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Dissertation

Isoflavones in the Rutaceae family

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Disertační práce

Isoflavony rostlin čeledi routovitých (Rutaceae)

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Declaration

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Signature

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SUMMARY

This study deals with the selective determination of isoflavonoids daidzein, genistein, biochanin A and their derivatives in plant extracts from the Rutaceae family. There were 20 species explored from these following five genera: *Citrus*, *Fortunella*, *Poncirus*, *Ruta* and *Severinia*. The combination of immunochemical methods, semipreparative high performance liquid chromatography and high performance liquid chromatography with mass spectrometry detector was used. All extracts were tested by five specific enzyme immunoassays and all extracts contained range of immunoreactive peaks. Free isoflavones were detected as well as their 7-*O*-glycosides. The most abundant glycoside was sissotrin, while the highest levels of aglycones were represented by formononetin. Generally 4´-methoxyisoflavones (i.e. formononetin and biochanin A) were more abundant than 4´-hydroxyisoflavones (i.e. daidzein and genistein). The content of individual isoflavonoids ranged from 0 to 2.6 ng/g (dry weight), the sum of all measured substances reached up to 5.9 ng/g.

Another object of this study was to search for IFS homologues in non-leguminous plants belonging to the Rutaceae, Cannabaceae and Solanaceae families, which have been previously shown to be isoflavonoid producers. With the help of molecular biology method PCR there were identified sequences in *Cannabis sativa, Humulus lupulus*, *Ruta montana, Ruta graveolens* and *Nicotiana tabacum* genom. New sequences were compared with complete IFS genes described in several species belonging to the Fabaceae family. Identities between all IFS genes and new sequences were more than 65% in all cases at the nucleotide level. In order to full understanding of function, structure and relationship between individual sequences it is necessary to identify the whole gene.

SOUHRN

Tato práce se zabývá selektivním stanovením isoflavonoidů daidzeinu, genisteinu, biochaninu A a jejich derivátů v extraktech rostlin čeledi routovitých. Bylo studováno celkem dvacet zástupců této čeledi z pěti různých rodů: *Citrus*, *Fortunella, Poncirus, Ruta* a *Severinia*. K analýzám bylo využito kombinace metod enzymové imunoanalýzy, semipreparativní kapalinové chromatografie a kapalinové chromatografie s hmotnostním detektorem. Extrakty všech rostlin byly testovány pěti specifickými imunochemickými metodami a obsahovaly řadu imunoreaktivních pásů. Detekovány byly jak volné isoflavony, tak jejich 7-*O*-glykosylované formy. Nejvíce zastoupeným 7-*O*-glykosidem byl sissotrin, zatímco nejvyšších hladin z řad neglykosylovaných isoflavonů dosahoval formononetin. Celkově pak methoxyisoflavony (např. formononetin a biochanin A) byly přítomny ve vyšších koncentracích než hydroxyisoflavony (např. daidzein a genistein). Obsah jednotlivých isoflavonů se pohyboval od 0 do 2,6 ng/g sušiny a celkový obsah isoflavonů v rostlinách dosáhl koncentrace téměř 6 ng/g sušiny.

Dalším cílem této práce bylo hledání homologů isoflavon syntázy v čeledích routovité, konopovité a lilkovité, které nepatří mezi typické producenty isoflavonoidů, ale jejich přítomnost u některých druhů již byla popsána. Pomocí molekulárně biologických metod PCR byly identifikovány sekvence v genomech *Cannabis sativa, Humulus lupulus*, *Ruta montana, Ruta graveolens* a *Nicotiana tabacum*. Nové sekvence byly srovnány s úplnými geny isoflavon syntáz rostlin z čeledi bobovité. Ve všech případech srovnání na úrovni nukleotidových bazí byla shoda větší než 65%. K úplnému pochopení funkce, struktury a vztahů mezi jednotlivými sekvencemi bude nutné identifikovat celý gen.

CONTENTS

1 INTRODUCTION

Isoflavones form a group of distinct secondary metabolites. Natural roles for isoflavones are in both positive and negative plant-microbial interactions. One role is in establishing the symbiotic relationship between a plant and rhizobial bacteria for the formation of nitrogen-fixing root nodules, where isoflavones act as chemoattractants for the rhizobial bacteria and as inducer of nod gene expression. Another major role is during the desease resistance response, where the biosynthesis of isoflavones is induced to provide defense compounds. However, isoflavones are produced predominantly in leguminous plants; they were also found in certain non-leguminous species.

Isoflavones have received much attention as a food component for their presumed activity in promoting better health. The first interest was attracted by the indication that their consumption may prevent the development of various cancers. Further health promoting effects under investigation are beneficial activities on the cardiovascular system, on bone metabolism, renal functions, etc. The mechanisms through which isoflavones may exert the above-mentioned functions are based not only on their estrogenic properties, but also on their role as inhibitors of protein kinases and topoisomerases. They function as regulators of gene transcription, modulators of transcription factors and antioxidants, as well as they alter some enzyme activities.

Isoflavonoids are classically assayed by using very accurate physico-chemical methods. The concentrations of these compounds in biological samples and food are usually determined by liquid or gas chromatography coupled with various detection methods.

The general aim of this study was to verify the occurance of isoflavonoids in the Rutaceae family, improve techniques of detection of isoflavonoids in plant material and increase number of study species using combination of semipreparative HPLC, HPLC-MS and immunochemical methods. Moreover, we tried to search for isoflavone synthase homologues in non-leguminous plants belonging to the Rutaceae, Cannabaceae and Solanaceae families, which have been previously shown to be isoflavonoid producers.

2 REVIEW OF THE LITERATURE

2.1 FLAVONOIDS AND ISOFLAVONOIDS

The flavonoids possessing a basic C_{15} skeleton of 1, 3-diphenylpropane are ubiquitous in all terrestrial plants except the Anthocerotopsida, and the number of known compounds, both as aglycones and glycosides, exceeds 6400[1]. Isoflavonoids (over 1000 structures) are a large subclass of flavonoids and are structurally different from other flavonoid classes because they contain a C_{15} skeleton based on 1, 2-diphenylpropane[2]. They are unlike the other flavonoids by the position of the phenolic ring B (**Fig 1**).

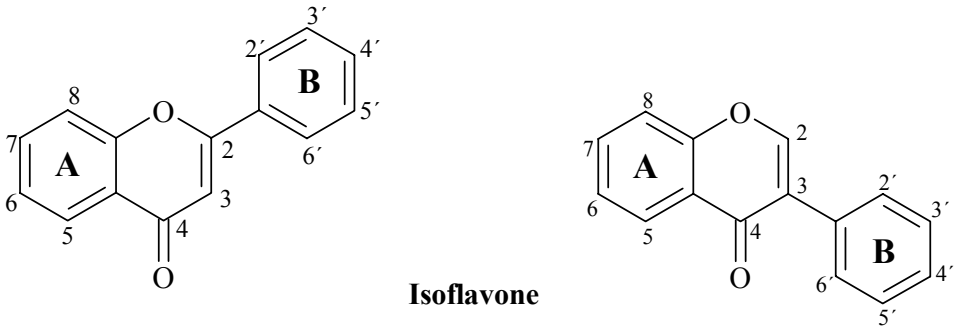


Fig. 1: General structure of flavone and isoflavone, the atom numbering and ring labelling.

In contrast to other groups of flavonoids, the distribution of isoflavonoids among plants is relatively sparse. In plants, isoflavonoids may be encountered as aglycones or as glycosides (with glucose, rhamnose or apiose as the usually occuring sugar components), but glycosidic derivatives are less common than the free-form[2]. The variability of molecules results from the possibility of carbon atoms of basic skeleton to carry additional substituents. An array of their modification leads to a wide range of compounds. The number of possible substituents on the basic structural skeleton (for example hydroxyl or methoxyl groups, as well as methyl-, prenyl-, isoprenyl- or methylendioxy- group, aromatic or aliphatic acids, and the presence of an extra heterocyclic ring) accounts for the multiplicity of subgroups among isoflavonoids.

Isoflavonoids can be divided into nine subclasses within the non-leguminous families. As in Leguminosae, the largest group consists of isoflavones. The other eight subclasses of isoflavonoids are presented in **Fig. 2**.

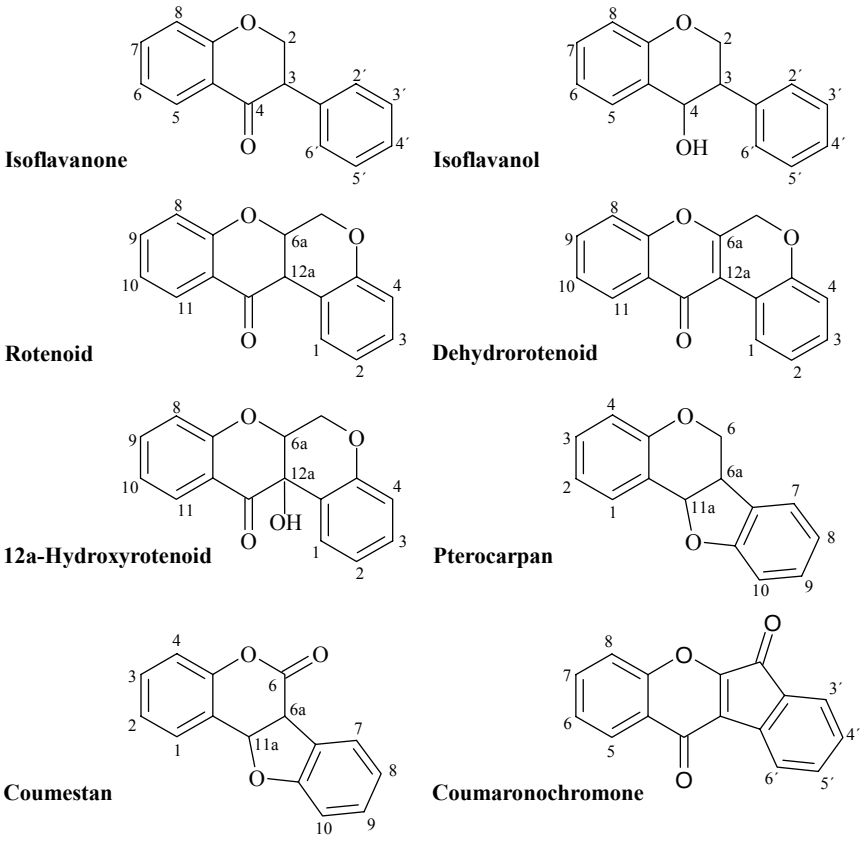


Fig. 2: Subclasses of isoflavonoids in non-leguminous families.

Isoflavonoids are known for their antifungal and insecticidal properties, they are considered as phytoalexins and chemosystematic markers too[2].

2.2 PRESENCE AND ROLE OF ISOFLAVONOIDS IN THE PLANT KINGDOM

Isoflavones are considered to be characteristic metabolites of legumes (Fabaceae family). They play major roles in different types of plant-microbe interactions, are important chemoattractants and signal molecules for symbiotic bacteria, and act together with other flavonoid compounds in legumes.

Stimulation of isoflavonoid biosynthesis in plants is a common response to an attack of many infecting organisms irrespective of whether they are bacteria, fungi, viruses or nematodes. Isoflavones occur even in response to abiotic stressors, such as heavy metal ions, DNA-intercalating chemicals, antibiotics, herbicides, chloroform, surfactants, ethylene, sulphur dioxide and ozone, freezing, mechanical injury and UV irradiation[3].

Simple isoflavones such as daidzein, genistein, biochanin A, formononetin and glycitein, are regarded generally as precursors of phytoalexins. They play key roles in non-specific plant defense against bacterial and fungal pathogens[4,5]. Daidzein inhibits the growth of fungi *Fusarium culmorum*, while glycitein and formononetin can reduce mycelial development in *Aspergillus ochraceus*[6]. VanEtten[7] found that formononetin was an efficient inhibitor of mycelial growth in *Aphanomyces euteiches* and *Fusarium solani*. Wang[8] reported the activity of isoflavones causing the resistance of pasture legumes to the destructive redlegged earth mite, *Halotydeus destructor*. Daidzein, genistein and coumestrol serve as signal molecules during the establishment of the symbioses between soybean and *Bradyrhizobium*, and between *Phaseolus* and *Rhizobium*. These three isoflavonoids specifically induce the transcription of nodulation genes in *Bradyrhizobium japonicum*[9] and *Rhizobium leguminosarum*[10].

Although the occurance of isoflavonoids is characteristic for legumes, the presence of these compounds has been reported in several other families. The spectrum of isoflavonoids producing taxa includes the representatives of four classes of multicellular plants, namely the Bryopsida, the Pinopsida, the Magnoliopsida and the Liliopsida. Reynaud *et al*.[2] summarized 164 compounds in altogether 31 angiosperm families, 3 gymnosperm families and one Bryophyte. One year later our group has updated this review by adding the literature evidence about 17 angiosperm families (**Attachment 1**). Most recently, one new isoflavane and pterocarpane have been found in roots of *Hildegardia barteri* (Sterculiaceae)[11]. The latest number of currently known isoflavone-producing families has reached 59[12].

2.3 BIOSYNTHESIS OF ISOFLAVONOIDS

Isoflavonoids originate from a flavanone intermediate. As a first step of the isoflavonoid pathway flavanones naringenin (5, 7, 4´-trihydroxyflavanone) or liquiritigenin (7, 4´- dihydroxyflavanone) undergo migration of the B-ring from the position 2 to the position 3 into the isoflavanones (**Fig. 3**). This reaction is accompanied by the hydroxylation at the position 2 and requires NADHP and molecular oxygen. The resulting 2-hydroxyisoflavanones are unstable and dehydrate either spontaneously or in enzyme-catalyzed reactions to the corresponding isoflavones, i. e. genistein and daidzein, respectively[13]. The enzyme responsible for this unique reaction is 2-hydroxyisoflavanone synthase, most often referred to isoflavone synthase (IFS) in literature.

The gene encoding the IFS enzyme has been identified from a variety of legumes, e.g. *Pisum sativum, Trifolium pratense, Trifolium repens, Vicia villosa, Vigna radiata, Medicago sativa, Lens culinaris, Cicer arietinum, Lupinus albus*[14], *Glycirrhiza echinata*[15], *Glycine max*[16] and in one non-leguminous species, i.e. *Beta vulgaris* (Chenopodiace)[14].

Jung[14] identified and characterized two IFS genes encoding functional proteins from soybean (IFS1 and IFS2). These two genes are mutually homologous to the high level (92.5% at the nucleotide level and 96.7% at the amino acid level). Both enzymes were shown to convert naringenin and liquiritigenin to isoflavones in yeast microsome assays, though at different efficiencies and both enzymes belong to the CYP93C subfamily of cytochrome P450.

