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**Department of Crop Sciences and Agroforestry**


# *In vitro* determination of anti-inflammatory activity of isoflavonoids

**M.Sc. Thesis**

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# DECLARATION

I, Veronika Škrovanová, declare that this thesis, submitted in partial fulfilment of the requirements for the degree of MSc, in the Institute of Tropics and Subtropics of the Czech University of life Sciences Prague, is wholly my own work unless otherwise referenced or acknowledged.

Prague, April 16, 2012 .....…….....

Veronika Škrovanová

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# ABSTRACT

Treatment of chronic inflammation is one of the most important issues in drug development. The inhibition of cyclooxygenase (COX) and lipoxygenase (LO) enzymes reduces the production of prostaglandins and leukotrienes, which are crucial mediators of inflammation. Based on the previous literary data, which described isoflavonoids as chemical compounds with many biological activities, but which have not been properly analyzed yet, we decided for screening-test of anti-inflammatory activity of 10 isoflavonoid compounds as inhibitors of COX-2 and 5-LO enzymes using enzymatic and cellular *in vitro* models, respectively. The results of COX-2 assay showed that none of the tested compounds was active at least half as much as indomethacin, a standard COX inhibitor. In contrast, all of isoflavonoid structures tested exhibited 5-LO inhibitory effect, whereas the most promising results were observed in biochanin A, which showed the lowest $IC_{50}$ value (29.03 µM), followed by 7,4'-dimethoxy-5-hydroxyisoflavone and 7-hydroxy-6-methoxyisoflavone ($IC_{50}$ values 30.29 µM and 32.10 µM, respectively). The results of this study suggest that the occurrence of one methoxyl group together with one or more hydroxyl groups in isoflavonoid structures may be responsible for their capacity to inhibit $LTA_4$ synthesis and therefore for suppressing inflammation. In summary, these compounds could be recommended for pharmaceutical or food industries as prospective structures for development of new herbal-based pharmaceutical preparations, nutraceuticals, food supplements, or functional foods with selective 5-LO-inhibitory effect.

Key words: Anti-inflammatory activity, Cyclooxygenase, Lipoxygenase, Isoflavonoids, Inhibition, *In vitro*

# ABSTRAKT

Zánětlivá onemocnění jsou jedním z nejzávažnějších problémů při vývoji léčiv. Inhibice enzymů cyklooxygenázy (COX) a lipoxygenázy (LO) snižuje produkci prostaglandinů a leukotrienů, které způsobují záněty. Na základě dřívějších studií, které popisují isoflavonoidy jako biologicky aktivní látky, které ale nejsou doposud dostatečně prozkoumány, jsme se rozhodli testovat 10 isoflavonoidů na možnou inhibici COX-2 v enzymatickém a 5-LO buněčném modelu *in vitro*. Při COX-2 testování bylo zjištěno, že žádná z testovaných látek není ani z poloviny tak účinná jako standardní inhibitor indomethacin. Na rozdíl od COX-2, všechny testované látky inhibovaly aktivitu 5-LO enzymu. Nejlepších výsledků dosáhl biochanin A s nejnižší hodnotou $IC_{50}$ (29.03 µM), následován 7,4'-dimethoxy-5-hydroxyisoflavonem a 7-hydroxy-6-methoxyisoflavonem ($IC_{50}$ 30.29 µM a 32.10 µM). Podle našich výsledků se předpokládá, že přítomnost jedné methoxylové skupiny a jedné nebo více hydroxylových skupin ve struktuře isoflavonoidu může být zodpovědná za inhibici $LTA_4$ syntézy a tím za protizánětlivé účinky. Výsledky testů naznačují možné využití těchto látek ve farmaceutickém anebo potravinářském průmyslu při vývoji přírodních farmaceutických přípravků, potravinových doplňků či funkčních potravin se selektivním 5-LO-inhibičním účinkem.

Klíčová slova: Protizánětlivá aktivita, Cyklooxygenáza, Lipoxygenáza, Isoflavonoidy, Inhibice, *In vitro*

# CONTENTS

# PREFACE

Musculoskeletal conditions are the most common cause of chronic disability around the world. In mammalian cells, inflammatory reaction causes severe diseases or pathological disturbances, such as cardiovascular diseases, rheumatoid arthritis, osteoporosis, bronchial asthma and even cancer. According to the statistics, these diseases belong also to the most frequent reasons of lower working capacity or disability pension in the Czech Republic. These disorders are commonly treated by conventionally manufactured drugs as e.g. glucocorticoids, disease-modifying antirheumatic drugs, non-steroidal anti-inflammatory drugs and others. However, use of these drugs is often associated to with acute side effect.

The anti-inflammatory capacity of crude plant extracts has been utilised since many years. Probably the best known anti-inflammatory compound of plant origin is salicin, isolated from white willow bark (*Salix alba* L.), whose synthetic derivative acetylsalicylic acid (Aspirin) is nowadays one of the most used non-steroidal anti-inflammatory drugs in the world. The valuable active compounds, such as estrogen-like compounds or even pure isoflavonoids, could be isolated from many broadly distributed plant species, prevalently belonging to subfamily Papilionoideae of family Fabaceae. The intake of isoflavonoids (plant secondary metabolites), which dually inhibit 5-LOX and COX enzymes, meliorates the symptoms of acute inflammatory as well as chronic inflammatory disorders. Isoflavonoids thus seem to be prospective targets for development of new safer non-steroidal anti-inflammatory preparations. This study intends to assess the inhibitory potential of isoflavonoids. Consequently, the results may contribute to the development of new and safe pharmaceuticals.

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| | |
|---|---|
| AA | Arachidonic acid |
| COX | Cyclooxygenase |
| DMARDs | Disease-modifying antirheumatic drugs |
| DMSO | Dimethylsulfoxide |
| EtOH | Ethanol |
| ETYA | Eicosatetraynoic acid |
| LO | Lipoxygenase |
| LTs | Leukotrienes |
| $Na_2EDTA$ | Disodium ethylenediamine tetraacetate |
| NSAIDs | Non-steroidal anti-inflammatory drugs |
| $PGG_2$ | Hydroperoxy-endoperoxide |
| $PGH_2$ | Hydroxy-endoperoxide |
| $PGI_2$ | Prostacyclin |
| PGHS | Prostaglandin $H_2$ synthase |
| PGs | Prostaglandins |
| TNF-α | tumor necrosis factor-α |
| $TXA_2$ | tromboxane $A_2$ |

# 1 INTRODUCTION

## 1.1 Inflammation

Inflammation is a biological process, induced by microbial infection or tissue injury (García-Lafuente *et al.*, 2009) and it is characterized by pain, redness, swelling, fever and loss of function. A main function of inflammation is to resolve infection and to repair the damage in order to achieve homeostasis within the immune system (Barton, 2008, Calixto *et al.*, 2003). Chronic inflammation leads to destruction of tissue and it is involved in the initiating and establishing of several pathological disturbances such as arteriosclerosis, cardiovascular diseases, pulmonary diseases, obesity, diabetes, Alzheimer's disease, neurodegenerative diseases and cancer (Aggarwal *et al.*, 2006; García-Lafuente *et al.*, 2009). Among a major enzymatic routes leading to inflammatory process in mammalian cells belong the cyclooxygenase (COX) and lipoxygenase (LO) pathways (González-Périz and Clària, 2007).

### 1.1.1 COX pathway

Prostaglandin $H_2$ synthase (PGHS), known as cyclooxygenase (COX) is the key enzyme required for the conversion of arachidonic acid (AA) to prostaglandins (PGs) and it has two distinct catalytic activities. Cyclooxygenase oxides AA to the hydroperoxy-endoperoxide $PGG_2$ and peroxidase subsequently reduces $PGG_2$ to the hydroxy-endoperoxide $PGH_2$. The unstable $PGH_2$ is than transformed by diverse tissue-specific synthases and isomerases into the prostanoids. It includes the prostaglandins ($PGE_2$, $PGF_2\alpha$, $PGD_2$), prostacyclin ($PGI_2$) and tromboxane $A_2$ ($TXA_2$) (Charlier and Michaux, 2003; DuBois *et al.*, 1998; Vane *et al.*, 1998). Prostanoids exert a broad spectrum of biological activities in both physiological and pathological conditions. PGs not only play a major role in inflammation, but also regulate diverse functions, including blood clotting, ovulation,

initiation of labor, bone metabolism, nerve growth and development, wound healing, kidney function, blood vessel tone, and immune responses (DuBois *et al.*, 1998).

