**: 575-579 (IF 2.379).

8.2 Conference contributions

Rondevaldova J, **Hummelova J**, Kokoska L. 2016. Demethyltexasin possesses *in vitro* anti-staphylococcal synergistic effect with ß-lactam antibiotics. *Trends in Natural Product research: a young scientist meeting of PSE and IUNG-PIB, Pulawy, Poland, May 30-June 2, 2016*, in Book of Abstracts, SL 5, p. 50.

Balastikova A, Rondevaldova J, **Hummelova J**, Kokoska L. 2014. *In vitro* synergistic anti-staphylococcal effect of demethyltexasin in combination with oxacillin. *42nd Annual General Meeting of the Australian Society for Microbiology, Melbourne, Australia, July 6-9, 2014*, in Delegate Handbook, Abstract no. 325.

Leuner O, Havlik J, **Hummelova J**, Hernandez Hernandez HJ, Kokoska L. 2014. Proyeccion de fitoestrogenes en legumbres no comunes, *9. Congreso Iberoamericano de Ingeniería de Alimentos, Valencia, Spain, January 13-16, 2014*, in Libro de Resúmenes, p. 420.

Hummelova J, Rondevaldova J, Kokoska L. 2012. Antimicrobial activity of selected isoflavones. *2nd International Conference on Antimicrobial Research, Lisbon, Portugal, November 21-23, 2012*, in Book of Abstracts, p. 171.

Hummelova J, Skrovanova V, Landa P, Kokoska L. 2011. Screening of Isoflavonoids as 5-lipoxygenase Inhibitors. *5th Scientific Conference of Institute of Tropics and Subtropics, Prague, Czech Republic, December 1, 2011*, in Book of abstracts, p. 29.

Hummelova J, Leuner O, Havlik J, Valterova I, Budesinsky M, Vrkoslav V, Lapcik O, Prokudina E, Kokoska L. 2011. Isolation and identification of rotenoids in Pachyrhizus tuberosus seeds, *59th International Congress and Annual meeting of the Society for Medicinal Plant and Natural Product Research, Antalya, Turkey, September 4-9, 2011*, abstract no. PG51. Planta Medica **77**: 1343 (IF 2.494).

Sklenickova O, Havlik J, **Hummelova J**, Kokoska L. 2010. Screening of isoflavonoids in tropical and subtropical neglected Fabaceae, *15th World Congress of Food Science and Technology, Cape Town, South Africa, August 22-26, 2010*, in Book of Abstracts, abstract no. P1104.