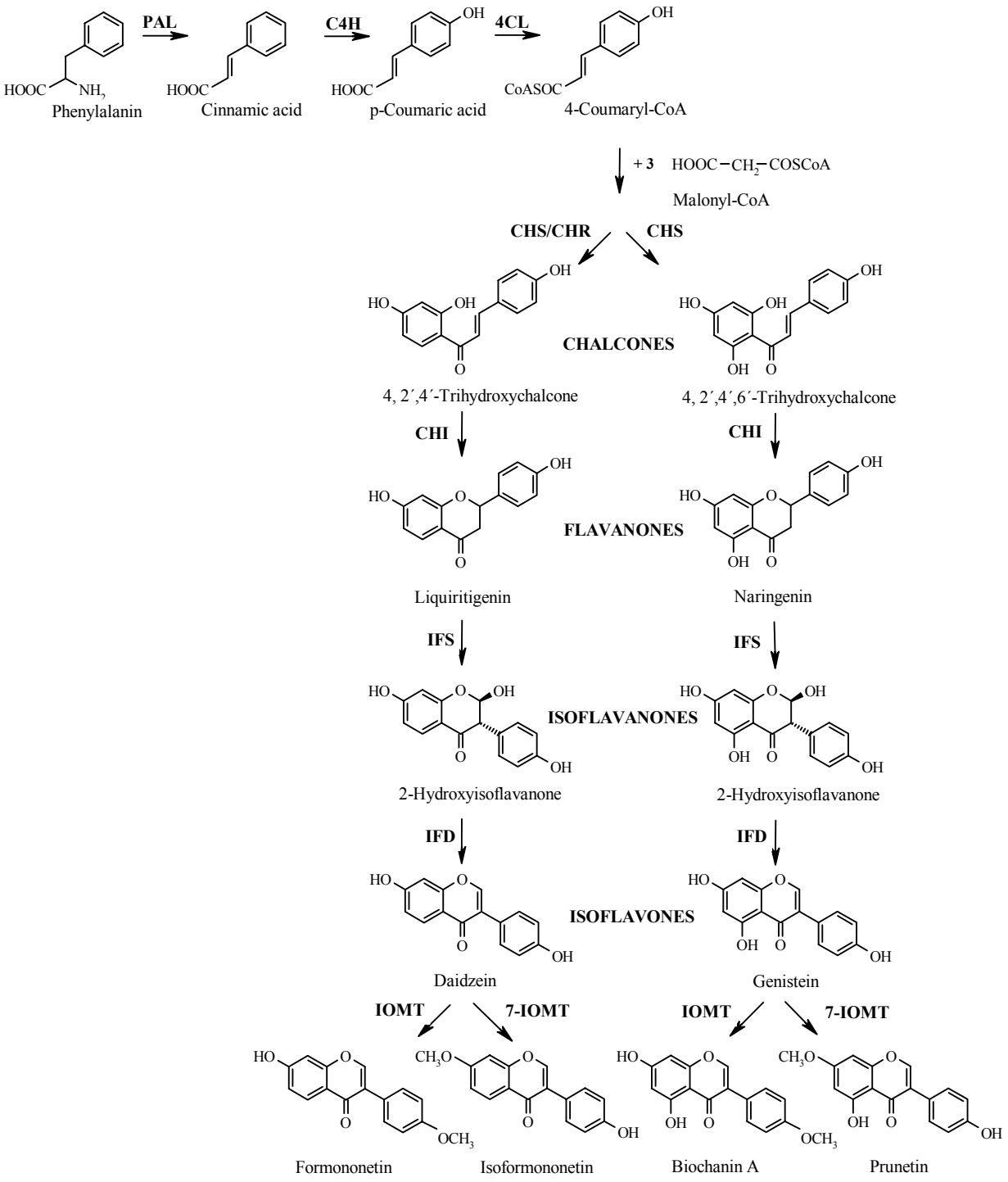


Fig. 3: Simplified diagram of the phenylpropanoid pathways showing intermediates and enzymes involved in isoflavone synthesis. **CHS**-chalcone synthase, **C4H**-cinnamate-4-hydrolase, **CHI**-chalcone isomerase, **CHR**-chalcone reductase, **4CL**-4-coumaroyl-CoA-ligase, **IFD**-isoflavone dehydrogenase, **IOMT**-isoflavone-*O*-methyltransferase, **IFS**-isoflavone synthase, **PAL**-phenylalaninammonia lyase.

2.4 ISOFLAVONE SYNTHASE, CYP ISOENZYMES AND CYTOCHROME P450

As mentioned above, isoflavone synthases fall into the most structurally diverse and functionally versatile superfamily of proteins, Cytochrome P450 monoxygenases. P450s are hemecontaining mixed-function oxidases and these enzymes are critical in many metabolic pathways in plants including the phenylpropanoid, terpenoid and alkaloid pathways. They catalyze hydroxylations of aliphatic and aromatic hydrocarbons. Other P450s of the flavonoid metabolism include methylene-dioxy bridge forming enzyme or cyclases producing glyceollins. In addition, P450s are involved in the detoxification of xenobiotic chemicals such as herbicides. P450s contain approximately 500 amino acids and have a molecular weight of approximately 55 kDa. P450s were found in all kingdoms but the largest number of P450 genes were discovered in plants. In the past years, considerable progress has been made in identifying P450s that are involved in isoflavonoid, glucosinolate and auxin biosynthesis, as well as homeostasis[17].

Consequently, P450 sequences form a large multigene superfamily that is divided into clans, families and subfamilies. All CYP isoenzymes in the same family have at least 40% structural similarity, and these in the same subfamily have at least 60% structural similarity.

The mechanism of the isoflavonoid biosynthesis was studied mainly with soybean cell cultures, and P450-dependent rearrangement of flavanone to isoflavone via 2-hydroxyisoflavone was demonstrated in the microsome preparations from elicitor-challenged cultures which produce pterocarpan-type isoflavonoid phytoalexins, glyceollins. Cell cultures of *Pueraria lobata* (Fabaceae) were also employed for the studies of the IFS, and experiments using $^{18}O_2$ demonstrated the incorporation of the isotope into the 2-hydroxyl group of 2-hydroxyisoflavone, firmly establishing the P450-dependent nature of the aryl migration[18]. A partial purification of the *Pueraria* enzyme has been performed by Hakamatsuka *et al*.[19]. Akashi *et al*.[15] obtained a full-length P450 cDNA, CYP Ge-8 (CYP93C2), from the cDNA library of elicited *Glycyrrhiza echinata* (Fabaceae) cells. When the flavanones liquiritigenin and naringenin were incubated with the recombinant yeast microsome expressing CYP93C2, major products emerged and were readily converted to the isoflavones daidzein and genistein by acid treatment. The aryl migration is an unusual reaction for P450. Both CYP93C and CYP93B employ (2*S*)-flavanone for the substrate and presumably abstract the hydrogen either at the C-2 (CYP93B) or the C-3 position (CYP93C) in the initial stage of the reaction. Then, in the CYP93C reaction, the side phenyl chain (B-ring) migrates to the C-3 position, and a hydroxyl group is introduced at C-2 in order to produce 2-hydroxyisoflavanone. The presence of an *in vitro* byproduct, 3-hydroxyflavanone,

in the recombinant microsomes supports this idea[20]. It has been demonstrated that the expression of soybean IFS in non-legume plants *Arabidopsis thaliana*[13], *Nicotiana tabacum* and *Zea mays*[21] results in the production of genistein and in maize daidzein too. However, the accumulation of genistein in both monocot and dicot transgenic tissues was closely related to the activity of phenylpropanoid pathway. Isoflavones were produced at significant levels only in tissues where the phenypropanoid pathway activity was elevated, such as in UV-treated tissues and in tissues where the ectopic expression of a heterologous transcription factor was used to activate genes of the phenylpropanoid pathway. Thus, when this pathway was activated, IFS was able to capture a small portion of its substrate, naringenin, from endogenous pools and convert it to genistein[22].

2.5 EFFECTS ON MAMMALIAN ORGANISMS

The biological effects of isoflavonoids on vertebrates were for the first time recognized because of "clover disease" in australian sheep in the 1940s. The sheep whose diet predominantly consisted of subterranean clover (*Trifolium subterraneum* L., Fabaceae) suffered from a reproductive disorder which reduced lambing rates and involved abnormal lactation, changes in sex organs, permanent infertility, prolapsed uterus and maternal dystocia[23]. These findings led to the investigation of estrogenic activity of isoflavonoids.

2.5.1 *Estrogenic effect*

Isoflavonoids are structurally similar to mammalian endogenous estrogens and may act as estrogen agonists or antagonists[24,25], depending on the tissue, estrogen receptors and concentration of circulating endogenous estrogens[26].

Isoflavonoids, in general, were found to be more potent estrogens than other flavonoids, but compared to estradiol estrogenic effect of isoflavones are weak[24, 27,28].

2.5.2 *Antioxidant effect*

The polyphenolic nature of isoflavones gives them the ability to act as antioxidants, and thus to inhibit or delay an oxidation of other molecules by preventing the initiation or the propagation of oxidizing chain reactions. The antioxidant activity is a fundamental property underlying a wide range of biological effects, including: antibacterial, antiviral, antiinflammatory, antiallergic, antithrombotic, antimutagenic, anticarcinogenic, antiaging, and vasodilatory effects[27].

The antioxidant activity of phytoestrogens has been reported so far *in vitro* as well as *in vivo*[29], and may help to lower the risk of cancer by protecting cells, for example against lipid peroxidation[30]. Genistein has been shown *in vivo* to increase the activity of antioxidant enzymes in mice: catalase, superoxide dismutase, glutathione peroxidase, and glutathione reductase[29,31].

2.5.3 *Interaction with enzymes*

Isoflavonoids may act different roles in biosynthesis and metabolism of steroids and fatty acids, the serum steroid binding proteins, the intracellular and transmembrane transfer of hormones to a membrane and nuclear receptors[27].

Protein tyrosine kinases (PTKs) are enzymes that catalyze phosphorylation of their own tyrosine residues, as well as those in other proteins, such as factors involved in cell

signal transduction and proliferation. Isoflavonoids, especially genistein, have been shown to inhibit cellular PTKs[32,33,34].

DNA topoisomerases are enzymes that catalyze topological changes in DNA and are required for DNA replication. Several studies have shown that genistein inhibits both topoisomerase I and II activity[35,36,37].

Aromatases are the enzymes that convert androgen to estrogen; therefore, these plant chemicals are thought to be capable of modifying the estrogen level in women. Because estrogens have a major effect on the breast cancer progression, an abnormal expression of aromatase in breast cancer cells may have a significant influence in breast tumor maintenance and growth in patients. Aromatase-inhibitor therapy is a second-line treatment for those who fail anti-estrogen therapy. Phytoestrogens such as isoflavones have been found to be competitive inhibitors of aromatase with respect to the substrate, suggesting that these compounds probably bind to the active site of the enzyme[38].

Several *in vitro* studies have demonstrated that phytoestrogens inhibit human 17-ß-hydroxysteroid dehydrogenase[39], 3-ß-hydroxysteroid dehydrogenase[40,41] and 5-α-reductase[42,43]. They also affect the activities of cyclooxygenase[44,45], lipooxygenase and cholesterol 7-α-hydroxylase[27], an enzyme implicated in the formation of primary biliary acids from cholesterol in the colon.

Daidzein, genistein, formononetin and biochanin A display a strong inhibition of human mitochondrial alcoholdehydrogenase; glucosides daidzin, genistin and puerarin (daidzein-8-C-glucoside) inhibit aldehyddehydrogenase[46]. Traditional Chinese medicine takes an advantage of this property in the alcoholism therapy[47,48].

2.6 HUMAN HEALTH AND PHYTOESTROGENS

The *in vitro* and *in vivo* laboratory experiments show a beneficial influence of isoflavonoids on human health[49], on bone metabolism, parameters of lipid and cholesterol metabolism, estrogen related parameters and vaginal histology.

2.6.1 *Breast and prostate cancer*

Adlercreutz[50,51,52] has reported the association between Western diets and "Western" diseases, such as breast and prostate cancer, colon cancer, and coronary heart disease. The link between some of these diseases and a lack of phytoestrogens in the Western diet (compared with an abundance of phytoestrogens in Asian diets) is well explored.

For example, Asian women who have low rates of breast cancer consume 30-50 times more soy products than do American women[53].

In several studies, a dietary intake and sources of isoflavones were studied in a Japanese population. Their daily total isoflavone intake *per capita* appears to be 20-50 mg. The four major soy products (tofu, miso, natto, and soybean paste) account for about 90% of the Japanese population's intake of daidzein and genistein. In a comparative study, it was discovered that average values of individual isoflavonoid in blood plasma were 7-110 times higher in Japanese men than in Finnish men[54].

Tofu consumption has been associated with a reduced risk of prostate cancer in Japanese men[55,56]. Japanese men, who consumed tofu more than five times a week, had half the risk of prostate cancer, compared with those who consumed tofu less than once a week. Furthermore, Asian people who have immigrated to Western countries and who generally adopt the dietary habits of the host country are at an increased risk of breast and other hormone-dependent cancers compared to those in their original countries[24,57].

2.6.2 *Symptoms of menopause*

After menopause, estrogen levels drop down dramatically, accompanied by menopause-related symptoms, e.g. osteoporosis, hot flushes, changes in vaginal histology and increased risk of cardiovascular diseases. In order to prevent menopause related complications, considerable number of women uses estrogen substitutes as a hormonereplacing therapy (HRT). Nevertheless, HRT appears double-edged, as it increases the risk of breast cancer[25,58,59].

The major symptoms of menopause have been assumed to occur universally. However, some menopausal experiences (e.g. hot flushes) vary from culture to culture. Only about 4 % of postmenopausal Japanese women use estrogen replacement therapy, compared

with about 30 % of postmenopausal women in United States[36,60]. Moreover, the incidence of hot flushes, hormone-related cancers and osteoporosis in Japan is reported to be one of the lowest in the world[61]. Although the difference in symptoms could be due to many factors, Adlercreutz[62] has suggested that the weakly estrogenic actions of a phytoestrogen-rich diet may explain the reduced frequency of menopausal symptoms in Japanese women.

Due to controversial opinions on HRTs, alternative therapies have been sought, such as phytoestrogens and SERMs (Selective Estrogen Receptor Modulators). Many studies have been focused on the determination of the efficacy of isoflavonoid phytoestrogens for menopausal symptoms elimination.

2.6.3 *Osteoporosis*

Osteoporosis is characterized by a loss of bone mass usually associated with aging, due to an increased bone resorption and reduced bone formation. As the one of menopausal symptoms, osteoporosis has been related to decrease of gonadal steroid production.

Estrogen replacement therapy has been shown to reduce the risk of osteoporosis in postmenopausal women[36]. Although the mechanisms are not entirely clear, it has been proposed that estrogen **A)** reduces a sensitivity of bone tissue to the resorptive effects of parathyroid hormone, **B)** blocks the release of interleukin-1, a potent bone resorption agent, or **C**) directly modulates an osteoblast activity. Hence, similar to estrogen and synthetic SERMs, soy isoflavones have been suggested to bring the beneficial effects of estrogen without its side effects[63,64].