Cyclooxygenase, as the key enzyme in the biosynthesis of prostaglandins from AA, exists in two isoforms, cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2). The constitutive isozyme COX-1 is present nearly in all cell types and its major function is to provide PG precursors for homeostatic regulation. COX-1 is involved in physiological functions such as blood platelet aggregation and homeostasis of the gastrointestinal tract and the kidney. The second isozyme, inducible COX-2, is almost undetectable under resting conditions. Its expression is induced in macrophages, fibroblasts, and vascular endothelial and smooth muscle cells in response to various pro-inflammatory stimuli such as cytokinines, endotoxins, growth factors and tumor promoters. The PGs produced by COX-2 are directly involved in many inflammatory reactions and are responsible for the characteristic inflammatory symptoms (redness, pain, edema, fever and loss of function). The inducible isozyme is implicated in pathological processes such as various cancer types and Alzheimer and Parkinson's diseases (Charlier and Michaux, 2003; Dubois *et al.*, 1998; Smith and Langerbach, 2001). COX-2 is also involved in important physiological functions in the central nervous system such as synaptic activity, long-term potentiation, long-term depression, memory consolidation, and neurovascular coupling during functional hyperaemia (Aïd and Bosetti, 2011). Like the central nervous system, the kidney, pancreas, intestine, blood vessels and airway cells contain constitutively expressed COX-2 (Warner and Mitchell, 2004). A third isoform COX-3 was discovered by Simmons *et al.* (2004). This isoform is an alternative splice variant of COX-1, which unlike COX-1 and COX-2 does not produce proinflammatory prostanoids but produces anti-inflammatory members of that family. It is supposed that COX-3 might represent a new therapeutic target (Willoughby *et al.*, 2000).

### 1.1.2 LO pathway

5-lipoxygenase (5-LO) is the key enzyme in leukotriene biosynthesis that catalyzes the initial steps in the conversion of AA to biologically active leukotrienes (Schneider and

Bucar, 2005). Beside 5-LO, that is found in mast cells, monocytes, macrophages, and granulocytes, in humans have been observed 12-LO and 15-LO isozymes. 12-LO has been found in platelets and some epithelial cell, and 15-LO in epithelial cells, but knowledge about their biological roles is limited. In contrast, 5-LO has been widely studied and it might be biologically most important LO (Calder, 2001; Charlier and Michaux, 2003).

5-LO catalyzes the incorporation of oxygen at C-5 of AA, leading to the formation of 5-hydroperoxyeicosatetraenoic acid, which is further metabolized by the same enzyme to 5-hydroxyeicosatetraenoic acid or to the key intermediate leukotriene $A_4$ ($LTA_4$) (Schneider and Bucar, 2005). Depending upon the available enzymes, this highly unstable epoxide may either undergo enzymatic hydrolysis into the dihydroxy-acid $LTB_4$ by $LTA_4$-hydrolase, or be conjugated with glutathion to form $LTC_4$ by $LTC_4$-synthase. This last compound can be metabolized into $LTD_4$ and $LTE_4$ by successive elimination of glutamic acid and glycine. These compounds ($LTC_4$, $D_4$ and $E_4$) are collectively referred to as cysteinyl- or peptido-LTs and in the past were referred to as the slow-reacting substances of prophylaxis (Charlier and Michaux, 2003; Schneider and Bucar, 2005; Werz, 2002). The conversion of AA by 5-LO adopted from Werz (2007) is shown in Figure 1.

Unlike prostanoids, LTs are produced by inflammatory cells only. While 5-LO is specifically expressed in cells of myeloid lineage, $LTA_4$ hydrolase and $LTC_4$ synthase are more widely distributed throughout the body. $LTA_4$ hydrolase is particularly abundant in the intestine, spleen, lung, kidney and erythrocytes, and $LTC_4$ synthase is expressed in mast cells, basophiles, eosinophils, endothelial cells and platelets (Charlier and Michaux, 2003).

$LTB_4$ is potent chemotactic and chemokinetic mediator and acts as leukocyte activator by stimulating migration and activation of granulocytes and T cells, leading to adherence of granulocytes to vessel walls, degranulation and release of cathelicidin LL-37 and superoxide. It also enhances the phagocytic activity of neutrophils and macrophages and stimulates secretion of immunoglobulins by lymphocytes. $LTB_4$ regulate the immune response and it has significant role in the pathogenesis of inflammatory diseases, such as arthritis, asthma or atherosclerosis (Pergola and Werz, 2010).

Cysteinyl-LTs ($LTC_4$, $D_4$ and $E_4$) play a pathophysiological role in immediate hypersensitivity reactions. They are potent constrictors of smooth muscles, particularly active in the airways leading to important bronchoconstrictors. They also stimulate mucus secretion and play an important role in bronchial smooth muscles cell proliferation. In the micro-vascular system, they increase the vascular permeability by contracting endothelial cells. In regard to these potent biological activities, LTs have prominent functions in pathophysiology and are connected to numerous disorders including bronchial asthma, allergic rhinitis, inflammatory bowel and skin diseases, rheumatoid arthritis, cancer, osteoporosis and cardiovascular diseases (Charlier and Michaux, 2003; Werz, 2007).

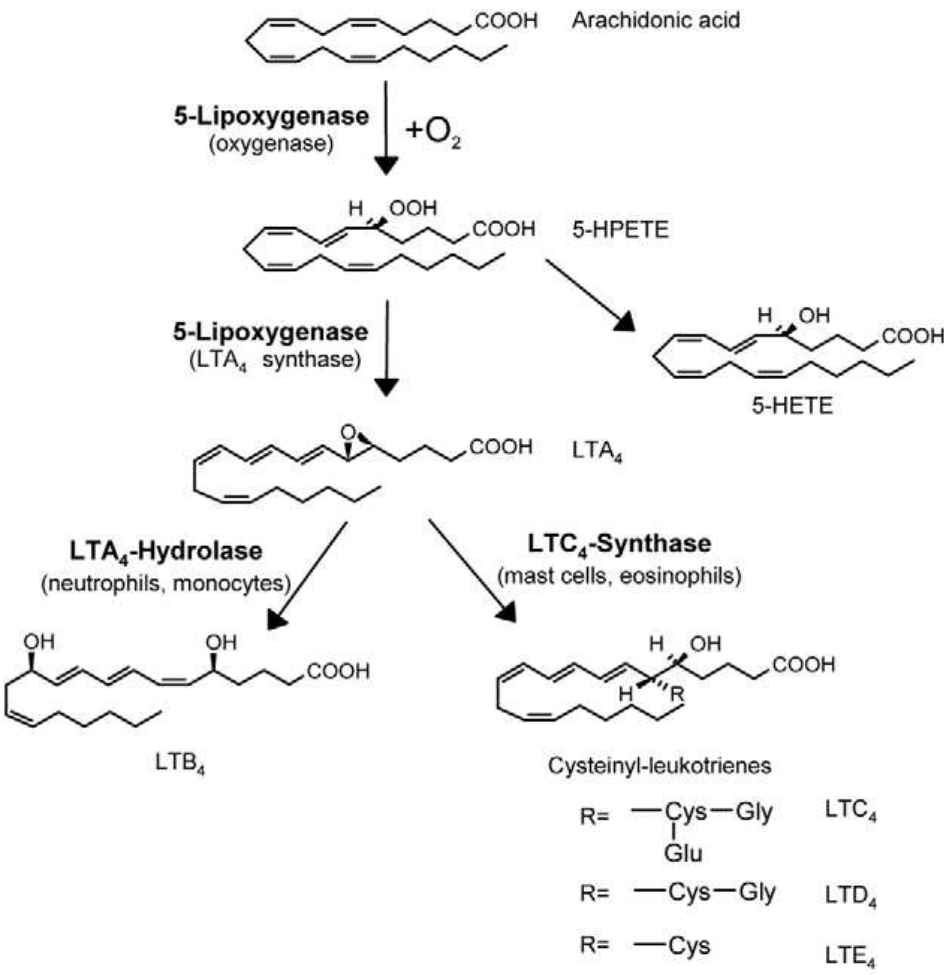


Fig. 1. Conversion of arachidonic acid by 5-LO (Werz, 2007)

### 1.1.3 Treatment of inflammation

Excessive inflammation is considered as a critical factor in many human diseases, including cancer and cardiovascular diseases (García-Lafuente *et al.*, 2009). Many therapies exist for treatment of inflammation-driven diseases such as asthma, rheumatoid arthritis, psoriatic arthritis, systemic lupus erythematosus, Crohn's disease, multiple sclerosis and systemic vasculitis (Gilroy et al., 2004). Among the most important therapies belong glucocorticoids, disease-modifying antirheumatic drugs and non-steroidal anti-inflammatory drugs (Pavelka *et al.*, 2005).

Glucocorticoids are widely used to treat both acute and chronic inflammations, including rheumatoid arthritis, inflammatory bowel disease, multiple sclerosis, psoriasis and eczema. They interact with the steroid receptor to downregulate the expression of specific genes that regulate the inflammatory process. Glucocorticoids inhibit the vasodilatation and increased vascular permeability that occurs at the site of inflammation. They decrease leukocyte migration into inflamed sites and also alter leukocyte distribution. On the other hand, long-term use of glucocorticoids is associated with side effects such as osteoporosis, metabolic disease and increased risk of cardiovascular disease (Coutinho and Chapman, 2010; Yamamoto and Gaynor, 2001).