Data from animal studies suggest that isoflavones could prevent bone loss that occurs as a result of estrogen deficiency[24,25]. For instance, two weeks of a genistin-rich treatment (1.0 mg/ day) in lactating ovariectomized rats was effective in maintaining bone tissue in comparison with ovariectomized control animals[65]. Furthermore, in the same report, genistin stimulated alkaline phosphatase activity of an osteoblast-like cell line, suggesting a positive effect on bone formation. Results from Yamaguchi's group[66] have shown that daidzein and genistein stimulated osteoblastic bone formation, and inhibited osteoclastic bone resorption. Most of the clinical studies suggest that phytoestrogens are somewhat effective in maintaining bone mineral density in postmenopausal women as well[67].

Soy intake has also been suggested to protect against bone loss by mechanisms independent of its estrogenic effects. First, soy foods are a good source of calcium. Miso and tempeh contain 92 and 77 mg of calcium per 0.5 cup serving, respectively. Tofu coagulated from soymilk by calcium salt has about 406 mg of calcium per quarter block. Second, a high soy-protein diet may prevent the urinary calcium loss compared with a high animal-protein diet[36].

2.6.4 *Cardiovascular diseases*

The leading cause of death in women in industrialized countries is coronary heart disease (CHD). In menopause the risk of CHD greatly increases and it is assumed that this is due to the deficiency of estrogen[68]. That phytoestrogens may be cardioprotective is supported by the low rates of CHD among Asian people compared with those of Westerners[53].

In a study of Crouse[69], naturally occurring isoflavones isolated with soy protein reduced the plasma concentrations of total and LDL cholesterol without affecting concentrations of triglycerides or cholesterol in mildly hypercholesterolemic volunteers. Teede[70] and Nestel[71,72] have reported a significant reduction in arterial stiffness in the case of people consuming purified isoflavones from soy and red clover. Vasodilatory effects of isoflavones or their metabolites on the microcirculation, suppression of adhesion molecules, antioxidant properties and inhibition of experimental atherosclerosis, may provide opportunities for pharmacological intervention[73,74].

2.7 DIETARY SOURCES OF ISOFLAVONES

Isoflavones enjoy a widespread distribution in the Leguminosae family, including such prominent high-content representatives as soybean (*Glycine max*), clover (*Trifolium* spp.), mung bean (*Vigna radiata*), alfalfa (*Medicago sativa*), peanut (*Arachis hypogaea* L.), licorice (*Glycyrrhiza* spp.), kudzu root (*Pueraria lobata*), chickpea (*Cicer arietinum*.)[75,76,77].

Soy is probably the most important dietary source of isoflavonoids. Raw soybeans contain 1.2-4.2 mg/g dry weight of isoflavones, while high protein soy ingredients like soy flour contain 1.1-1.4 mg/g dry weight[36]. The principal isoflavones found in soy proteins and soy foods are daidzein, genistein, and glycitein. Each of them is found in the unconjugated form (aglycone) and conjugated forms: glucoside (daidzin, genistin, and glycitin), acetylglucoside, and malonylglucoside[78].

Glycine max

Trifolium pratense

2.8 METABOLISM AND ABSORPTION IN HUMAN ORGANISM

When consumed by humans, phytoestrogens undergo several metabolic transformations. The conjugated forms of isoflavonoids are probably hydrolysed in part by gastric acid and they also undergo enzymatic hydrolysis by intestinal microflora and β-glucosidases in food. Intestinal bacterial microflora cleaves the sugar moieties and releases the biologically active aglycones, which can be further biotransformed into specific metabolites. Biochanin A and formononetin can be converted by 4′-*O*-demethylation to the more potent phytoestrogens genistein and daidzein[79]. These compounds may be absorbed or further metabolized in the distal intestine. Daidzein is metabolized to dihydrodaidzein, which is further metabolized to both equol and *O*-desmethylangolesin. Genistein metabolises to dihydrogenistein and then to 6′-hydroxy-*O*-desmethylangolesin and hormonally inert *p*-ethylphenol[24,36,80] (**Fig. 4**).

After absorption in the small intestine, isoflavonoids are transported via portal venous system to liver, where the isoflavonoids and their metabolites are efficiently conjugated with glucuronic acid and sulfate by hepatic phase II enzymes (UDP-glucuronosyltransferases and sulfotransferases). Isoflavone conjugate profiles in human urine suggest that glucuronic acid is the primary moiety[81]. Like endogenous estrogens, isoflavones are excreted through both urine and bile and undergo entherohepatic circulation. After excretion into bile, conjugated isoflavonoids can be deconjugated once again by intestinal bacteria. Deconjugation may promote reabsorption, further metabolism, and degradation in the lower intestine. Human metabolism and excretion of isoflavonoids after soy consumption show considerable individual variation[82, 83, 84].

Although *O*-demethylation of isoflavones has been attributed to metabolism by intestinal microflora, the study by Tolleson[79] demonstrates that human hepatic enzymes can perform the same transformation and may play a key role in the conversion of 4′-*O*-methylated isoflavones to more potent phytoestrogens.

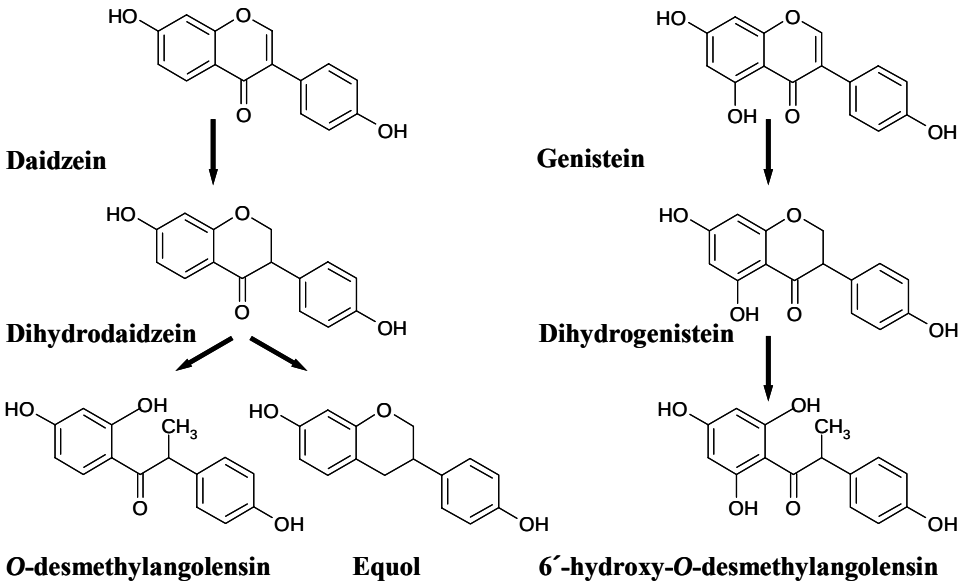


Fig. 4: Metabolic products of daidzein and genistein.

3 METHODS OF EXTRACTION AND IDENTIFICATION OF ISOFLAVONOIDS

3.1 EXTRACTION METHODS

Isoflavones are found in biological material in different forms. As mentioned above, they occur in plants and in many food products as glycosidic conjugates or free aglycones; in biological fluids (blood, urine) they are found as aglycones, β-glucuronides and sulphate esters[85].

Isolation and purification of an analyte of interest from the plant, food or physiological matrices is very often a tricky problem. Matrices could be simplified by carrying out acid or enzymatic hydrolysis[86,87]. Commonly used methods for isolation of isoflavones include a simple maceration sample in a convenient organic solvent, Soxhlet extraction[88], ultrasonic extraction[89], solid phase extraction (SPE)[90], supercritical fluid extraction (SFE)[91,92]. Frequently used polar liquid solvents for extraction of isoflavonoids from homogenized samples are aqueous solutions of methanol, ethanol and acetonitrile.

3.2 ANALYTICAL METHODS

The interest in analytical methods for plant metabolites has risen sharply in the past 20 years. Common techniques used for the determination of isoflavones are chromatographic and electromigration methods.

Thin-layer chromatography (TLC) is the simplest and inexpensive method and it is used for rough qualitative analysis of compounds[93].

Gas chromatography (GC) is valuable for biological samples (such as urine, faeces and blood) containing low concentrations of phytoestrogens. A disadvantage of this method is needful derivatization of isoflavones prior to the GC analysis. Increase of the volatility of an analyte (especially for those possessing a hydroxyl, carboxyl or amino group) involves the production of trimethylsilyl ether derivatives with *N*,*O*-bis-(methylsilyl)-trifluoroacetamide containing 1% trimethylchlorosilane. Other silylating reagents, such as hexamethylsilazane-trimethylchlorosilane have also been reported[94,95].

High performance liquid chromatography (HPLC) coupled with an electrochemical, UV-Vis diode array detector, fluorescence detector, electrochemical detector and/or mass spectrometric detector (MS) is one of the widely used methods for the determination of

phytochemical substances. Separation by the HPLC obviates the need for derivatization of isoflavonoids.

Reversed-phase HPLC/UV-Vis diod array analysis of the most common isoflavonoids (daidzein, genistein, formononetin, biochanin A and coumestrol), which allowed quantification of phytoestrogen levels in legumes, has been reported[96,97]. Analyses are very often performed by reversed-phase C_8 and C_{18} bonded silica using isocratic or gradient separation system. A mobile phase for isocratic or gradient system often consists of a mixture of methanol, acetonitrile, *n*-propanol, tetrahydrofuran or ethanol with aqueous buffer (acetic acid, formic acid, phosphoric acid, trifluoroacetic acid, ammonium acetate, and ammonium formate or phosphate buffer). Individual analytes are predominantly detected by UV at 254-280 nm. The identification of analytes is based on the comparison of retention times or on adition of an internal standard. The comparison of retention times and UV-Vis-DAD absorbtion spectra of standards from a library enhances the information value. UV-Vis spectra characterization is useful tool for the identification of individual isoflavones, but this determination efficiency is limited. On the other hand, many isoflavones have similar spectra (e.g.: daidzein and formononetin or genistein and biochanin). Another weakness of these detection methods is their non-specifity, leading to the possibility of sample matrix interference. Therefore the arrangement of the HPLC and the MS detector become very popular[98].

Barnes *et al.*[99] have described for the first time the application of HPLC-mass spectrometry with ***heated nebuliser-atmospheric pressure chemical ionization*** (HN-APCI) and IonSpray interface to the analysis of isoflavones. This combination of the HPLC-APCI allows an efficient separation and identification of isoflavones with greater efficiency than the HPLC with UV detection alone[100].

The HPLC method using the ***coulometric electrode array detection*** (CoulArray detector) allows a determination of very low (ng-pg) amount of samples in complex matrices without a previous preparation[101].

Capillary electrophoresis (CE) is a powerful technique that enables rapid, high-resolution separation (10^4-10^6 theoretical plates), while requiring less sample than the traditional HPLC. The technique is applicable to a wide range of analytes present in a buffered aqueous solution as charged species. The CE offers shorter analysis times and higher efficiences too. The separation is based on differences between charge-to-mass ratios. Analytes migrate into discrete zones at different velocities. Anions and cations are separated by the CE due to electrophoretic migration and the electro-osmotic flow, while neutral species co-eluate with electro-osmotic flow[102].

Micellar electrophoretic capillary chromatography (MEKC) is a modified capillary electrophoresis, which requires the addition of a surfactant such as sodium dodecyl sulphate, at a concentration above its critical micelle concentration to the running buffer. Surfactants are molecules that contain both polar and lipophilic head groups. The hydrophilic polar head groups point outward, whereas the hydrophobic non-polar tails point toward the center of the micelles. During the MEKC separation, non-polar portions are free and migrate at the electro-osmotic flow velocity. The distribution coefficient between the micellar and non-micellar phase greatly influences the migration velocity of the analytes[103].

Matrix-assisted laser desorption/ionization-time of flight-mass spectroscopy (MALDI-TOF-MS) is a soft ionization technique. This method allows the analysis of biomolecules (biopolymers such as proteins, peptides and sugars) and large organic molecules (such as polymers, dendrimers and other macromolecules), which tend to be fragile and fragmented when ionized by other conventional ionization methods. It resembles the most to the electrospray ionization both in relative softness and the ion-type production (although it causes much fewer multiply charged ions). In this technique, samples are co-crystallized with a matrix, usually an organic acid, which absorbs energy from the laser pulses and allows a soft desorption ionization of the sample. The sample ions are then analyzed with a TOF mass analyzer. MALDI-TOF-MS has advantages over other methods, including the speed of analysis, sensitivity, tolerance toward contaminants, and the ability to analyze complex samples. Wang and Spornes[104] presented the first example of using MALDI-TOF-MS in order to identify isoflavones in soy samples. This work demonstrates that MALDI-TOF-MS can produce isoflavone profiles of biological samples and serve as a powerful tool to identify and study processing-induced changes of isoflavones in soy products.

Immunoassays

Immunochemical methods offer the advantages of specificity, a high rate of sample throughput and comparatively low cost. Application of immunoassays to human serum and tissues, where sample volumes are necessarily small, allows studies of phytoestrogen absorption and metabolism. Most of recently published immunoassays employed polyclonal rabbit antibodies, raised against protein conjugates with an isoflavone[105,106,107]. Based on the nature of detection, three types of immunoassays for isoflavonoids have been developed: the radioimmunoassay (RIA), the enzyme-linked immunosorbent assay (ELISA), and the time-resolved fluoroimmunoassay (TR-FIA).

In **RIA** methods, one of immunoreagents is labeled with a radionuclide. The most used radionuclides are ^{125}I and ^{3}H. The ^{125}I-isoflavone-tyrosine-methylester conjugate and ^{3}H-isoflavone-leucine conjugate were reported as competitive radioligands.

RIA analysis of formononetin in blood plasma and rumen fluid of wethers fed on red clover was reported by Wang[108]. Other RIA modifications for analysis of daidzein and genistein, using ^{125}I-labeled tracer, were published by Lapčík[109,110]. These methods were used for determination of isoflavones in plant extracts, beer, human plasma and urine.

Creeke[111] reported indirect competitive **ELISA** for determination of daidzein and equol in plasma, based on polyclonal antibodies against hapten conjugates with keyhole limpet hemocyanin. Other indirect competitive ELISAs were developed, by use of polyclonal antibodies raised against daidzein, genistein, formononetin[112] and equol[113] coupled with bovine serum albumin (BSA). Conjugates with thyreoglobulin or BSA were used for coating of microtitration plates. These methods were used for determination of isoflavones in soy food, urine and plasma[114,115].

TR-FIA combines the advantages of reagent stability and lack of radiation over RIA. This technique also provides a comparable or even better sensitivity and assay range in comparison with conventional ELISA and FIA methods. Analytes are labelled with chelates of lanthanoids, particularly europium, samarium, terbium.

The choice of analytical method depends on the required sensitivity, on the complexity of the biological matrix (as it relates to the time spent working up the samples prior to analysis), on the chromatographic resolution required, and on the expense. The attributes of each technique are summarized in **Table I**.

Table I: Comparison of analytical methods for analysis of phytoestrogens[116].

Technique	Sensitivity	Specificity	Pros	Cons
GC–MS	50 fmol	High	High resolution Good for unknowns	Complex work up Difficult chemistry
HPLC –UV (and DAD)	2 pmol	Moderate better with DAD	Good for soy food and conjugates	Low sensitivity Less specific
–Fluorescence	200 fmol	Good	Sensitive	Limited to fluorescent analytes
–ED (and array)	20 fmol	Better with detection array	Suitable for biological samples	Cannot determine novel compounds
–MS	1–500 fmol	High	Ease of use and sensitive	Limited chromatographic resolution
CE –UV (DAD),	50 fmol	Moderate better with DAD	High separation resolution Excellence mass sensitivity	Limited injected sample volume Poor concentration sensitivity
–Fluorescence	1–5 fmol	Moderate	Sensitive	Limited fluorescent analytes
–ED	1–2 fmol	Moderate	Sensitive	Limited specificity
–MS	100 amol	High	Sensitive	Difficult interface Low resolution
UVand IR spectroscopy	NA	Fair	High throughput	Lack of specificity
MALDI-TOF-MS	100 fmol	High	High throughput	Lack of quantitation
Immunoassay	1–100 fmol	Good	High throughput	Cross reactivity

4 RUTACEAE FAMILY

The Rutaceae is a large family of plants of the order Sapindales that includes 150 genera with about 900 species. Many of them are of significant economic importance as fruit bearing, medicinal and ornamental species. They range in form and size, from herbs to shrubs and small trees. The most economically important genus in the family is *Citrus*, which includes the orange, lemon, lime, grapefruit, etc.

Ruta (Rue) is 20-60 cm tall strongly scented evergreen subshrub, native to the Mediterranean region, Macaronesia and southwest Asia. Different authors distinguish between 8-40 species in the genus including *Ruta angustifolia* - Egyptian rue, *R.chalepensis* - Fringed rue, *R. corsica* - Corsican rue, *R. graveolens* - Common rue, *R. montana* - Mountain rue.

The best known species is the yellow flowering Common rue. Twigs of rue were often used as a kind of bookmark in ancient times due to its strong scent and natural bug repellent properties. Formerly bitter taste rue was also used extensively in Middle Eastern cuisine. The rue plant was an ancient herbal remedy for faintness, cramp, hysteria and womb diseases, but it has been also used as an abortifacient and emmenagogue drug[117] and as the "antimagic" herb for centuries. It was considered a reliable defense against witches. Rue has sometimes been called "herb-of-grace" in literary contexts. It is one of the flowers distributed by the mad Ophelia in William Shakespeare's Hamlet (act IV. scene V.):

“There's fennel for you, and columbines: there's rue
for you; and here's some for me: we may call it
herb-grace o' Sundays: O you must wear your rue with
a difference. There's a daisy: I would give you
some violets, but they withered all when my father
died: they say he made a good end,--“

Juice from the fresh *Ruta* plant contains furocoumarins (xanthotoxin, bergapten), which are responsible for photosensibilization of human skin[118]. They may cause severe dermatitis to sensitive people with water blisters and blotchy skin. Ingesting large amounts of rue can cause violent stomach pain, vomiting, and convulsions[119].

Rue is considered a national plant of Lithuania and it is the most frequently referred herb in Lithuanian folk songs, as an attribute of young girls, associated with virginity. A bride traditionally wears a little crown made of rue, which is a symbol of maidenhood.

During the wedding, where the crown is burned, it is to symbolize the loss of careless childhood and the entrance into the world of adulthood.

An attractive detail about the rue plant is that rue leaf was the model for the suit of clubs in playing cards[120].

Ruta graveolens

Ruta corsica

Citrus is a genus of trees and shrubs, originating in tropical and subtropical Southeast Asia. It is undoubtedly the most important genus in the whole family with 60 known species, of which most are cultivated, e.g. lemon (*C. limon*), citron (*C. medica*), edible or sweet orange (*C. sinensis*), Seville orange (*C. aurantium*), mandarins (*C. reticulata*), limes (*C. aurantifolia*), pomelo (*C. grandis*) and grapefruit (*C. paradisi*).

Citrus limon

Citrus sinensis

They are rich in vitamin C, flavonoids, acids and volatile oils. An infusion from the leaves and the flowers is used in the treatment of stomach problems, sluggish digestion, etc. Fruits are antiemetic, antitussive, carminative, diaphoretic, digestive and expectorant[121]. The juice contains a high quantity of citric acid giving them their characteristic sharp sour flavour. Citrus fruits are notable for their fragrance, partly due to flavonoids and limonoids contained in the rind. Essential oils obtained from the peel; petals and leaves are used as food flavouring and in perfumery, and are also used in aromatherapy. They have antifungal and antibacterial properties.

Poncirus trifoliata (Trifoliate Orange) is a genus closely related to *Citrus*. It is a Chinese and Korean native plant, and is also known as the Chinese Bitter Orange. Because of the relative hardiness is used as a rootstock for *Citrus* genus. *Poncirus trifoliata*[122] has many traditional uses for medicinal remedies. In Korea, the dried fruits were used as a digestive and to allay eczema and fever. In China, the fruits were also employed as having antiemetic, laxative, antispasmodic, stimulant, and diuretic properties. The fruits were also used as a medication for rheumatism. A decoction of thorns from the tree was good for toothaches, and of the bark as a cold remedy.

The genus ***Murraya*** comprises several species, including the Curry Tree. The Curry Tree or Curry-leaf Tree (*Murraya koenigii*; syn. *Bergera koenigii*) is a tropical to sub-tropical tree, which is native to India. Its leaves are highly aromatic and are used as an herb.

Phellodendron or Cork-tree is a genus of deciduous tree, native to east and northeast Asia. The name refers to the thick and corky bark of some species in the genus. The bark in some species is thick, resembling that of the Cork Oak, but is not thick enough for commercial cork production. It has been used to produce a yellow dye. One species, Amur cork tree (*Phellodendron amurense*) is one of the 50 fundamental herbs used in traditional Chinese medicine. Recently, *Phellodendron* has also attracted the attention of the pharmaceutical community because of the phytochemicals it produces. Berberine[123] has antibacterial and anti-fungal properties. Jatrorrhizine[124] may be anti-mutagenic. Phellodendrine is promising as an immune suppressant. Palmatine[125] may be a vasodilator.

Pilocarpus is a genus comprising about 13 species, native to the neotropics of South America. Various species are important pharmacologically. Many of the species have the common name Jaborandi. Jaborandi is a perfect example of a plant which made the transition from Amazonian indigenous tribal use to folklore use, and then into modern medicine that is based upon natural chemicals found in the plant. An alkaloid called pilocarpine, which was discovered in 19th century, stimulates acetylcholine receptors in lacrimal gland and cause increased secretion of tears[126]. The medicinal properties of pilocarpine, including its ability to stimulate sweating and salivation, as well as to lower intraocular pressure in the eyes, have been recognized for many centuries by the Tupi Indian tribe of northern Brazil. Pilocarpine was introduced into conventional ophthalmology for the treatment of glaucoma. The mixture of pilocarpine and another natural product, physostigmine, remains to this day one of the mainstay drugs in ophthalmology for the treatment of symptoms of dry mouth from salivary

gland hypofunction caused by radiotherapy for cancer of the head and neck; and the treatment of symptoms of dry mouth in patients with Sjogren's syndrome[127].

Casimiroa edulis is native to the tropical highlands of Central and South America. The fruit was well known to the Aztecs. The leaves, bark and seeds contain the glucoside casimirosine, which lowers blood pressure. In large doses, glucoside acts as a sedative, and alleviates rheumatic pains [128].

Psilopeganum is a monotypic genus, with only a single species: *Psilopeganum sinense*. *P. sinense* is another one of the 50 fundamental herbs from the Rutaceae family used in traditional Chinese medicine.

Ptelea trifoliata (Hoptree) is a deciduous shrub or a small tree with a broad crown. By several Native American it has been used as herbal medicine for different ailments.

The fruit of several species of ***Zanthoxylum*** genus is used to make the spice Sichuan Pepper. *Z. macrophyllum* tree is sometimes called "toothache tree" or "tingle tongue" because of the numbness of the mouth, teeth and tongue that is induced by chewing its leaves. The leaves of *Zanthoxylum simulans* are pounded and applied to cuts and wounds as a styptic and they also speed the healing process. The external application of the leaves is also used in the treatment of scabies. The leaf juice is dropped into wounds in order to kill germs. A decoction of the root is used in the treatment of malaria. Different tissues of *Z. ailanthoides* have several health benefits, such as myocardium disorder attenuation, bone-injury alleviation, and cold resistance[129].

Aegle marmelos (bael) is a fruit-bearing tree, commonly known as Bel, or Beli fruit, Bengal quince, Stone apple, or Wood apple. The bael fruit possesses important medicinal properties to treat dyspepsia, diarrhoea and dysentery. The root bark extract of this plant has been reported to be beneficial to cure intermittent fever, mental diseases, pericarditis and angina pectoris; the leaves of bael have hypoglycaemic effect[130].

Agathosma is a genus of about 135 species, native to the southern part of Africa. Many of the species are highly aromatic and the name of genus means "good fragrance". The essential oils and extracts of the leaves are used as flavoring for teas and candy. The two main oil compounds of *A. betulina* are isomenthone and disphenol. The leaves have traditionally been steeped in brandy, vinegar, and tea water, and are said to relieve gastrointestinal and urinary tract ailments.

Dictamnus albus is single species of this genus. *D. albus* is known variously as Burning-bush derives from the volatile oils produced by the plant, which can catch fire readily in hot weather.

It leads to comparisons with the burning bush of the Bible, including the suggestion that this is the plant involved there[131]. The root was considered a sure remedy for epilepsies, and other diseases of the head. The plant is known to have emmenagogic properties[132].

5 AIMS OF THE STUDY

The assumption about the presence of the isoflavonoid metabolic pathway throughout the Rutaceae family has been reported for the first time in 2004[133].

The general aim of this study was to verify the occurance of isoflavonoids in the Rutaceae family, to improve techniques of detection of isoflavonoids in plant material and to increase number of study species using combination of semipreparative HPLC, HPLC-MS and nonradioactive immunochemical ELISA methods. Furthermore the aim was to try to describe isoflavonoids pathway in preselected non-leguminous plants, based on literary and experimental data and using molecular biology methods.

6 MATERIAL AND METHODS

6.1 Plant material and chemicals

6.1.1 *Plant material*

Fresh plant material (stalks, leaves, flowering tops) was collected from the plants growing in the subtropical greenhouse of the Czech University of Agriculture in Prague. All Rutaceae species were authenticated by Ladislav Kokoška and the voucher specimens have been deposited in the Institute of Tropical and Subtropical Agriculture Herbarium. Plant material for extraction of DNA was obtained in the Institute of Tropical and Subtropical Agriculture Herbarium or in the Institute of Experimental Botany ASCR, v.v.i.

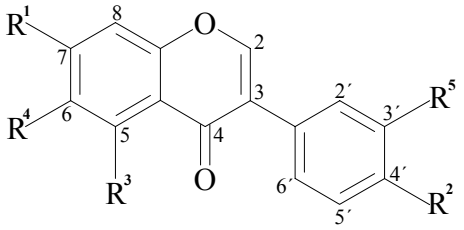
6.1.2 *Chemicals for extraction, HPLC and HPLC-MS*

HPLC-grade acetonitrile was purchased from Merck (Darmstadt, Germany); methanol, ethanol and purified acetic acid from Penta (Chrudim, Czech Republic); formic acid was obtained from Sigma-Aldrich (Buchs SG, Switzerland).

6.1.3 *Standards*

Isoflavones standards (**Table II**) were obtained from following suppliers: 6,7,4´-trihydroxyisoflavone; 7,3´,4´-trihydroxyisoflavone; 5-hydroxy-7,4´-dimethoxyisoflavone (genistein-4,7´-dimethyl ether); glycitin glycitein; genistein; sissotrin; formononetin; daidzein; and 3´-hydroxydaidzein were purchased from Indofine (Somerville, NJ, USA); biochanin A and genistin from Sigma-Aldrich; prunetin from Fluka (Buchs SG, Switzerland); daidzin was a generous gift from Dr. Yasuda (Hokkaido Institute of Pharmaceutical Science, Japan); 5-methoxygenistein was a generous gift from Wei Guang Ma (Faculty of Agriculture, Hokkaido University, Sapporo, Japan)[134] and isoformononetin was prepared by selective methylation of daidzein by Ivan Černý (Institute of Organic Chemistry and Biochemistry, Prague)[135].

Table II: Structure of isoflavones under study.



	R^1	R^2	R^3	R^4	R^5
Daidzin	OGlc*	OH	H	H	H
Genistin	OGlc*	OH	OH	H	H
Daidzein	OH	OH	H	H	H
Sissotrin	OGlc*	OCH_3	OH	H	H
Genistein	OH	OH	OH	H	H
Formononetin	OH	OCH_3	H	H	H
Isoformononetin	OCH_3	OH	H	H	H
Prunetin	OCH_3	OH	OH	H	H
Biochanin A	OH	OCH_3	OH	H	H
6,7,4´-Trihydroxyisoflavone	OH	OH	H	OH	H
7,3´,4´-Trihydroxyisoflavone	OH	OH	H	H	OH
5-Hydroxy-7,4´-dimethoxyisoflavone	OCH_3	OCH_3	OH	H	H
3´-Hydroxydaidzein	OH	OH	H	H	OH
5-Methoxygenistein	OH	OH	OCH_3	H	H
Glycitin	OGlc*	OH	H	OCH_3	H
Glycitein	OH	OH	H	OCH_3	H

*OGlc (*O*-glucoside)

6.1.4 *Immunoreagents*

Haptens

All carboxymethylderivatives of daidzein, genistein and biochanin A were synthesized by Nawaf Al-Maharik at the Department of Chemistry, University of Helsinki[136]. Resulting haptenic analogues were: biochanin A-7-carboxymethylether, genistein-4'--carboxymethylether, genistein-7-carboxymethylether, daidzein-4'-carboxymethylether, daidzein-7-carboxymethyl-ether.

Conjugates

Immunization conjugates of BSA with daidzein (daidzein-4´-BSA and daidzein-7-BSA), genistein (genistein-4´-BSA and genistein-7-BSA) and biochanin A (biochanin A-7-BSA) were prepared by Oldřich Lapčík (ICT, Prague). Conjugates of ovalbumin with the same haptens were prepared by Michaela Vítková[137].