Disease-modifying antirheumatic drugs (DMARDs) have been shown to improve signs and symptoms of rheumatoid arthritis, an idiopathic autoimmune disorder, in addition to reducing or preventing joint destruction (Van de Putte *et al.*, 2004). This group includes methotrexate, D-penicillamine, sulphasalazine, gold salts, antimalarial drugs and immunosuppressant drugs. They are associated with a wide range of side effects and variable efficacy (Lambert, 2012), therefore the new generation of biological DMARDs that inhibit cytokine tumor necrosis factor-α (TNF-α) and that plays a significant role in brain immune and inflammatory activities were developed. TNF-α is produced in the brain in response to various pathological processes such as infectious agents, ischemia and trauma and its inhibition is used for treatment of inflammatory diseases (Feuerstein *et al.*, 1994). Clinical studies indicate that TNF-α-neutralizing therapy can cause several

complications, such as increased infection rates and should not be given to patients with cardiac failure or a history of demyelinating disease (Gilroy *et al.*, 2004).

Non-steroidal anti-inflammatory drugs (NSAIDs) provide their therapeutic activities through inhibition of cyclooxygenase (COX), the enzyme that makes prostaglandins (Vane and Botting, 1998). NSAIDs are widely used in the treatment of rheumatic disorders and other degenerative and inflammatory joint diseases, and as multi-purpose analgesics. Although steroidal anti-inflammatory drugs and NSAIDs are currently used to treat acute inflammation, these drugs have not been entirely successful in curing chronic inflammatory disorders while such compounds are accompanied by unexpected side effects including gastric ulceration and renal failure, which limit their use in some patients (Mitchell *et al.*, 1995, Yoon and Baek, 2005).

#### 1.1.3.1 Non-steroidal anti-inflammatory drugs

NSAIDs have been used since more than 2000 years, since the analgesic and antipyretic effect of extract from willow bark has been discovered. In China and other parts of Asia, as well as in North America and South Africa, salicylate-containing plants were being applied therapeutically. Salicin, the active compound of willow bark, was identified in Europe in the 17th century and salicylic acid was synthesized for commercial purposes in 1874. Acetylsalicylic acid (aspirin), the more palatable form of salicylic acid, was introduced into the market by Bayer company in 1898. The main therapeutic actions of aspirin were recognized as its antipyretic, anti-inflammatory, and analgesic effects by the early 1900s (Pavelka *et al.*, 2005; Vane and Botting, 1998).

Later, several other drugs, namely antipyrine, phenacetin, acetaminophen (paracetamol), phenylbutazone, fenamates, indomethacin and naproxen, that shared some or all of these actions, were discovered and called NSAIDs. Despite the diversity of their chemical structures, these drugs have the same therapeutic properties as well as similar side effects like gastric upset, delaying of the birth process in high doses, and damage of the kidney in overdose (Vane and Botting, 1998). Traditional NSAIDs were known to

inhibit both isoforms of COX and their adverse gastrointestinal toxicities were attributed to the inhibition of gastroprotective PGs produced via COX-1 pathway. Thereafter, selective COX-2 inhibitors (the Coxibs) were developped because of their superior anti-inflammatory and analgesic properties with reduced adverse effects compared to traditional NSAIDs (Rao and Knaus, 2008). The first two NSAIDs developed as selective COX-2 inhibitors were Celecoxib (Celebrex™) and rofecoxib (Vioxx™). These joined some existing NSAIDs like etodolac (Lodine™), meloxicam (Mobic™, Mobicox™), and nimesulide (Aulin™, Mesulid™, Nimed™, and others), that display some level of COX-2 selectivity. Recently the number of therapeutically available COX-2-selective agents has been increased by the addition of second generation COX-2 inhibitors such as valdecoxib (Bextra™) and etoricoxib (Arcoxia™), and also lumiracoxib (Prexige™) (Warner and Mitchell, 2004; Simmons *et al.*, 2004). By decreasing $PGI_2$ production, COX-2 antagonists may tip the natural balance between prothrombotic thromboxane $A_2$ and antithrombotic $PGI_2$, potentially leading to increased thrombotic cardiovascular events (Mukherjee et al., 2001). It has led to withdrawal of Merck's Vioxx® (rofecoxib) and Pfizer's Bextra® (valdecoxib) from the world-wide market. Celecoxib was allowed to remain in the market place, but with a warning indicating a risk of adverse cardiovascular events (Dogné et al., 2005, Melnikova, 2005).

A promising alternative to avoiding the adverse reactions of NSAIDs, that are caused by the inhibition of COX-1, could be the development of dual inhibitors that simultaneously inhibit COX-2 and 5-LO with improved efficacy and reduced side-effects when compared with selective COX inhibitors (Fiorucci *et al.*, 2001). COX-2 and 5-LO enzymes display similar expression patterns and converging functions and have been involved in the development and progression of numerous types of cancer such as pancreatic, lung, colorectal and prostate. So, the use of dual inhibitors opens up new perspectives in the prophylactic treatment of this disease (Charlier and Michaux, 2003). This dual COX-2/5-LO inhibition has been reported to provide analgesic efficacy and excellent gastrointestinal safety in a variety of *in vivo* tests (Janusz *et al.*, 1998).

### 1.1.3.2 5-LO inhibiting drugs

5-LO is important enzyme in the biosynthesis of leukotrienes, and it is a potential target in the treatment of asthma, atherosclerosis, prostate cancer, and allergy (Rossi *et al.*, 2010; Song *et al.*, 2010). Zileuton, a benzothiophene *N*-hydroxyurea, is the only 5-LO inhibitor in market for the treatment of chronic and persistent bronchial asthma. Zileuton suppresses PG biosynthesis by interference with AA released in macrophages and is often utilized as a selective tool to evaluate the role of 5-LOX and LTs *in vitro* as well as *in vivo* models of inflammation (Rossi *et al.*, 2010; Song *et al.*, 2010). However, Zileuton exhibits hepatic toxicities and drug interactions, and thus, its clinical use is limited by the need to monitor hepatic enzyme levels (Rossi *et al.*, 2010). Other adverse effects include dyspepsia, abdominal pain and nausea (Scow *et al.*, 2007).

RBx 7796 (Clafrinast) is a distinct chemical entity from N-hydroxyurea that inhibits 5-LO in a competitive manner and exhibits efficacy in several experimental animal models of airway inflammation (Shirumalla *et al.*, 2006). A selective COX-2 inhibitor Celecoxib, that is used in the therapy of inflammatory and painful conditions, inhibits 5-LO in *in vitro* as well as *in vivo*. This inhibition is unique amongst the other coxibs (Maier *et al.*, 2008). It is obvious that many inhibitors of 5-LO also affect the related 12- and 15-LOs and/or COX enzymes because of sharing the same substrate AA (Pergola and Werz, 2010). The plant kingdom is a valuable source for new 5-LO and dual COX-2/5-LO inhibitors. The possibility of finding leading structures might be the basis for synthetic modifications with the aim of optimizing the bioavailability and pharmacokinetics, thus considerably improving the efficiency of plant constituents for therapy (Schneider and Bucar, 2005).

### 1.1.3.3 Anti-inflammatory phytochemicals

Natural products, including those derived from higher plants have, over the years contributed greatly to the development of modern therapeutic drugs. Most plant-derived secondary metabolites are known to interfere directly or indirectly with various

inflammatory mediators (Calixto *et al.*, 2003). Bark from the white willow tree (*Salix alba* L.) is one of the oldest herbal remedies for pain and inflammation, as an analgesic and antipyretic agent. The mechanism of action is similar to that of aspirin which is a nonselective inhibitor of COX-1 and COX-2 (Gulati, 1999). Salicin from white willow bark is converted to salicylic acid by the liver and is considered to have fewer side effects than aspirin (Maroon *et al.*, 2010).