Antibodies

Polyclonal rabbit antisera were raised against daidzein-4´-BSA (Ab No. 3, Ab No. 4) daidzein-7-BSA (Ab No. 24), genistein-4´-BSA (Ab No. 13, Ab No. 14), genistein-7-BSA (Ab No. 23), biochanin A-7-BSA (Ab No. 29).

Other chemicals for ELISA and all information about the synthesis of conjugates, their characterization, and immunisation, were completely described by Vítková[138,139].

6.1.5 *Chemicals for DNA extraction*

Chloroform, isoamyl alcohol, and ethanol: LACH-NER, Neratovice, Czech Republic; isopropanol, NaCl, cetyltriethylammonium bromide (CTAB): Sigma-Aldrich; ethylendiamin tetraacetic acid (EDTA): Serva, Heidelberg, Germany;

2-Amino-2-(hydroxymethyl)-1, 3-propanediol (Tris-BASE): DucheFa, Haarlem, Netherlands or Invisorb Spin Plant Mini Kit (Invitek, Berlin, Germany).

6.1.6 *PCR chemicals and solutions*

The PCR reactions were performed with aliquots of genomic DNA, dNTP (premixed solution containing the sodium salts of dATP, dCTP, dGTP, dTTP, each at concentration of 10 mM in water (Promega, Medison, WI, USA)), specific primers (Invitrogen Corporation-California, USA, GENERI BIOTECH-Hradec Králové, Czech Republic), reaction buffers, Taq DNA polymerases (Chemos-Prague, Czech Republic; Phusion-Biolabs, Ipswich, MA, USA; Top-Bio-Prague, Czech Republic) and $MgCl_2$ (Promega).

Agarose gel electrophoresis

Agarose gel:	1% (w/v) volume agarose gel (Serva, Heidelberg, Germany)
10 x Gel loading Buffer:	0.5% (w/v) orange G (Sigma-Aldrich)
	50% (v/v) glycerol (Sigma-Aldrich)
10 x TAE buffer:	4.84% (w/v) Tris-BASE
	3.72% (w/v) Sodium acetate (trihydrate), (Sigma-Aldrich)
	0.372% (w/v) EDTA

pH was adjusted to 8.2 with glacial acetic acid.

Ethidium bromide (Sigma-Aldrich)

Reference DNA molecular ladder, standard weight 100 bp and 1 Kbp (Fermentas, Glen Burnie, MD, USA)

Media for the Growth of Bacteria

Luria Bertani (LB) media: per liter Bacto-tryptone, Bacto-yeast extract, NaCl
pH adjusted to 7.0 with 1 M NaOH
Autoclaved 15 min at 120°C

LB media was solidified by the addition of 1.5% w/v agar and autoclaved as above.

Other material

Purification gel kit Qiagenkit (Qiagen, Venlo, Netherlands)

pGEM-T Easy Vector (Promega, Medison, WI, USA)

E. coli DH5α cells (JM109, Promega, Medison, WI, USA)

Ampicilin (Serva, Heidelberg, Germany)

X-Gal (5-Bromo-4-chloro-3-indolyl-galactoside, Sigma-Aldrich)

IPTG (Isopropyl-1-thio-ß-D-galactoside, Sigma-Aldrich)

6.2 METHODS AND TECHNICAL EQUIPMENT

6.2.1 *Sample preparation for HPLC and ELISA*

Plant material was frozen immediately after collection and kept at -20°C until lyophilization (FreeZone 1, Labconco, Kansas City, MO, USA). Dry samples were pulverized in an electric mill (Fex IKA A 11, IKA Werke, Staufen, Germany). A sheet of filter paper was processed in the same way as the samples, in order to get a blank.

Powdered material was extracted by maceration with use of 80% ethanol in water as the extracting solvent (20 mL/g) for one week with occasional stirring. Plant extracts (4 mL) were concentrated to dryness with use of a Labconco CentriVap concentrator. The residue was dissolved in 2 mL of mobile phase, filtered through a 0.45 µm teflon syringe filter (Iso-Disc, PTFE-13-4, Supelco, St. Luis, MO, USA) and applied to the semi-preparative column.

6.2.2 *CHROMATOGRAPHIC METHODS*

HPLC Semi-preparative system

The HPLC fractionation of the isoflavones was performed on a Merck C18 reverse phase column (LichroCART 250-10, Purospher STAR RP-18e; 5 µm). The semi-preparative system included a model LC-10AD binary pump, a FRC-10A fraction collector, a SIL-10AD auto injector, a SCL-10A system controller, and a SPD-M10A photodiode array detector (Shimadzu, Kyoto, Japan). UV detection was performed at a wavelength of 254 nm and the injection volume was 500 µL. The mobile phase used for analysis was methanol (solvent A) and 0.5% acetic acid in water (solvent B), both degassed with helium. The linear gradient elution program was: initial concentration 50:50 (A:B), taken to 40% B in 5 min, to 30% B in 20 min, to 20% B in 25 min, to 0% B in 45 min and held for 5 min to wash the column, and finally increased to 50% B in 55 min. The flow rate was 2.5 mL/min and separations were carried out at ambient temperature. 50 fractions of volume 2 mL were collected and evaporated to dryness. Dried samples were reconstituted in phosphate buffer saline-Tween 20 (PBS-Tw) for ELISA.

HPLC Analytical system

Some fractionations were performed on an analytical column-Merck C18 reverse phase, LichroCART 125-4, Purospher STAR RP-18e; 5 µm. Linear gradient elution program was: **A** (0.5% AcOH):**B** (MeOH) - 0′ 60:40, 5′ 48:52, 20′ 30:70, 25′ 0:100, 45′ 0:100, 50′ 60:40, 55′ STOP. The flow rate was 0.8 mL/min. Injection volume was 50 µL, 22 fractions were collected.

HPLC-MS

The HPLC-MS system consisted of a HP 1100 (Hewlett Packard, Waldbronn, Germany) equipped with a vacuum degasser (model G1322A), a binary pump (G1312A), an autosampler (G1313A), a column thermostat (G1316A) and a PAD (G1315A). The system was coupled on-line to a HP Mass selective detector (model G 1946A) and controlled by ChemStation software (revision A 07.01). An Agilent (Palo Alto, CA, USA) Zorbax SB C18 analytical column (100 × 2.1 mm i.d.; 3 μm) was used. Gradient elution was carried out employing mobile phase, consisting of acetonitrile (solvent A) and 0.2% acetic acid in water (solvent B), as follows (all steps linear): 0 min, 15:85 (A:B); 10 min, 23:77; 15 min, 50:50; 20 min 85:15. The flow rate was 0.3 mL/min and the temperature of column oven was 36°C. The volume of sample applied to the column was 50 μL. The effluent from the chromatograph was introduced directly into the quadrupole mass spectrometer operating in positive ESI mode. The nebulizer gas pressure was 60 psi, the drying gas was at a flow rate of 12 L/min and temperature of 300°C, and the capillary voltage was 3500 V. Individual isoflavones and their conjugates were identified by comparing their retention times (t_R), molecular ions $[M+H]^+$ and characteristic fragments with those of standards[133, 140].

6.2.3 *IMMUNOCHEMICAL METHODS*

ELISA

Five competitive enzyme-linked immunosorbent assays for the determination of individual isoflavones, i.e. daidzein, genistein, biochanin A and their derivatives were used. The specificity of the systems reflected the position used for immunogen construction for the creation of antibodies. Daidzein-4′ and genistein-4′ ELISA systems recognized also the 4′-methoxyderivatives, formononetin and biochanin A. The methods based on 7-position of the hapten molecule were sensitive to 7-*O*-glucosides of daidzein, genistein and biochanin A, namely daidzin, genistin and sissotrin, and to aglycone 7-methoxyisoflavone (prunetin).

Polystyrene microplates (Costar, cat. No.9018; Corning Incorporated (New York, NY, USA)) were filled with BSA-isoflavone conjugates diluted in carbonate-bicarbonate buffer pH 9.6 (100 μL/well) and incubated overnight at laboratory temperature for adsorption. Ovalbumin-hapten was used as the coating conjugate instead of BSA-hapten for the genistein-7 ELISA. The coated plates were washed four times with 0.01M PBS containing 0.05% Tween 20 with use of a Labsystem Multiwash (New York, NY, USA) to remove unbound antigens. Aliquots (50 μL) of the antigen solution in PBS-Tw (isoflavone standard solution in the range of 80 ng/mL-20 pg/mL, or diluted samples) and 50 μL of antibody solution in PBS-Tw with 0.1 % BSA were added to the wells.

After incubation for 1.5 h at laboratory temperature and washing four times with PBS-Tw, the conjugate of the swine anti-rabbit IgG with horseradish peroxidase diluted 1:2000 in PBS-Tw was added (100 μL). Plates were incubated for 1 h at laboratory temperature and washed four times with PBS-Tw. A 100 μL aliquot of a solution of peroxidase substrate with chromogenic compound (50 mg of *o*-phenylendiamine (OPD) in 100 mL of 0.1M citrate/phosphate buffer pH 5.0 containing 0.03% hydrogen peroxide) were added to each well. After incubation at laboratory temperature for 15 min, the enzyme reaction was stopped by addition of 50 μL of 2M sulphuric acid and absorbance was measured at 492 nm by a Labsystem Multiscan MCC/340 (Helsinki, Finland). The absorbance was inversely proportional to the amount of antigen in solution. Data were processed using Microsoft Excel software (Microsoft Corporation, Redmond, WA, USA)[141].

Cross-reactivity

The cross-reactivity of individual methods is the additional tool for identification of certain analytes. To assess cross-reactivity with structurally related compounds, standard curves for the compounds of interest were compared with standard curve of antibody-specific isoflavone performed on the same plate.

Cross-reactions were calculated as a percentage using the equation:

$$CR(AG2) = \frac{I_{50}(AG1)}{I_{50}(AG2)} \cdot 100(\%)$$

,

where $I_{50}(AG1)$ was the 50% intercept of the calibration curve obtained for corresponding immunogen and $I_{50}(AG2)$ was the 50% intercept of the calibration curve of cross-reacting antigen. In the table of cross-reactivity (**Table III**), each of the methods used in this study is specific for several isoflavonoids. These methods overlap in certain cases. In such cases the positive signal obtained by the second method may be considered to be independent evidence. No cross-reactivities were recorded with flavonoids**,** lignans, chalcones and steroids. Identities of main immunoreactivities were subsequently verified by HPLC-MS.

Table III: Cross-reactivities (%) of individual immunoassays.

	Biochanin A-7	**Daidzein-4´**	**Daidzein-7**	**Genistein-4´**	**Genistein-7**
			Cross-reactivity (%)		
Biochanin A	**100**	0.50	< 0.01	178	0.40
Daidzein	< 0.01	**100**	**100**	14.70	9.30
Genistein	2.80	0.50	3.50	**100**	**100**
Sissotrin	**116**	0.02	5.0	0.10	4.80
Daidzin	< 0.01	0.03	**41.0**	< 0.01	1.20
Formononetin	1.60	**192**	6.0	6.50	< 0.01
Isoformononetin	< 0.01	0.20	**342**	< 0.01	37.0
3´-OH-daidzein	< 0.01	3.50	3.40	0.10	2.70
Genistin	< 0.01	0.03	**46.0**	< 0.01	**32.0**
Prunetin	5.00	< 0.01	**30.0**	2.40	**215**
5- Methoxygenistein	< 0.01	< 0.01	5.20	0.15	0.40
7,4´-Dimethoxygenistein	**46.50**	< 0.01	< 0.01	< 0.01	0.20
Glycitin	0.02	< 0.01	2.54	< 0.01	0.01
Glycitein	< 0.01	0.11	1.82	0.32	0.10
6,7,4´-Trihydroxyisoflavone	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
7,3´,4´-Trihydroxyisoflavone	0.02	2.53	1.68	0.36	0.62
Apigenin	0.09	0.01	0.23	0.05	0.08
Naringenin	0.03	0.16	0.42	0.13	< 0.01
Naringin	< 0.01	< 0.01	0.40	0.13	0.34
Kaempferol	0.59	< 0.01	1.89	0.18	0.48
Steroids*	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
O-desmethylangolensin	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01

*Tested steroids: testosterone, epistestosterone, 17α-hydroxy-pregnenolone, cortisol, estradiol

6.2.4 *MOLECULAR BIOLOGY METHODS*

6.2.4.1 PRIMER DESIGN

The Consensus-Degenerate Hybrid Oligonucleotide Primer (CODEHOP) design is a method used for isolation of an unknown gene by the polymerase chain reaction (PCR). It relies on **degenerate** primers consisting of a pool of primers that contain most or all of the possible nucleotide sequence encoding a conserved amino acid motif. It also relies on **consensus** primers consisting of single reverse and forward primers containing the most common nucleotide at each codon position within the motif.

The National Centre for Biotechnology Information (NCBI) protein database provides information about completely or partially sequenced genes encoding IFS enzymes isolated from 16 species (*Astragalus membranaceus* var. mongholicus-partial gene, *Cicer arietinum*-part., *Glycine max*-complete gene, *Glycine soja*-compl., *Lens culinaris*-part., *Lupinus albus*-part., *Medicago sativa*-part., *Medicago truncatula*-compl., *Pisum sativum*-compl., *Pueraria montana* var. lobata-compl., *Trifolium pratense*-compl., *Trifolium repens*-part., *Vicia villosa*-part., *Vigna radiata*-compl., *Vigna unguiculata*-compl.) of the Fabaceae family and one from non-leguminous species *Beta vulgaris* (Chenopodiaceae) (checked 30.9.2008).

Alignment of all cDNA sequences encoding IFS has revealed extremely conservative parts, which were used for primer design (**Table IV**). Alignment was carried out in the program Vector NTI 9 and primers were designed in on-line available program Primer3 v. 0.4.0[142].

Table IV: CODEHOP PCR primers.

***PRIMER* (5´-> 3´)**	
F	
CGACCCTGTCGTTGAAAGGGTCATCAAGAA	***consensus***
YGAYCCTRTCRTTGAAAGGGTCATCAAGAA	***degenerate***
R BLACK	
CTGATGTGGTCCTTGGTGATTTTGATCTCC	***consensus***
YYGRTKTGBTCYTTGGTGATTTTGATCTCC	***degenerate***
R BLUE	
AATAAGAGATGCAAGAAGTGTTGCCATTCC	***consensus***
RAKWAKWGATGMAAGAAGTGTTGCCATTCC	***degenerate***
R BROWN	
AACACAGACAAGACTATGTGCCCTTGGAAC	***consensus***
AACACANMYRAGACTATGTGCCCTTGGAAC	***degenerate***
R GREEN	
TGCAACGCCGATCCTTGCAAGTGGAACACA	***consensus***
WGCDRSRCYDDBCCTTGCAAGTGGAACACA	***degenerate***

Primers consist of a relatively short (12 nucleotides) 5´degenerate core and a longer (18 nucleotides) 3´non-degenerate consensus clamp. Hybridization of the 5´degenerate core with the target template is stabilized by the 3´non-degenerate consensus clamp, which allows higher annealing temperatures without increasing the degeneracy of the pool.