Diosgenin, a saponin aglycon, can be found in several plant species such as *Balanites aegyptica* [L.] Delile, *Costus speciosus* (Koenig), *Dioscorea* species, *Solanum xanthocarpum* Schrad & Wendl., *S. incanum* L. and *Trigonella foenum graecum* L. In the 1940s, diosgenin became an intermediate for the chemical synthesis of certain corticosteroids and structurally related fertility regulants and nowadays it is the major starting material used in the industrial production of steroidal hormones. Many investigations have shown that diosgenin has a large variety of biological functions including anti-inflammatory activity and it has been traditionally utilized to treat rheumatism and arthritis (Bruneton, 1999; Dawson, 1991; Hirai *et al.*, 2010; Jung *et al.*, 2010; Olayemi and Ajaiyeoba, 2007). Curcumin is a naturally occurring yellow pigment present in the rhizomes of the plant *Curcuma longa* L. The plant originates in southern Asia and apart from its use as a coloring and flavouring spice in foods; curcumin has also been widely used in both Ayurvedic and Chinese medicine as an anti-inflammatory agent and in wound healing. Recently, many reports suggest that curcumin exerts an anti-inflammatory action in models of atherosclerosis, Alzheimer's disease, arthritis and pancreatitis, including the inhibition of macrophage activation, and inhibition of LO and COX-2, metabolite production via arachidonic acid pathway (Calixto *et al.*, 2003). *Boswellia serrata* Roxb. and *B. carterii* Birdw. contain boswellic acid that selectively inhibits 5-LO. *Boswellia* species originate in India, Ethiopia, Somalia, and the Arabian Peninsula and are better known as frankincense called olibanum. Its resin contain boswellic acid that can inhibit the leukotriene biosynthesis in neutrophilic granulocytes by inhibiting 5-LO and it possesses anti-inflammatory, anti-arthritic, and analgesic properties (Calixto *et al.*, 2003; Gupta *et al.*, 2000; Safayhi *et al.*, 1992; Safayhi *et al.*, 2000). Capsaicin can be found in *Capsicum annum* L., a small spreading shrub originally cultivated in the tropical Americas. The fruit has been used for various medicinal purposes

by native peoples of the American tropics for hundreds of years. Capsaicin produces highly selective local anesthesia by causing degeneration of capsaicin-sensitive nociceptive nerve endings which can produce significant and long-lasting increases in nociceptive thresholds (Maroon *et al.*, 2010).

Flavonoids, compounds that are broadly distributes in plants, have been reported to display marked *in vitro* and *in vivo* anti-inflammatory properties (Calixto *et al.*, 2003). They have mild if any side-effects when consumed medicinally and therefore referred to as „tender drugs“ (Chi *et al.*, 2001).

# 1.2 Isoflavonoids

The discovery of isoflavonoids as natural products has its origin in the mid-nineteenth century, when both Reinsch (1842) and Hlasiwetz (1855) obtained ononin from the roots of the legume *Ononis spinosa* L. Almost one hundred and fifty years later, at the end of 2004 it was known as many as 1600 examples of isoflavonoids (Veitch, 2007). Isoflavonoids are known for their antifungal and insecticidal properties, they are considered as phytoalexins and also their estrogen-like properties have been reported (Reynaud *et al.*, 2005).

## 1.2.1 Characteristics

The isoflavonoids are structurally distinct from other flavonoid classes in that they contain a $C_{15}$ skeleton based on 1,2-diphenylpropane (Tahara and Ibrahim, 1995). In contrast to flavonoids, isoflavonoids possess a 3-phenylchroman skeleton that is biogenetically derived from the 2-phenylchroman skeleton of the flavonoids (Birt *et al.*, 2001). The formation of isoflavonoids consists of two steps. The first one is the oxidation of a flavanone and its rearrangement and the second one is the elimination of water molecule, which yields isoflavone (e.g., (2*S*)-liquiritigenin to daidzein). The first step is catalyzed by isoflavone-synthase and leads to a 2-hydroxyisoflavanone, that is an intermediates (Bruneton, 1999).

All isoflavonoids are believed to be derived from a restricted number of simple isoflavones, either daidzein (7,4'-dihydroxyisoflavone) or genistein (5,7,4'-trihydroxyisoflavone) (Tahara and Ibrahim, 1995, Veitch, 2007). There are several subclasses of isoflavonoids according to the number of substituents on the basis 3-phenylchroman skeleton and according to the different oxidation level of the central pyran ring, namely isoflavones, pterocarpanoids, rotenoids, isoflavanones, isoflavans, coumestans, 3-arylcoumarins, isoflavonoids oligomers, coumaronochromones and isoflav-3-enes (Tahara and Ibrahim, 1995). In all types, there is high frequency of prenylated

derivatives, and consequently, of furan-, dihydrofuran-, and pyran-type structures (Bruneton, 1999).

The most frequently reported of all isoflavonoid subclasses are isoflavones, which occur in the free state, or, less commonly, as glycosides (*O*-glycosides, or exceptionally *C*-glycosides). In plants, isoflavonoids may be found as aglycones or as glycosides. Glucose, rhamnose or apiose are common sugar component of glycosides (Reynaud *et al.*, 2005).

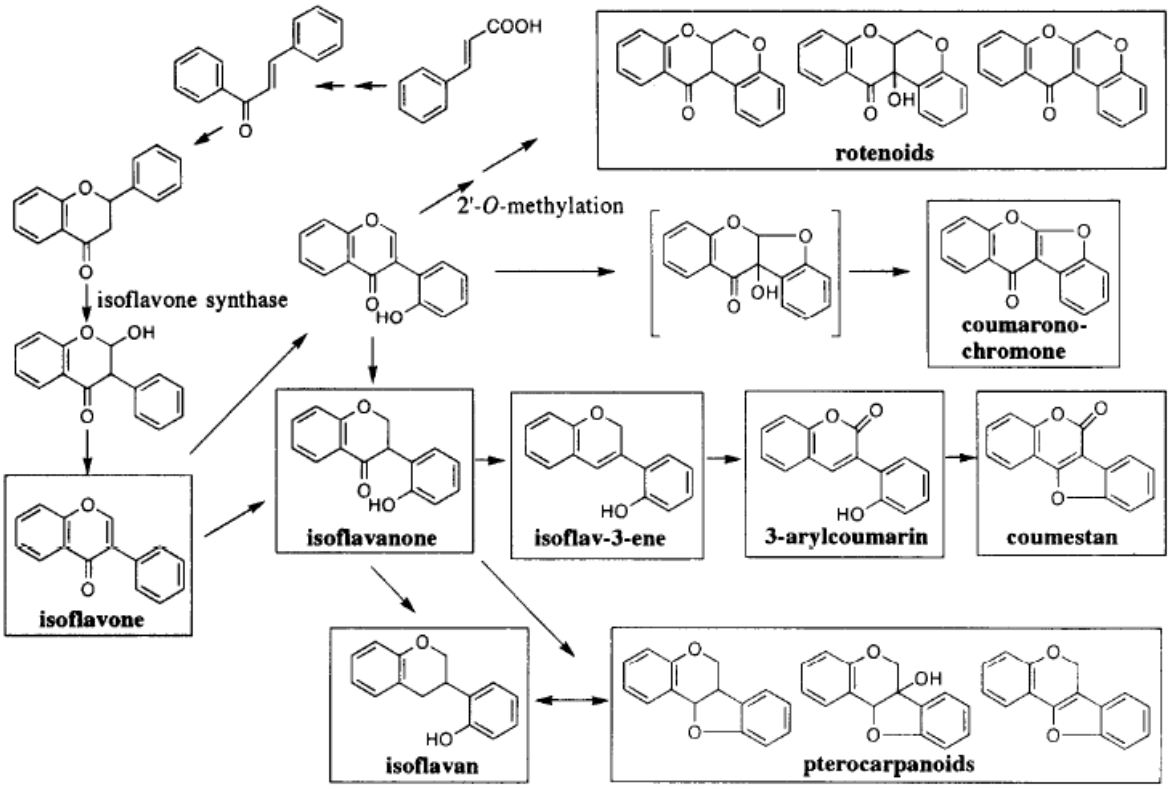


Fig. 2. Biosynthetic relationships of major isoflavonoid classes (Tahara and Ibrahim, 1995)

## 1.2.2 Taxonomical distribution

Isoflavonoids are found mainly within the subfamily Papilionoideae of the Leguminosae family but they are also occasionally found in some other angiosperm families (Gikas *et al.*, 2008, Veitch, 2007). There are over 164 types of isoflavonoids

reported in 31 non-leguminous angiosperm families, but also 15 structures in three gymnosperm families (Cupressaceae, Podocarpaceae and Araucariaceae) and one Bryophyte possessing three isoflanonoids (Reynaud *et al.*, 2005).

Among the monocots, six families have been reported to produce isoflavonoids. The major source of isoflavonoids in this group are the Iridaceae with 52 different compounds described followed by Liliaceae (4 compounds), Stemonaceae (3 compounds), Zingiberaceae (2 compounds), Poaceae and Eriocaulaceae (1 compound each) (Reynaud *et al.*, 2005). In addition to these six families, isoflavones have been reported in two *Juncus* species from family Juncaceae (Abdel-Mogib, 2001), in *Aloe vera* (L.) Burm. fil. from family Asphodelaceae, (Saxena and Sangeeta, 2000) and *Smilax glabra* L. from family Smilacaceae (Yi *et al.*, 1998).