In the **Fig. 5** are presented expected sizes of the PCR products arranged by designed primers.

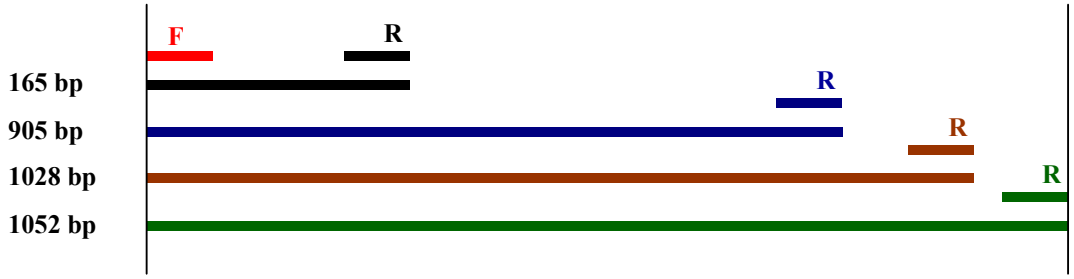


Fig. 5: Expected size of the PCR products.

6.2.4.2 PREPARATION OF GENOMIC DNA

Plant genomic DNA was extracted either according to CTAB DNA extraction protocol (constitution of CTAB extraction buffer is listed in **Table V**) or by use of commercial kit Invisorb Spin Plant Mini Kit.

Around 100 mg of preselected fresh plant leaf material was placed into a tube with glass beads and it was frozen in liquid nitrogen. The tissue was grinded for 12 second with use of homogenizer (Silamat S5, VIVADENT, Lichtenstein). Then, CTAB extraction buffer (250 µL) was added and vortexed briefly. The extract mixture was incubated for 15-20 minutes at room temperature. 250 µL of chloroform: isoamyl alcohol mixture (24:1) was added to the extract to precipitate cell debris. The aqueous and organic phases were separated by centrifuging the tubes at 13 000 RPM (revolutions per minute) in an Eppendorf centrifuge (Hamburg, Germany) for 10 minutes. Then upper aqueous layer was transferred to a new tube containing 140 µL of isopropanol and left for 5 minutes at room temperature. The mixture was spun for 7 minutes at 13 000 RPM and the supernatant was discarded. The pellet was washed with 1 mL of 70% ethanol and spined for 7 minutes again. Ethanol was sucked off and the pellet was dried under vacuum for 5-20 minutes (depending on the pellet size). Product was dissolved in 30-50 µL double distilled water (dd H_2O) and incubated at 55°C for 5 minutes. The quality of the genomic DNA was determined on an agarose gel.

Table V: CTAB DNA extraction buffer.

1.4 M NaCl	(5 M stock solution)	28 mL
20 mM EDTA	(0.5 M stock solution)	4 mL
100 mM Tris pH 8	(1 M stock solution)	10 mL
3% w/v CTAB	(6% stock solution)	50 mL
Double distilled water		8 mL
Final volume		**100 mL**

6.2.4.3 AMPLIFICATION OF DNA BY PCR

Specific DNA fragments were amplified by PCR. A typical PCR reaction mixture was prepared as follows (**Table VI**):

Table VI: PCR reaction mixture.

10x Taq DNA polymerase buffer	2.5 µL
2mM dNTP´s	0.5 µL
DNA template	1 µL
$MgCl_2$	2 µL
5´primer (F	1 µL
3´primer (R)	1 µL
ddH_2O	15 µL
Taq DNA polymerase	2 µL
Final volume	**25 µL**

The above mentioned 25 µL of reaction mixture was filled into a 0.5 mL tube. The tube was placed in a thermal cycler (XP cycler, Eppendorf).

6.2.4.4 TOUCHDOWN PCR

The Touchdown PCR is a modification of conventional PCR. The Touchdown PCR uses a cycling program with varying annealing temperatures. It is a useful method to increase the specificity of PCR products. The annealing temperature in the initial cycle should be 5-10°C above the melting temperature (T_m) of the primers. In subsequent cycles, the annealing temperature is decreased in steps of 1-2°C/cycle until temperature is reached that is equal to, or 2-5°C below, the *T*m of the primers. The Touchdown PCR enhances the specificity of the initial primer-template duplex formation and hence the specificity of the final PCR product.

Each cycle consisted of the following steps: 96°C for 15 seconds-DNA template denaturates; 60°C for 25 seconds-primer annealing; 72°C for 60 seconds-synthesis of DNA fragment; each cycle was followed by a 1°C decrease of the annealing temperature, and the DNA fragments were amplified about 35 cycles.

Neutral agarose gel was used to quantify and asses the quality of DNA fragments amplified using PCR. Agarose was dissolved in 1× TAE buffer and heated to boil using a microwave and then cooled to 60°C. Finally ethidium bromide was added to final concentration of 0.01 µL/mL. The liquid agarose with ethidium bromide was poured into perplex tray with an appropriate comb and allowed to solidify at room temperature. When the agarose gel was solid, the comb was removed forming wells in the gel and the tray was submerged in an electrophoresis tank containing 1× TAE buffer. Rests of agarose were washed out from the wells by agitation.

Gel loading buffer (10×) was added to PCR products or to DNA samples. These mixtures were loaded into individual wells and electrophoresed at 8 V/cm. Quantification of the amount (ng) and size (Kb) of DNA fragments was carried out by comparison of their mobility with mobility of the reference DNA molecular weight standard 100 bp and 1 Kbp ladder. In the end electrophoresis gels were photographed on a transilluminator. The bands of interest were cut out of the gel and purified using a commercial Qiagenkit according to Qiagen clean kit protocol. The purified products were cloned into pGEM-T Easy Vector.

6.2.4.5 CLONING OF cDNA FRAGMENT

The pGEM-T Easy Vector System (**Fig. 6**) is a convenient system for the cloning of PCR products. The vector is available in linear form that was prepared by the producer by cutting the pGEM-T Easy Vector with an enzyme *EcoR* V and by adding 3´ terminal thymidine to both ends. These single 3´-T overhangs at the insertion site greatly improve the efficiency of ligation of a PCR product into the plasmids by preventing recirculization of the vector and providing a compatible overhang for PCR products generated by certain thermostabile polymerases.

The pGEM-T Easy Vector System includes a 2× Rapid Ligation Buffer for ligation of PCR products, pGEM-T Easy Vector and T4 DNA Ligase. The ligation was set up according the protocol for the use of pGEM-T Easy Vector as described below (**Table VII**). The reactions were mixed by pippeting and incubated overnight at 4°C.

Table VII: pGEM-T Easy Vector System protocol.

2x Rapid Ligation Buffer	5 µL
pGEM-T Easy Vector (50 ng)	0.5 µL
PCR product	3.5 µL
T4 DNA Ligase (3 units/ µL)	1 µL
Final volume	**10 µL**

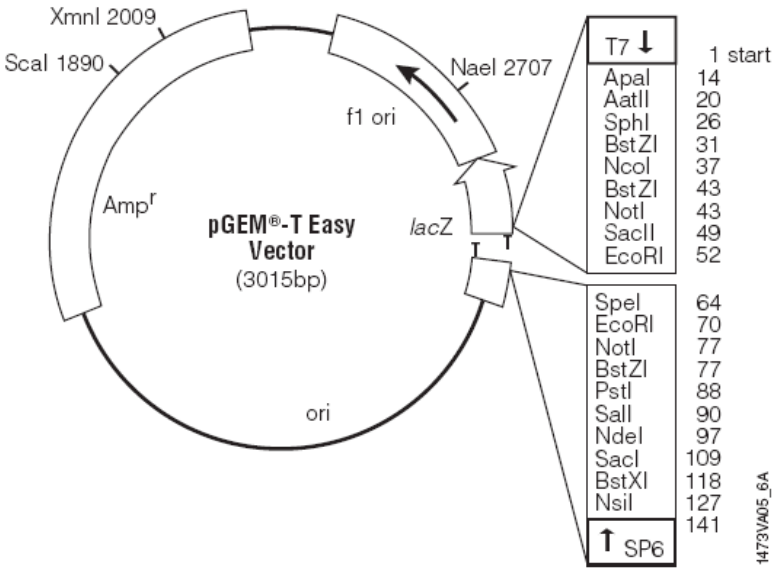


Fig. 6: pGEM-T Easy Vector circle map and sequence reference points[143].

pGEM-T Easy Vector sequence reference points:	
T7 RNA polymerase transcription initiation site	1
Multiple cloning region	10-128
SP6 RNA polymerase promoter (-17 to +3)	130-158
SP6 RNA polymerase transcription initiation site	141
pUC/M13 Reverse Sequencing Primer binding site	176-197
*lac*Z start codon	180
lac operator	200-216
ß-lactamase coding region	1337-2197
phage f1 region	2380-2835
lac operon sequences	2836-2996, 166-395
pUC/M13 Forward Sequencing Primer binding site	2949-2972
T7 RNA polymerase promoter (-17 to +3)	2999-3

6.2.4.6 BACTERIAL TRANSFORMATION - Transformation of Escherichia coli with plasmid DNA

The competent *E. coli* DH5α cells were thawed on ice and divided into 25 µL aliquots. Each aliquot was pipetted into pre-chilled microcentrifuge tubes. For the transformation of ligation products, 10 µL of each ligation was mixed with one aliquot of competent cells. The cells were incubated on ice for 30 min and then heat-shocked at 42°C for 50 sec. After the heat shock, each aliquot of cells was added to 0.75 mL of LB medium and incubated at 37°C with shaking for 1 hour. Finally, the mixture was shortly centrifuged, 2/3 of supernatant were removed and all of the cells were spread onto a selective LB agar plate with ampicilin (0.1 mg/mL), which was let to dry for 10 minutes and then incubated at 37°C overnight.

6.2.4.7 IDENTIFICATION OF RECOMBINANT PLASMIDS IN TRANSFORMED COLONIES

When cloning into pGEM-T Easy Vector System, a blue-white selection was used to identify recombinant colonies. The vector contains a gene LacZ that codes enzyme beta-galactosidase. LacZ is made up of two fragments, alpha and omega. In case that this enzyme is not interrupted by insert DNA, it is functional and it metabolizes galactose producing lactose and glucose. Beta-galactosidase could convert other substrates, such as colorless modified galactose sugar X-Gal (5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside), into a colored product (blue product). In order for the gene to be actively transcribed from the DNA and for the enzyme to be produced, an activator called IPTG (Isopropyl-1-thio-ß-D-galactoside) must be added. Both X-Gal and IPTG are delivered to the bacteria through the growth medium. The transformed cells were plated on a LB agar plate with ampicilin supplemented with 100 µL of water solution IPTG (100 mM) and 20 µL X-Gal (50 mg/mL) in DMSO. Within the LacZ gene there are multiple cloning sites where the plasmid may be cut and DNA may be added. This produces a plasmid with foreign DNA, located within the LacZ gene. When transcription of the gene is activated by IPTG, the foreign DNA that has been inserted is transcribed as well. When the gene is later translated into the enzyme, the inserted DNA is translated as well. Because of its location within the enzyme, the foreign DNA's translated protein product disrupts activity and function of the enzyme. The disrupted enzyme activity is observed as a white bacterial colony. If the enzyme is functioning, each colony has a blue color. LB agar plate with ampicilin, IPTG, X-GAL and transformed cells were incubated at 37°C overnight.

6.2.4.8 COLONY PCR AND GROWTH OF CULTURES

A 25 μL aliquot of a PCR reaction mixture in a 0.5 mL tube was inoculated with a selected single white bacterial colony with use of a sterile toothpick. The standard Touchdown PCR reactions were set up as described in section *6.2.4.4.*, with either pGEM-T Easy Vector primers or primers specific for the fragment being cloned. The PCR products were visualized on a TAE agarose gel with appropriate DNA markers.

Bacteria still present on the toothpick were streaked out onto LB agar plates with antibiotic selection and incubated at 37°C overnight. White streaks of colonies were used to inoculate 5 mL of liquid LB medium containing the appropriate selective antibiotic (ampicilin) and growth over night in orbital shaker at 37°C. 4 mL viable bacterial cultures were pelleted in a centrifuge. Plasmids were isolated by mini-prep kit Qiagen. Their nucleotide sequences were analyzed on the genetic analyzer ABI 3100 Avant in the DNA laboratory at the Department of Botany, Faculty of Science, Charles University.

6.2.4.9 LONG TERM STORAGE OF BACTERIAL CULTURES

Bacterial cultures were maintained for a long period of time by transferring 0.5 mL of dense bacterial culture to a tube. An equal volume of sterile 50% v/v glycerol was added to the culture, the content vortexed and the tube stored at -80°C.

7 RESULTS AND DISCUSSION

7.1 Immunoreactivity of isoflavonoids in crude extracts

Occurrence of isoflavonoids has been previously presented in six species belonging to three Rutaceous genera (*Fortunella obovata*, *Murraya paniculata*, *Citrus aurantium*, *C. grandis*, *C. limonia* and *C. sinensis*) using radioimmunoassay[Chyba! Záložka není definována.]. In this study the number of Rutaceae plants shown to produce isoflavonoids has been extended by further 20 species belonging to five genera, i.e. *Citrus*, *Fortunella*, *Poncirus*, *Ruta* and *Severinia*.

Water-ethanolic extracts were analyzed by immunoassay for initial screening. ELISA of crude extracts yielded semi-quantitative data (**Table VIII**), enabling the optimisation of the amount of material for further analysis and the exclusion of negative samples. Daidzein-, genistein- and biochanin A-like immunoreactivities were found in the extracts of all tested samples of rutaceous plants.

The highest immunoreactivities were determined by genistein-7-method. With regard to the sensitivity of this method, a high content of derivatives in position 7 could be assumed. All representatives of the Rutaceae family were fractionated for detail analysis of isoflavones distribution.

Table VIII: Levels of isoflavones (ng/g dry weight) measured in crude extracts of representative species Rutaceae family using ELISA.