In dicots, there are five non-leguminous families with relative abundance in isoflavonoids, namely Asteraceae (21 compounds), Chenopodiaceae and Nyctaginaceae (19 compounds each), Moraceae (18 compounds) and Ochnaceae (17 compounds). However, presence of isoflavonoids has been reported only in few species among these families. Isoflavonoid formononetin can be found in the species *Cimicifuga racemosa* (L.) Nutt. of the family Ranunculaceae (Reynaud et al., 2005).

Although there are many other non-leguminous families with occurrence of isoflavonoids (Reynaud et al., 2005), they are restricted almost entirely to the family Leguminosae. Among non-papilionoid, there have been reported only few species as sources of isoflavonoids, namely *Apuleia leiocarpa* (Vogel.) J. F. Macbr., *Cassia javanica* L. subsp. *nodosa* (Buch.-Ham. ex Roxb.) K. Larsen & S. S. Larsen (syn. *C. nodosa* Buch.-Ham. ex Roxb.) and *Senna siamea* (Lam.) H. S. Irwin & Barneby (syn. *Cassia siamea* Lam.) (subfamily Caesalpinioideae each), and from subfamily Mimosoideae, there have been reported *Albizia procera* (Roxb.) Benth. and *Prosopis juliflora* (Sw.) D. C.. On the other hand, in Papilionoideae, the largest of the three Leguminosae subfamilies, isoflavonoids are widely distributed, which emphasize the importance of this subfamily (Veitch, 2007).

## 1.2.3 Biological properties

Flavonoids are substances that show a variety of biological effects *in vitro* as well as *in vivo*. They exert antimicrobial, antiviral, anti-inflammatory, antioxidant, antihepatotoxic, antihypertensive, hypolipidemic and antiplatelet activities in numerous mammalian cell systems (Formica and Regelson, 1995). Their value in plant disease resistance as well as their function in symbiotic plant-rhizobial interactions in crops, where they serve as inducers of the nodulation genes of symbiotic *Rhizobium* bacteria, is also remarkable (Dixon and Sumner, 2003).

Isoflavonoids belong to the group of phytoestrogens. They are naturally occurring non-streroidal compounds of plants which promote estrogenic activity in mammals. The isoflavones daidzein, genistein, formononetin and biochanin A and the coumestan coumestrol have been identified as the most common estrogenic compounds in plants (Mazur, 1998, Reynaud *et al.*, 2005). Since the circulating concentration of isoflavone aglycone may be 100-fold greater than estradiol, isoflavones and flavonoids may be antiestrogenic, depending on the level of natural estrogens. Isoflavonoids and/or flavonoids might counteract endogenous estrogens through competitive binding to estrogen receptors (Birt *et al.*, 2001).

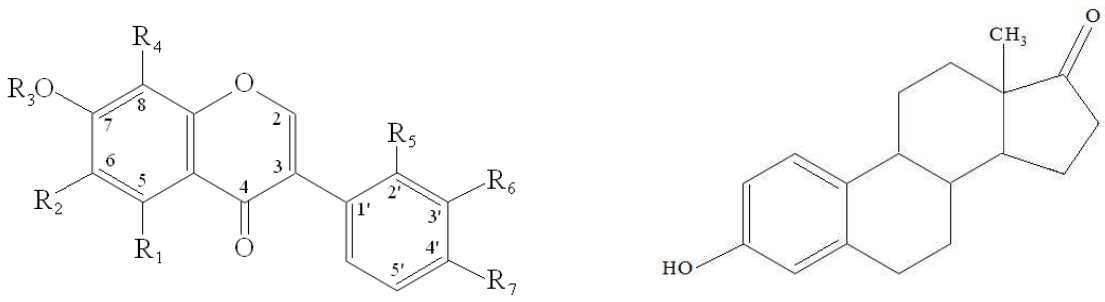


Fig. 3. Chemical structures of isoflavone (left) compared with estrogen (right)

Major sources of isoflavones for humans are seed products of soybean and chickpea. Soybean (*Glycine max* L.) is the main source of daidzein and genistein, chickpea (*Cicer arietinum* L.) is the main source of biochanin A and its daily intake is very high to oriental population (Dixon and Sumner, 2003; Wu *et al.*, 1998). It might be a reason why incidence of breast, colorectal and prostrate cancer is lower in peoples of Asia compared to the inhabitants of industrialized Western world (Adlercreutz, 1998; Adlercreutz and Mazur, 1997; Mazur, 1998). The isoflavone genistein is a phytoestrogen found in high levels in soy products. It is reported to prevent a wide range of disorders including cancer and osteoporosis as well as cardiovascular diseases and postmenopausal symptoms (Kim *et al.*, 2003; Verdrengh *et al.*, 2003).

### 1.2.4 Anti-inflammatory effects

A number of investigations have shown that a variety of flavonoids exhibit anti-inflammatory activity both *in vitro* and *in vivo* (García-Lafuente *et al.*, 2009; Ibrahim, 2001; Takano-Ishikawa *et al.*, 2006), including several isoflavonoids such as genistein, daidzein, formononetin or biochanin A (Bao *et al.*, 2011; Duan *et al.*, 2003; Hämäläinen *et al.*, 2007; Hooshmand *et al.*, 2007; Jun *et al.*, 2005; Kalhan *et al.*, 2007; Krishnan *et al.*, 2007; Mahesha *et al.*, 2007; Richard *et al.*, 2005; Verdrengh *et al.*, 2003). Among them, the genistein is one of the most studied isoflavonoid compounds. Its anti-inflammatory effect has been evaluated on a guinea pig model of asthma. In this model of airway inflammatory disease, genistein markedly attenuates ovalbumin-induced bronchoconstriction, pulmonary eosinophilia and airway hyperresponsiveness (Duan *et al*, 2003). In another study, Verdrengh *et al.* (2003) demonstrated that genistein exert evident anti-inflammatory properties on *in vivo* cell-mediated responses in mice. Study of Hooshmand *et al.* (2007) indicate that genistein suppresses the production of proinflammatory molecules such as COX-2 in human chondrocytes, while it has no effect on COX-1 production; therefore, using it as dietary supplement can be an attractive and viable therapy for treatment or prevention of osteoarthritis. Research of Krishnan *et al.* (2007) shows that genistein regulates the expression of key genes in the prostaglandin pathway, suggesting that it

exhibits anti-inflammatory activity in prostate cancer through the inhibition of the prostaglandin pathway. Findings of Bao *et al.* (2011) suggest that soy isoflavone as nutritional supplement may also provide a novel means for the treatment of asthma and airway inflammatory disease through inhibition 5-LO and decreasing $LTC_4$ synthesis.

Among others, Jun *et al.* (2003, 2005) reported that genistein, daidzein and biochanin A isolated from *Pueraria lobata* Ohwi significantly suppressed AA release which may contribute to the anti-inflammatory properties. Biochanin A displayed the most active inhibition on AA release of about 70%, which was verified by Dixon and Sumner (2003). This compound is predominantly found in weedy Red Clover (*Trifolium pratense*) where it is reported as the most abundant isoflavone (Delmonte *et al.*, 2006), and in chickpea (*Cicer arietinum*).

As far as anti-inflammatory action of other isoflavonoids is considered, isoflavone aglycones such as daidzein, biochanin A, and formononetin also significantly suppressed AA release using *in vitro* assay (Jun *et al.*, 2005). Epidemiological studies as well as *in vitro* and animal data suggest that an increase in dietary isoflavones lowers the risk several cancers including prostate cancer via inhibition of inflammation. Ingesting modest amounts of soy can achieve adequate concentrations of genistein and other isoflavones in the prostate to mediate suppression of inflammation and proliferation by modulating gene expression within the prostate (Swami *et al.*, 2009).

Above-mentioned examples suggest that isoflavonoids might be able to interact with COX and LO enzymes in site of inflammation, which afford the occasion to test these compounds for their anti-inflammatory capacity. Thus, it can be supposed that systematic investigation of isoflavonoids can lead to clarification of mechanism of their anti-inflammatory action as well as to determination of structure-activity relationship of these compounds.

# 2. OBJECTIVES

The aim of this thesis is to study 10 isoflavonoids for their potential to inhibit COX-1, COX-2 and/or 5-LO using cellular and enzymatic models *in vitro* in order to assess in detail their anti-inflammatory activity and to determine their structure relationship. Additional aim of the thesis is to summarize data about inflammatory process and possibilities of its treatment by non-steroidal anti-inflammatory drugs, LO-inhibiting drugs and anti-inflammatory phytochemicals. Taxonomical distribution of isoflavonoids, their biological properties and especially their anti-inflammatory effects are also reviewed.

# 3. MATERIALS AND METHODS

## 3.1 Tested compounds

5,4'-dihydroxy-7-methoxyisoflavone (Prunetin), 5,7,4'-trimethoxyisoflavone, 5,7,4'-trihydroxyisoflavone (Genistein), 5,7-dihydroxy-4´-methoxyisoflavone (Biochanin A), 6,7,4'-trimethoxyisoflavone, 7,4'-dimethoxy-5-hydroxyisoflavone, 7,4'-dimethoxyisoflavone, 7,8,4'-trihydroxyisoflavone, 7-hydroxy-6-methoxyisoflavone, 7-methoxyisoflavone were obtained from Indofine Chemical Company (USA). The structures of all above mentioned compounds are described in Fig 4 and Table 1.