Plant species	**Voucher specimen No.**	biochanin 7	daidzein 4	daidzein 7	genistein 4	genistein 7
Citrus sinensis (L.) Osb. *x Poncirus trifoliata* (L.) Raf.	**RP 2**	210.5	137.5	423.9	310.5	1447.9
Citrus asahikan Hort. ex Tanaka	**RP 5**	144.5	59.6	115.9	78.7	524.8
Citrus bergamia Risso	**RP 7**	63.7	47.9	44.1	57.1	361.9
Citrus glaberrima Hort. Ex Tanaka	**RP 10**	135.7	100.7	166.5	253.0	1119.7
Citrus hassaku Hort. Ex Tanaka	**RP 16**	140.8	77.1	268.0	280.5	1177.7
Citrus ichangensis Swingle	**RP 17**	95.8	129.3	165.1	179.5	625.6
Citrus jambhiri Lush.	**RP 19**	269.3	187.9	296.3	320.9	1359.2
Citrus junos Siebold ex Tanaka	**RP 20**	166.7	96.3	149.3	116.1	844.0
Citrus macrophylla Wester	**RP 36**	252.1	82.8	96.9	173.9	435.7
Citrus macroptera Montrous.	**RP 37**	159.6	86.9	109.3	172.3	741.1
Citrus x paradisi Macf.	**RL 48**	149.0	148.4	197.3	237.1	2448.8
Citrus taitensis Risso	**RP 86**	212.8	102.0	215.1	381.2	942.6
Citrus wilsonii Tanaka	**RP 99**	90.3	79.6	69.9	112.4	828.2
Fortunella japonica Swingle	**RL 102**	137.6	61.9	111.1	146.6	608.2
Poncirus trifoliata (L.) Raf.	**RP 110**	175.4	67.1	63.1	94.8	598.1
Ruta angustifolia Pers.	**RV 119**	283.5	83.3	86.6	84.0	358.2
Ruta corsica DC.	**RV 112**	468.1	43.7	74.4	54.8	253.0
Ruta graveolens L.	**RV 113**	436.1	80.1	213.2	153.1	654.8
Ruta montana Mill.	**RV 114**	278.0	100.4	169.3	98.2	318.4
Severinia buxifolia Ten.	**RP 115**	689.7	207.7	524.1	349.3	604.1

7.2 ELISA of HPLC fractions

Aliquots of the extracts were evaporated under vacuum to dryness and the residues were dissolved in the mobile phase for fractionation. Following HPLC separation, 50 fractions were obtained from each plant. Fractions were evaporated to dryness and analyzed by a set of 5 immunoassays. Information about the distribution of immunoreactivities on the chromatogram was obtained (**Fig. 7**). Extracts from all plants under study contained numerous immunoreactive peaks distributed through the whole chromatogram from polar substances (supposedly the glycosides of isoflavones) up to far less polar.

The chromatographic mobilities of immunoreactive fractions were compared with those of authentic standards.

Considering the specifity of the biochanin-7-method, sissotrin (fractions 20, 21) and biochanin A (fraction 38) were detected. The content of biochanin A was confirmed by the genistein-4´-method. Genistein (fractions 23, 24, 25) immunoreactivity was determined when measured with genistein-7-method as well. This method had broader spectrum of cross-reactivities. Besides genistein, its 7-*O*-glucoside (genistin-fractions 10, 11) and prunetin (fraction 37) were determined. The one of daidzein specific methods, daidzein-4´-method, indicated daidzein-corresponding immunoreactivity (fraction 18) and formononetin-corresponding immunoreactive peak (fractions 30, 31). Another independent immunoassay for daidzein analysis (daidzein-7 method) indicated daidzein-corresponding immunoreactivity, daidzin (fraction 8) and at the 30% of prunetin immunoreactivity. Immunoreactivities of isoformononetin (fractions 27, 28) and 7,4´-dimethoxygenistein (fraction 47) were not observed.

Fig. 8 and **Table IX** summarize the obtained values of isoflavonoids in fractions of all tested Rutaceae plants. The most abundant glycoside was sissotrin. The highest levels of aglycones were represented by formononetin. Although quantitative, as well as qualitative, differences were recorded between individual species, certain isoflavonoid immunoreactivities have been detected in all samples under study. Free aglycones, as well as 7-*O*-glycosylated isoflavones, were detected; methoxyisoflavones appeared more abundant than hydroxyisoflavones (**Attachment 2**).

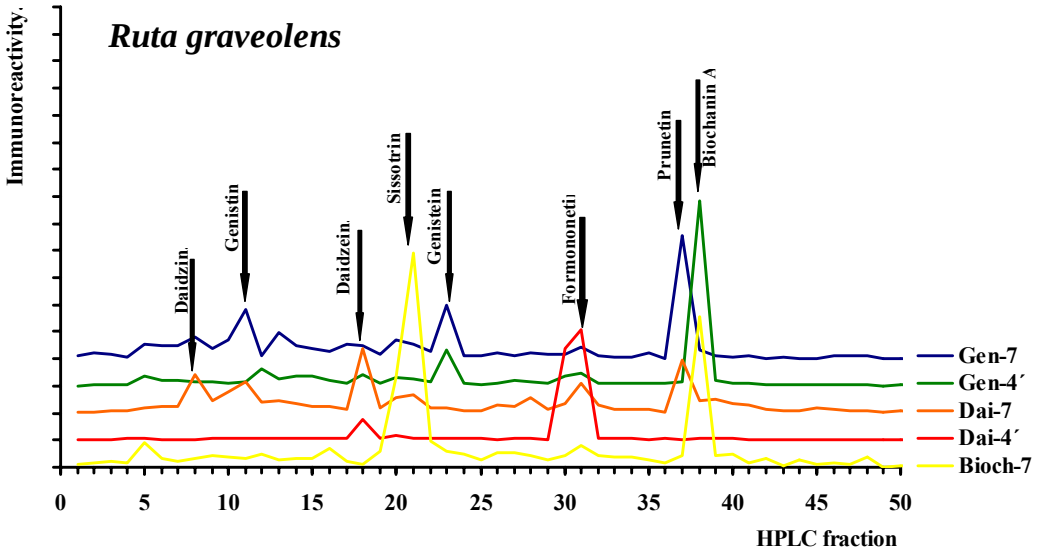


Fig. 7: Distribution of immunoreactivities of chromatographic fractions in *Ruta graveolens.*

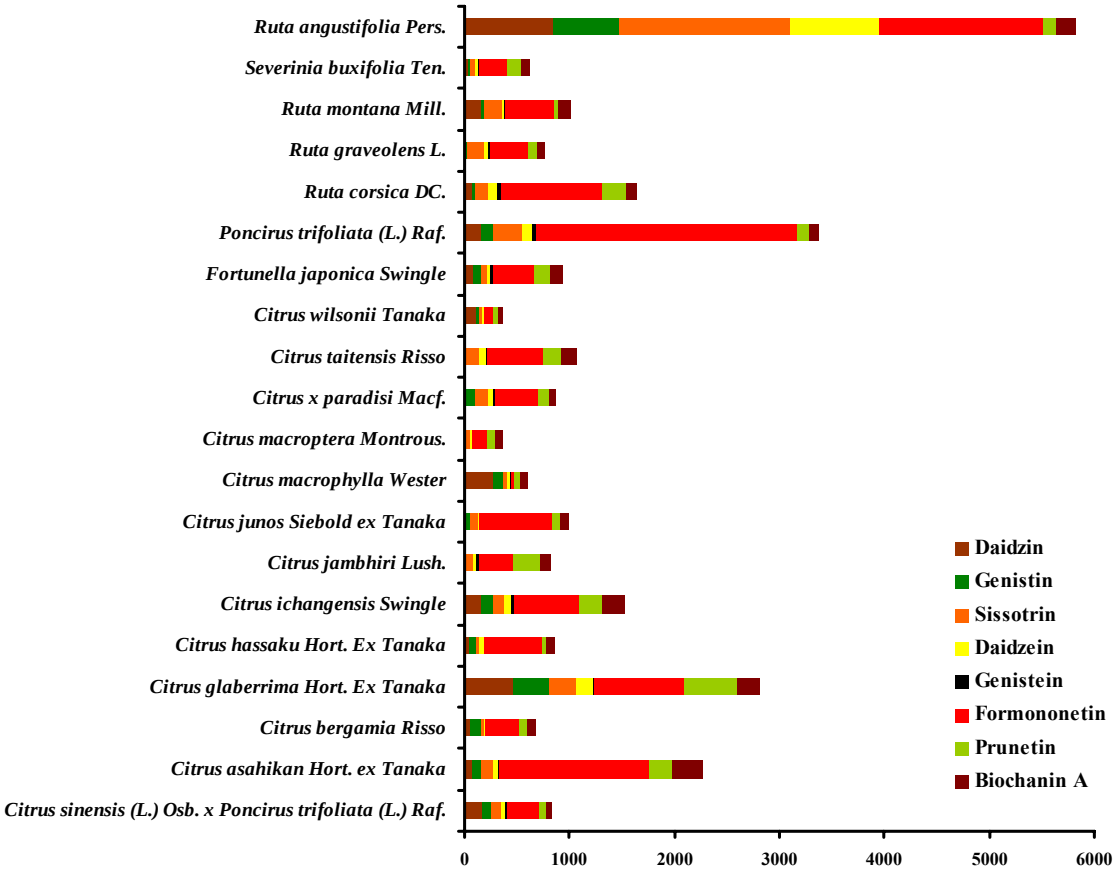


Fig. 8: Isoflavone-levels (ng/g dry weight) detected in the Rutaceae family with use of HPLC-ELISA.

Table IX: Levels of isoflavones (ng/g dry weight) measured in representative species of the family Rutaceae using HPLC-ELISA.

Plant species	Voucher specimen No.	Daidzin	Genistin	Sissotrin	Daidzein	Genistein	Formononetin	Prunetin	Biochanin A
Citrus sinensis (L.) Osb. *x Poncirus trifoliata* (L.) Raf.	RP 2	177.9	88.7	76.4	41.9	14.8	299.9	83.1	48.2
Citrus asahikan Hort. ex Tanaka	RP 5	76.8	74.8	124.7	34.5	15.4	1440.0	211.5	292.4
Citrus bergamia Risso	RP 7	52.6	106.5	23.5	15.4	20.8	319.1	70.8	87.6
Citrus glaberrima Hort. Ex Tanaka	RP 10	461.7	341.4	257.9	164.4	18.6	844.0	505.8	220.5
Citrus hassaku Hort. Ex Tanaka	RP 16	38.6	78.2	32.7	31.5	11.0	554.7	39.7	83.6
Citrus ichangensis Swingle	RP 17	164.2	107.9	98.1	70.4	39.6	621.4	217.4	205.7
Citrus jambhiri Lush.	RP 19	0.0	15.1	70.2	26.0	27.9	318.7	265.5	93.7
Citrus junos Siebold ex Tanaka	RP 20	3.3	58.1	67.8	14.2	6.4	680.0	82.7	79.8
Citrus macrophylla Wester	RP 36	276.4	99.4	34.1	26.2	16.3	25.3	53.8	79.9
Citrus macroptera Montrous.	RP 37	0.0	20.0	43.7	8.8	16.5	151.0	62.4	68.6
Citrus x paradisi Macf.	RL 48	0.0	107.5	127.2	42.3	13.7	410.2	110.9	50.6
Citrus taitensis Risso	RP 86	0.0	20.4	122.0	56.0	20.5	531.1	173.7	147.9
Citrus wilsonii Tanaka	RP 99	108.5	30.9	34.6	6.6	8.6	91.8	29.7	42.9
Fortunella japonica Swingle	RL 102	92.2	63.5	55.2	35.1	27.6	387.0	168.1	106.3
Poncirus trifoliata (L.) Raf.	RP 110	153.8	121.5	268.5	107.7	24.1	2491.6	115.9	98.1
Ruta corsica DC.	RV 112	79.3	21.4	136.1	79.1	25.0	972.4	234.9	90.9
Ruta graveolens L.	RV 113	0.0	24.9	160.6	38.9	25.5	355.8	80.6	83.1
Ruta montana Mill.	RV 114	158.0	36.0	169.2	16.4	9.2	459.8	38.5	117.8
Severinia buxifolia Ten.	RP 115	33.3	25.6	46.5	27.4	17.6	259.0	127.0	79.5
Ruta angustifolia Pers.	RV 119	847.4	628.5	1620.8	856.5	11.0	1556.2	133.5	179.2

7.3 HPLC-MS

Main immunoreactivities were subsequently verifying by HPLC-MS-SIM (**Fig. 9**). Data confirmed the presence of isoflavonoids in all the plants under the study. Together with the verification of main immunoreactive peaks, additional isoflavonoids were found, namely ononin and glycitein in concentration around 200 ng/g dry weights.

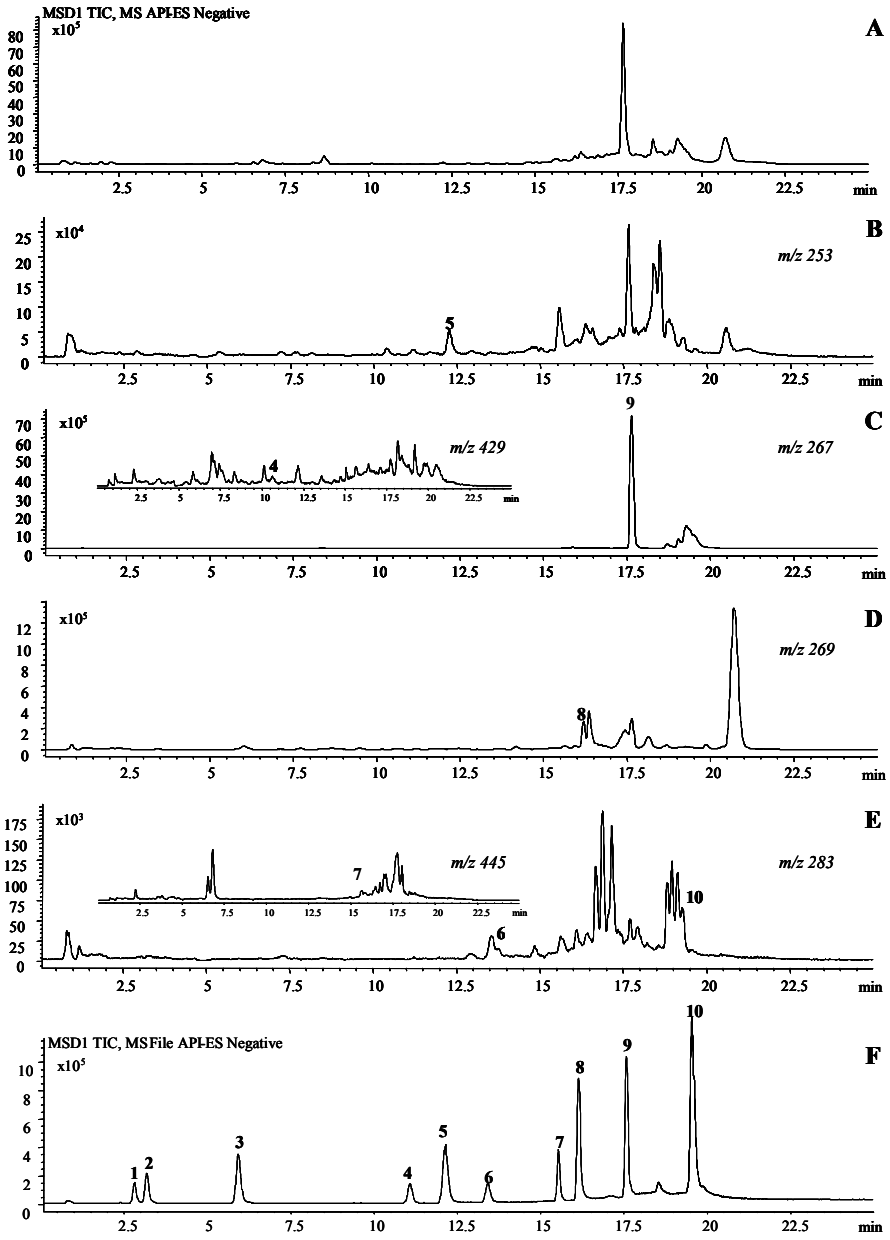


Fig. 9: HPLC/MS chromatograms of an extract from *Ruta graveolens*.