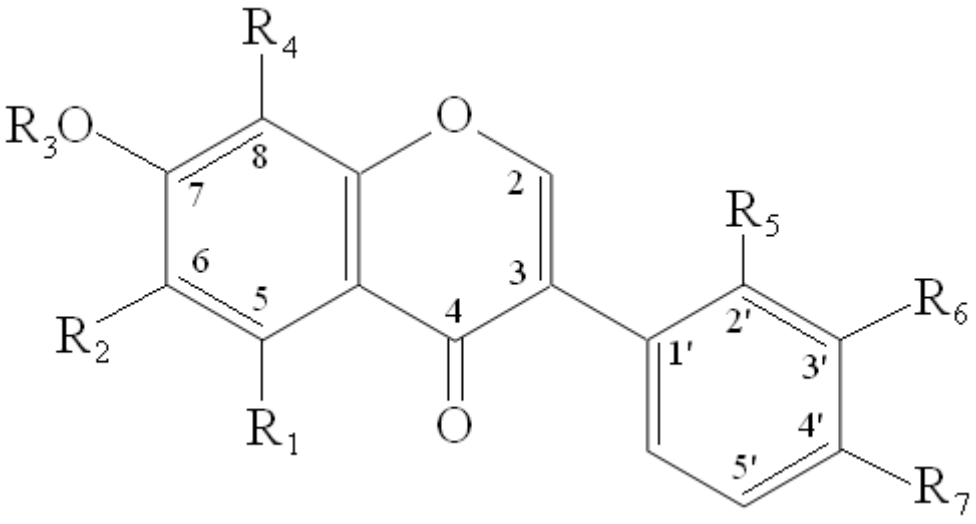


Fig. 4. Basic chemical structure of isoflavonoids

Table 1. Structures of tested isoflavonoids

| **Systematic name of isoflavone** | **Purity [%]** | **$R_1$** | **$R_2$** | **$R_3$** | **$R_4$** | **$R_5$** | **$R_6$** | **$R_7$** |
|---|---|---|---|---|---|---|---|---|
| 5,4'-dihydroxy-7-methoxyisoflavone | 98+ | OH | H | $CH_3$ | H | H | H | OH |
| 5,7,4'-trimethoxyisoflavone | 98 | $OCH_3$ | H | $CH_3$ | H | H | H | $OCH_3$ |
| 5,7,4'-trihydroxyisoflavone | 99+ | OH | H | H | H | H | H | OH |
| 5,7-dihydroxy-4'-methoxyisoflavone | 99+ | OH | H | H | H | H | H | $OCH_3$ |
| 6,7,4'-trimethoxyisoflavone | 98 | H | $OCH_3$ | $CH_3$ | H | H | H | $OCH_3$ |
| 7,4'-dimethoxy-5-hydroxyisoflavone | 98 | OH | H | $CH_3$ | H | H | H | $OCH_3$ |
| 7,4'-dimethoxyisoflavone | 99,4 | H | H | $CH_3$ | H | H | H | $OCH_3$ |
| 7,8,4'-trihydroxyisoflavone | 98+ | H | H | H | OH | H | H | OH |
| 7-hydroxy-6-methoxyisoflavone | 98 | H | $OCH_3$ | H | H | H | H | H |
| 7-methoxyisoflavone | 99 | H | H | $CH_3$ | H | H | H | H |

## 3.2 COX assay

Porcine hematin, L-epinephrine, disodium ethylenediamine tetraacetate ($Na_2EDTA$), dimethylsulfoxide (DMSO), adrenochrome, hydrochloric acid (HCl), AA and human recombinant COX-2 were purchased from Sigma-Aldrich (Czech Republic), Tris. HCl from Bio-Rad (PA, USA).

*Principle:* The purified enzyme and COX-2 were incubated with a defined concentration of test sample and AA. After stopping the enzymatic reaction by addition of formic acid (Fluka) and dilution, the produced $PGE_2$ was quantified by means of a $PGE_2$ EIA kit (Assay Designs).

*Sample preparation:* The test samples were dissolved in DMSO. Compounds were tested in concentrations of 50, 100, and 150 µM for screening.

*Incubation procedure:* The assay was performed according to a procedure described by Reininger and Bauer (2006) in a microtiter scale with purified COX-2 from sheep placental cotyledones or human recombinant COX-2. COX-2 (0.5 unit/reaction) was added to 180 µl of incubation mixture containing 100 mM Tris buffer (pH 8.0), 5 µM porcine hematin, 18 mM L-epinephrine and 50 µM $Na_2EDTA$. 10 µl of the test substance dissolved in DMSO or pure DMSO in case of blank was added and the mixture was preincubated for 5 min at room temperature. The reaction was initiated by adding 10µl of 5µM AA and the reaction mixture was incubated at 37°C for 20 min. The reaction was stopped by addition of 10 µl of 10% formic acid.

*$PGE_2$ EIA:* A competitive $PGE_2$ EIA kit (Assay Designs, MI, USA) was used to determine the concentration of $PGE_2$, the main AA metabolite in this reaction. All samples were diluted 1:15 in EIA buffer. The EIA was incubated for 2 hours at room temperature on a plate shaker. After a washing step, 200 µl of substrate solution were added to each well, and the EIA was incubated for 45 min at room temperature. After addition of 50 µl stop solution, the EIA was evaluated photometrically by microplate reader Tecan Infinite M200 (Tecan Group, Switzerland) at 405 nm. The results were expressed as percentage inhibition of $PGE_2$ formation against untreated samples (blanks).

## 3.3 5-LO assay

Sodium chloride (NaCl), potassium chloride (KCl) and sodium hydroxide (NaOH), were purchased from Lachema a.s. (Czech republic), eicosatetraynoic acid (ETYA), ethanol (EtOH), calciumionophor A23, waterfree D-glucose, trypan blue and gentian violet from Sigma-Aldrich (Czech Pepublic), dextran T-500 from Roth (DE), calcium chloride dihydrate ($CaCl_2.2H_2O$), acetic acid ($CH_3COOH$) from Penta (Czech republic), amonium chloride ($NH_4Cl$) from Lach-ner s.r.o. (Czech republic) and zileuton from Farmak a.s. (Czech republic).

*Principle:* Activated neutrophile granulocytes with 5-LO activity were incubated with a defined concentration of test sample and AA. After stopping the enzymatic reaction by addition of formic acid and centrifugation to remove cellular fragments, the produced $LTB_4$ was quantified in the supernatant by means of an $LTB_4$ EIA Kit (Assay Designs).

*Isolation of human neutrophile granulocytes:* The assay was performed according to a process described by Adams *et al.* (2004). A Vacutainer™ system containing a 0.129 M pre-analytical citric acid solution was used to collect 45 mL of venous human blood from healthy volunteer donor. After immediate transfer to a falcon tube containing 20 mL of sedimentation solution (1 % NaCl, 6 % dextran T-500), the blood was left to separate for 60 min at 4 °C. While most of the dense erythrocytes sink into the dextran layer, the lighter blood fractions remain in the upper layer which was then removed and centrifuged at 1600 rpm at 4 °C for 10 min to concentrate the leukocytes, the plasma supernatant was discarded, the pellet was resuspended in 20 mL of a wash buffer (7.4 % $CaCl_2$ dihydrate; 0.1 % anhydrous D-glucose; 0.2 % $MgCl_6$ $H_2O$; 0.04 % KCl; 1.75 % Tris; with the pH adjusted to 7.6 with 1 N HCl). After centrifugation at 1600 rpm at 4 °C for 10 min and removal of the supernatant the resulting pellet was resuspended in 14 mL of hypotonic lysis buffer (0.17 % $NH_4Cl$; 0.2 % Tris; pH 7.2) and gently shaken for 5 min at room temperature to destroy remaining erythrocytes. The suspension was submitted to another centrifugation at 1400 rpm at 4 °C for 5 min. The pellet was resuspended in 10 mL of wash buffer and then centrifuged at 1400 rpm at 4 °C for 15 min. The resulting pellet was

resuspended in 3 mL of Assay buffer (1.75 % Tris, 0.9 % NaCl, pH 7.4), tested for vitality, quantified and then diluted to a cell concentration of 4500 cells/µL with Assay buffer.

*Cell vitality test:* 20 µL of cell suspension and 10 µL of (0.4 %) trypan blue solution were mixed and then 10µL of this suspension were examined and quantified in a „Bürker" chamber 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" chamber.

*Sample preparation:* Samples used for tests of COX inhibition were used also for tests of 5-LO giving final concentrations 38 µM and 19 µM. They were dissolved in an appropriate amount of DMSO and added to the test mixture for screening. Zileuton, the commercially available specific 5-LO inhibitor, was used as a positive control at a concentration of 50 µM.

*Incubation procedure:* The incubation mixture in a microtitre plate consisted of 225 µL leukocyte suspension, with 10 µL $CaCl_2$, 10 µL ETYA as an inhibitor of the 12- and 15-LO pathways, 10 µL Ca ionosphere A 23187 and 5 µL AA, the substrate of the 5-LOX pathway, along with 5 µL of sample or inhibitor dissolved in DMSO or 5 µL of pure DMSO in control wells. After incubation for 10 min at 37 °C in waterbath, the reaction was stopped by addition of 20 µL 10 % formic acid. The microtitre plate was centrifuged for 15 minutes 4 °C and 1400 rpm to separate free $LTB_4$ from cellular particles before the supernatant is diluted 50-fold and applied to a $LTB_4$ EIA Kit.

*$LTB_4$ EIA:* A competitive $LTB_4$ EIA kit (Assay Designs, MI, USA) was used according to the producer's instructions to determine the concentration of $LTB_4$, the main AA metabolite in this reaction. After incubation on an orbital shaker for 2 hours at room temperature, the plate was washed by 400µL three times and 200 µL of substrate solution were added to each well. After EIA incubation for 2 hours at room temperature, 50 µL of Stop solution were added and the EIA was evaluated photometrically by microplate reader Tecan Infinite M200 at 405 nm.

*Data analysis*: The results were expressed as percentage inhibition of $LTB_4$ formation against untreated samples (blanks). Mean $IC_{50}$ value was calculated from two different isoflavonoid concentrations, each concentration was analysed in six repetitions. $IC_{50}$ values were determined by regression analysis.

# 4. RESULTS AND DISCUSSION

## 4.1 COX inhibition

The results of COX-2 assay showed that any of 10 compounds assayed in this study did not exhibited inhibitory action higher than 40% within the concentration range (50, 100 and 150 μM) tested. As it is shown in Fig. 5, none of the tested compounds was active at least half as much as indomethacin that inhibited COX-2 enzyme at concentrations 50, 100 and 150 μM by 86.4%, 95.7% and 94.4%, respectively. Nevertheless, the greatest activity was evidenced in genistein followed by 7,4'-dimethoxy-5-hydroxyisoflavone and 7-methoxyisoflavone. The highest inhibition of 7,4'-dimethoxy-5-hydroxyisoflavone of 22.5% was registered at 50 μM. At 100 μM concentration, the highest COX-2 inhibitions was recorded for genistein and 7-methoxyisoflavone at 39.9% and 17.3%, respectively, On the other hand, the lowest inhibitory activity was observed in biochanin A and 5,7,4'-trimethoxyisolfavone followed by 7,4'-dimethoxyisoflavone, prunetin and 7,8,4'-trihydroxyisoflavone. Because all these compounds were inactive or only slightly active on COX-2 inhibition, we decided do not perform COX-1 assay. This decision was based on the well-known functions of COX-1 and -2 enzymes. It means that COX-2, an inducible isozyme, is implicated in many pathological processes and almost undetectable under resting conditions. Its inhibition is therefore desirable because it leads to suppression of proinflammatory molecules in contrast to COX-1 inhibition. COX-1, a constitutive isozyme, is present nearly in all cell types and is involved in the regulation of the “housekeeping functions” and its inhibition is associated with many side effects.

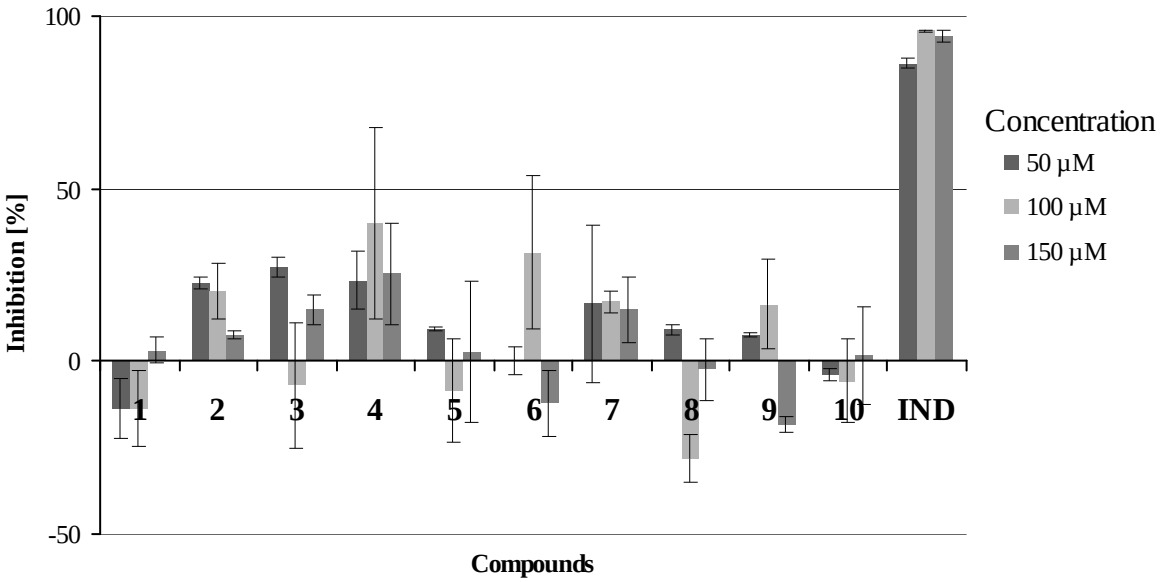


Fig. 5. COX-2 inhibition of studied isoflavonoids: 1 = 5,7-dihydroxy-4'-methoxyisoflavone (Biochanin A), 2 = 7,4'-dimethoxy-5-hydroxyisoflavone, 3 = 7-hydroxy-6-methoxyisoflavone, 4 = 5,7,4'-trihydroxyisoflavone (Genistein), 5 = 5,4'-dihydroxy-7-methoxyisoflavone (Prunetin), 6 = 7,4'-dimethoxyisoflavone, 7 = 7-methoxyisoflavone, 8 = 7,8,4'-trihydroxyisoflavone, 9 = 6,7,4'-trimethoxyisoflavone, 10 = 5,7,4'-trimethoxyisolfavone, IND = indomethacine

With exception of genistein and biochanin A, there are no previous reports describing effect of isoflavonoids tested in this study on COX activity. Considering anti-inflammatory action of genistein, the findings of Hooshmand *et al.* (2007) indicate that this compound suppresses the production of COX-2 in lipopolysaccharide (LPS)-induced chondrocytes *in vitro*. In this study, primary cultures of normal human chondrocytes were treated with genistein and then stimulated by 1 µg/ml LPS. After harvesting of cells, the cytosolic fraction was isolated for assessment of COX-1 and COX-2 protein levels. This data indicate that genistein has the ability to suppress COX-2, while it has no effect to COX-1. Another study conducted by Swami *et al.* (2009) reported that COX-2 mRNA levels were suppressed in cells derived from human peripheral zone prostate tissue after exposure to 10 µM genistein. Secreted $PGE_2$ levels were quantified using $PGE_2$ enzyme

immunoassay. Moreover, a significant decrease in mRNA expression of a PG receptor, which is commonly detected in normal and cancer-derived cultures, was observed after the genistein treatment. Genistein have also been associated with lowered human breast cancer risk by reducing inflammatory prostanoids, COX-2 activity *in vitro* (Horia and Watkins, 2007). *In vivo* study performed by Verdrengh *et al.* (2003) on mice revealed that joints destruction was less frequent in genistein-treated than in control animals, which corresponds to our results where genistein possessed some activity in concentration range. In addition, Wang *et al.* (2000) investigated anti-inflammatory activity of genistein, using microsomal suspensions of COX-1 and AA as substrate. In this study, genistein showed the highest COX-1 inhibitory activity with an $IC_{50}$ value of 80 μM. Despite the above mentioned studies suggesting significant anti-inflammatory properties of genistein, our results showed its weak direct 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 $PGE_2$. In case of biochanin A, the study of Wang *et al.* (2000) reports an inhibitory activity of biochanin A with $IC_{50}$ value of 350 μM in COX-1 assay. This previous finding together with results of our assay, where biochanin A was not active at lower concentrations than 150 μM, suggest that this isoflavonoid has no direct inhibitory effect on COX enzyme activity.

# 4.2 LO inhibition

Similarly as in previous test, the production of $LTB_4$, was measured to assess the 5-LO inhibition by 10 naturally occurring isoflavonoids using *in vitro* assay with activated granulocytes. The results of all compounds studied are listed in Table 2.

Table 2. *In vitro* inhibitory effect of naturally occurring isoflavonoids on LO

| **Compound** | **$IC_{50}$ [µM][1]** |
|---|---|
| 5,7-dihydroxy-4'-methoxyisoflavone | 29.03 (± 2.81) |
| 7,4'-dimethoxy-5-hydroxyisoflavone | 30.29 (± 6.53) |
| 7-hydroxy-6-methoxyisoflavone | 32.10 (± 1.39) |
| 5,7,4'-trihydroxyisoflavone | 32.70 (± 2.18) |
| 5,4'-dihydroxy-7-methoxyisoflavone | 33.28 (± 5.06) |
| 7,4'-dimethoxyisoflavone | 33.67 (± 3.36) |
| 7-methoxyisoflavone | 33.90 (± 8.26) |
| 7,8,4'-trihydroxyisoflavone | 36.74 (± 6.77) |
| 6,7,4'-trimethoxyisoflavone | 37.98 (± 1.78) |
| 5,7,4'-trimethoxyisolfavone | 45.17 (± 5.42) |
| Zileuton[2] | 5.27 (± 1.73) |

[1]Half-maximal inhibitory concentration, [2] positive control

In this test, all compounds possessed certain degree of 5-LO inhibitory effect represented by values of $IC_{50}$ ranging from 29.03 to 45.17 µM, however, no compound tested in our assay was observed to be as active as Zileuton ($IC_{50}$ = 5.27 µM)

The most promising results were observed for biochanin A, which showed the lowest $IC_{50}$ value of 29.03 µM, followed by 7,4'-dimethoxy-5-hydroxyisoflavone and 7-hydroxy-6-methoxyisoflavone with $IC_{50}$ value of 30.29 µM and 32.10 µM, respectively. For genistein, the inhibitory activity was observed at $IC_{50}$ equal to 32.70 µM. Similar results possesses 5,4'-dihydroxy-7-methoxyisoflavone with $IC_{50}$ value of 33.28 µM. Compounds with relatively low inhibitory effect on $LTB_4$ synthesis have 7,4'-dimethoxyisoflavone, 7-methoxyisoflavone, 7,8,4'-trihydroxyisoflavone, 6,7,4'-trimethoxyisoflavone, 5,7,4'-trimethoxyisolfavone with $IC_{50}$ values of 33.67 µM, 33.90 µM, 36.74 µM, 37.98 µM and 45.17 µM, respectively.

Biochanin A, genistein and daidzein are only isoflavonoids previously tested for their effect on LO activity. According to Jun *et al.* (2003, 2005) biochanin A possesses strong inhibitory activity at 1 µM concentration, which correspond to our results in that biochanin A possess the most promising results even at the lowest concentration tested (19 µM). Since biochanin A is widely distributed in common plant species it may be attractive to further investigate for its anti-inflammatory properties. On the other hand, 5-LO inhibitory activity in murine macrophages of 90%, 81% and 69%, in terms of LO-induced lipid peroxidation, was evidenced at 110 µM concentration of daidzein, genistein and biochanin A, respectively. These compounds were tested also in intact cancer cell line at final concentration of 50 µM where biochanin A displayed the most active inhibition on AA release of about 70% in contrast to genistein and daidzein that showed inhibition by 33% and 25%, respectively.

The anti-inflammatory effect of the isoflavones may be important in immune modulation and the prevention of bone loss and cancer (Huang *et al.*, 2005) and in the treatment of asthma (Kalhan *et al.*, 2007). It was evidenced that genistein inhibits human blood eosinophil $LTC_4$ synthesis; this compound is a key effector cell in asthma and other allergic diseases. The synthesis is inhibited as a results of blockade of 5-LO activation; genistein blocks 5-LO translocation specifically to the nuclear membrane in eosinophils.

Consequently, it was registered that dietary soy isoflavone supplementation reduces eosinophil $LTC_4$ synthesis and eosinophilic airway inflammation (Kalhan *et al.*, 2007). A similar study of the effects of soy isoflavones on airway disorders revealed that genistein at the dose of 30 mg/kg had a remarked effect on inhibition of the infiltration of inflammatory cells in airways (Bao *et al.*, 2011). Furthermore, Gottstein *et al.* (2003) registered that genistein in comparison with daidzein showed greater inhibitory activity in human platelet aggregation *in vitro* at concentrations of ≥ 25 μM and ≥ 50 μM, respectively.

According to our best knowledge, there is no available evidence about anti-inflammatory activity of 7,4'-dimethoxy-5-hydroxyisoflavone, 7-hydroxy-6-methoxyisoflavone. For other tested compounds, namely for 7,4'-dimethoxy-5-hydroxyisoflavone, 7-hydroxy-6-methoxyisoflavone, 5,4'-dihydroxy-7-methoxyisoflavone (prunetin), 7,4'-dimethoxyisoflavone, 7-methoxyisoflavone, 7,8,4'-trihydroxyisoflavone, 6,7,4'-trimethoxyisoflavone, 5,7,4'-trimethoxyisolfavone, no reported study is available. Because of their potential to significantly inhibit $LTB_4$ production, they might be prospective in the treatment of severe diseases. Nevertheless, there is a need to examine their occurrence in a plant kingdom as well as mechanism of their action. In summary, the most potential tested isoflavonoid is biochanin A. However all tested compounds are considered to be capable to inhibit 5-LO enzyme.

In regards to the structure activity, Jun *et al.* (2003, 2005) suggested that occurrence of 4'-hydroxyl group in both daidzein and genistein might indicate a structure activity relationship. Furthermore, it was proposed that the 5,7-dihydroxyl groups in both 5,7-dihydroxy-4'-methoxyisoflavone (biochanin A) and 5,7,4'-trihydroxyisoflavone (genistein) and the 4'-methoxyl group in biochanin A and 7-hydroxy-4'-methoxyisoflavone (formononetin) might be key functional groups of isoflavones in suppressing AA release. Moreover, the results of our assay revealed that compounds possessing one or more hydroxyl groups together with one methoxyl group (5,7-dihydroxy-4'-methoxyisoflavone, 7,4'-dimethoxy-5-hydroxyisoflavone, 7-hydroxy-6-methoxyisoflavone, 5,4'-dihydroxy-7-methoxyisoflavone) showed the highest activity. While isoflavonoids with only hydroxyl or methoxyl groups (7,4'-dimethoxyisoflavone, 7-methoxyisoflavone 7,8,4'-

trihydroxyisoflavone, 6,7,4'-trimethoxyisoflavone, 5,7,4'-trimethoxyisolfavone) showed significantly weaker inhibitory activity. Surprisingly, 5,7,4'-trihydroxyisoflavone, that possesses only hydroxyl groups, was according to our results exceptionally active. Nevertheless, it is recommended to assess the inhibitory activity of this compound in further laboratory investigation.

# 5. CONCLUSIONS

Ten different isoflavonoids were tested for their capacity to inhibit COX-2 and 5-LO enzymes using *in vitro* enzymatic and cellular model, respectively. The results of COX-2 assay showed that none of the compounds analyzed in this study exhibited inhibitory action higher than 40% within the whole concentration range (50, 100 and 150 μM) tested. Our results suggest that direct inhibition of COX enzyme is probably not responsible for its previously reported anti-inflammatory effect.

In contrast to COX assay, it is better to assess the inhibitory activity of isoflavonoids by 5-LO assay. This is confirmed also by the results of our study, since all tested compounds showed 5-LO inhibitory activity. In 5-LO assay, it was demonstrated that even at low concentrations isoflavonoid biochanin A significantly inhibits activity in AA release. It is hence considered a promising compound in fighting inflammatory diseases. Moreover, this compound is commonly found in Red Clover and chickpea. A significant inhibitory activity was also registered in 7,4'-dimethoxy-5-hydroxyisoflavone and 7-hydroxy-6-methoxyisoflavone, till the present time unknown for their anti-inflammatory capacity. As far as structure-activity relationship is considered, the occurrence of one methoxyl group together with one or more hydroxyl groups in isoflavonoid structures may be responsible for their capacity to inhibit $LTA_4$ synthesis and therefore for suppressing inflammation.

The results of this study suggest isoflavonoids as promising and effective agents against inflammatory health disorders. Unlike in the NSAIDs, glucocorticoids or other drugs, no side effects were evidenced in these estrogen-like compounds. Thus, the isoflavonoids can be considered as prospective substances for development of new herbal-based nutraceuticals, food additives and pharmaceutical preparations acting as 5-LO selective inhibitors.

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# APPENDIX A

## List of publications related to the thesis

*Symposium*: Hummelová J., Kokoška L., Landa P., Škrovanová V. Screening of Isoflavonoids as 5-lipoxygenase Inhibitors. Proceeding of symposium: 5th Scientific Conference of Institute of Tropics and Subtropics, Czech University of Life Sciences Prague, December 2, 2011. p. 28-30.