A: TIC chromatogram of *Ruta graveolens*, **B-E**: SIM chromatograms for selected *m/z*, **F**: TIC chromatogram of standards. Peak identification: **1**-daidzin, **2**-glycitin, **3**-genistin, **4**-ononin, **5**-daidzein, **6**-glycitein, **7**-sissotrin, **8**-genistein, **9**-formononetin, **10**-biochanin A.

7.4 ISOFLAVONES IN *ARABIDOPSIS THALIANA*

Arabidopsis thaliana (Brassicaceae), sometimes called mouse-ear cress, a small flowering plant related to cabbage and mustard, is one of the model organisms useful for studying plant sciences, including genetics and plant development. This study aimed to frame occurance of isoflavonoids, except from Fabaceae family, into gene context. It focused on *A. thaliana* because this herb is considered to be isoflavone non-producer and that is supported by knowledge of complete genom.

A. thaliana has several advantages that made it the model for understanding the genetic, cellular, and molecular biology of flowering plants. The small size of its genome made it useful for genetic mapping and sequencing. At about 125 million base pairs of DNA, distributed in five chromosomes, it presents a small genome for plant species. There were found about 27,235 protein coding genes. Plant's small size and rapid life cycle are also its advantages. For many commonly used lab strains, it takes about six weeks from germination to mature seed. The small size of this plant is convenient for cultivation in a small space and the plant produces many seeds. Further, the selfing nature of this plant assists genetic experiments. Each individual plant can produce several thousands seeds making genetics studies easier. Finally, plant transformation in *Arabidopsis* is a routine that uses *Agrobacterium tumefaciens* as the vector to introduce foreign genes to the plant genome. In accord with previously published data, no homologue was found to known isoflavone synthases from the Fabaceae plants[144].

Arabidopsis thaliana[145]

ELISA of A. thaliana

Water-ethanolic extracts from leaves and inflorescence stalks from *A. thaliana* were tested for the occurance of isoflavones by using ELISA. The isoflavonoids-like immunoreactivity was found out in all extracts, thus extracts were fractionated by HPLC and all fractions were analysed by all five immunoassays. The chromatogram created on the base of immunoreactivity of fractions is given in the **Fig. 10**, while the retention times corresponded to those of authentic standards. The main immunoreactive peak was comprised by formononetin and prunetin followed by biochanin A and daidzein.

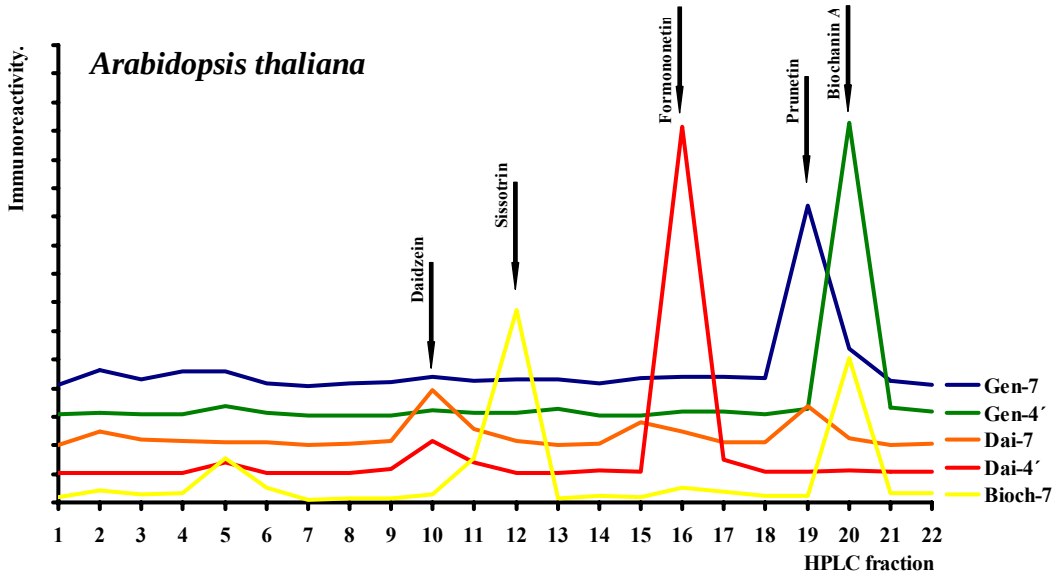


Fig. 10: Distribution of immunoreactivities of chromatographic fractions in *A. thaliana.*

The HPLC-MS-SIM analysis confirmed the presence of all immunoreactive isoflavones; moreover, ononin, for which an immunoassay was not available, was detected (**Attachment 3**).

Despite the absence of a homologue to leguminous IFS in *A. thaliana* genom, the spectrum of isoflavones in wild type *A. thaliana* was observed. This observation is supported by the demonstration of isoflavones in other brassicaceous species, namely *Lepidium sativa*[146], *Brassica oleracea*[147]. The necessity to explain this discrepancy makes hypothesize that other gene(s) must be responsible for the aryl migrating activity in Brassicaceae.

7.5 MOLECULAR BIOLOGY

7.5.1 *Touchdown PCR with consensus primers*

All primers (**Table IV.**) were tested on plant DNA of *Pisum sativum* L., *Phaseolus vulgaris* L., *Trifolium pratense* L. and *Vigna radiata* L. by the Touchdown PCR method. Using the consensus F and R_{black} primers, PCR product (~160-200 bp) was observed in the case of all legumes plants (signed in black oval in **Fig. 11**). PCR of all legume DNAs with F, R_{blue} consensus primer only produced one PCR product (~800 bp, blue oval) and those with R_{green} consensus primer produced one PCR product (~800 bp, green oval) too, both were present in the case of *Phaseolus vulgaris* L.

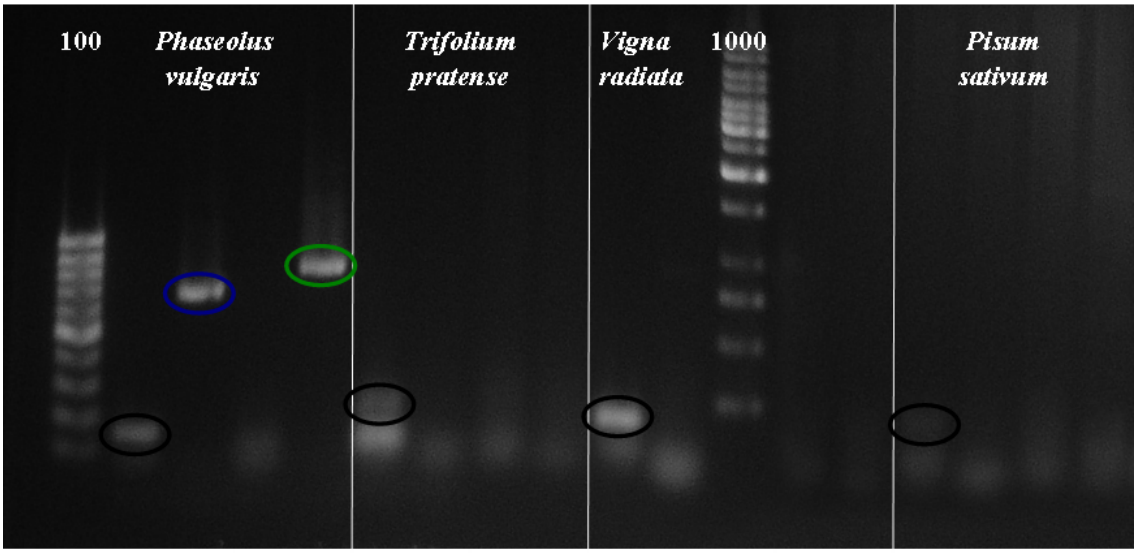


Fig. 11: Electrophoresis of PCR products using consensus primers. Line 1, 100 bp ladder; line 2, R_{black}; line 3, R_{blue}; line 4, R_{brown}; line 5, R_{green}; line 6, R_{black}; line 7, R_{blue}; line 8, R_{brown}; line 9, R_{green}; line 10, R_{black}; line 11, R_{blue}; line12, 1 kbp ladder; line 13, R_{brown}; line 14, R_{green}; line 15, R_{black}; line 16, R_{blue}; line 17, R_{brown}; line 18, R_{green}.

7.5.2 *Touchdown PCR with degenerate primers*

More PCR products were obtained employing all degenerate primers. Apart from applying R_{brown} primer with DNA from *Phaseolus vulgaris* and R_{green} primer with DNA from *Vigna radiata*. PCR products of expected lengths are highlighted in **Fig. 12** (black PCR product ~200 bp, blue PCR product ~800 bp, brown PCR product ~900 bp and green PCR product ~1000 bp).

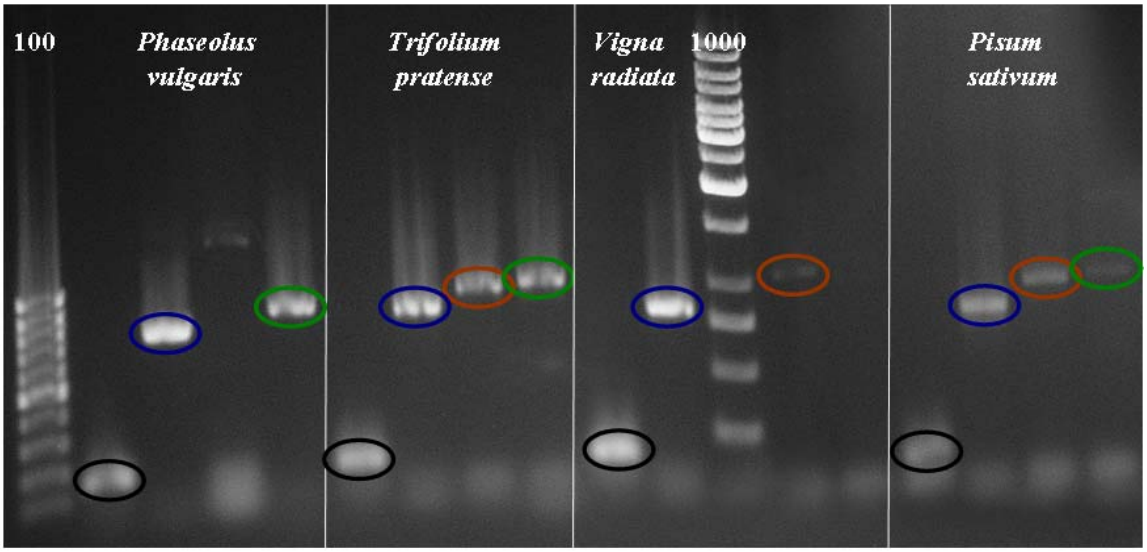


Fig. 12: Electrophoresis of PCR products using degenerate primers. Line 1, 100 bp ladder; line 2, R_{black}; line 3, R_{blue}; line 4, R_{brown}; line 5, R_{green}; line 6, R_{black}; line 7, R_{blue}; line 8, R_{brown}; line 9, R_{green}; line 10, R_{black}; line 11, R_{blue}; line12, 1 kbp ladder; line 13, R_{brown}; line 14, R_{green}; line 15, R_{black}; line 16, R_{blue}; line 17, R_{brown}; line 18, R_{green}.

The choice of F, R_{blue} degenerate primers was chosen for all further experiments. It was based on the length, intensity and frequency of PCR products in Fabaceae family.

7.5.3 *PCR screening non-legume species*

For searching of desired IFS gene, the Touchdown PCR method was used for the screening of broader spectrum of non-legumes plants. DNA from 32 plant species from 12 families was used for basic overview. In the **Table X**, all tested species are listed. Some experiments brought positive and some negative results, independently on a plant family. It could be caused either by low quality of DNA extraction or by absence of any homologue specified by primers.

Subsequently, research was focused only on selected species, i.e.: *Cannabis sativa*, *Humulus lupulus* (Cannabaceae), *Ruta montana*, *Ruta graveolens* (Rutaceae), *Nicotiana tabacum* (Solanaceae) and the comparative legume sample *Phaseolus vulgaris*.

Species	Family	Y/N	Species	Family	Y/N
Cannabis sativa	**Cannabaceae**	**Y**	*Calendula officinalis*	**Asteraceae**	**N**
Humulus lupulus		**Y**	*Achillea filipendulina*		**Y**
Aralia manshurica	**Araliaceae**	**Y**	*Dahlia vulgaris*		**N**
Ruta graveolens	**Rutaceae**	**Y**	*Helichrysum brachteatum*		**N**
Ruta chalapensis		**Y**	*Helianthus annuus*		**Y**
Ruta divaricata		**Y**	*Cosmos bipinnatus*		**Y**
Ruta montana		**Y**	*Inula helenium*		**Y**
Urtica dioica	**Urticaceae**	**N**	*Rudbeckia angustifolia*	**Rosaceae**	**N**
Morus alba	**Moraceae**	**Y**	*Mespilus germanica*		**Y**
Broussonetia papyrifera		**N**	*Fragaria vesca*		**Y**
Maclura pomifera		**N**	*Cydonia oblonga*		**Y**
Papaver somniferum	**Papaveraceae**	**N**	*Eryobotrya japonica*		**Y**
Nicotiana tabacum	**Solanaceae**	**Y**	*Rubus idaeus*		**N**
Lycopersicum esculentum		**N**	*Rubus fruticosus*		**N**
Cucurbita maxima	**Cucurbitaceae**	**N**	*Chaenomeles japonica*		**N**
Vitis vinifera	**Vitaceae**	**Y**	*Arabidopsis thaliana*	**Brassicaceae**	**N**

Table X: Overview of tested plants (Yes/No-positive/negative result).

7.5.4 *Touchdown PCR with selected species*

DNA of selected samples was tested with all of degenerate primers again. PCR products were observed in all cases using F, R_{black} degenerate primers, F, R_{blue} degenerate primers and in the case of *Phaseolus vulgaris* with F, R_{brown} degenerate primers (**Fig. 13**). **Fig. 14** shows PCR product of *Humulus lupulus* with use of only F, R_{blue} degenerate primers. Following electrophoresis, the specific bands (~800bp) were cut out of the gel and purified with use of a Qiagen Gel Extraction Kit. The purified products were cloned into pGEM-T Easy Vector. Chemically competent *E. coli* DH5α cells were transformed with plasmid DNA and blue-white selection based on ß-galactosidase activity was used to identify recombinant colonies (**Fig. 15**).

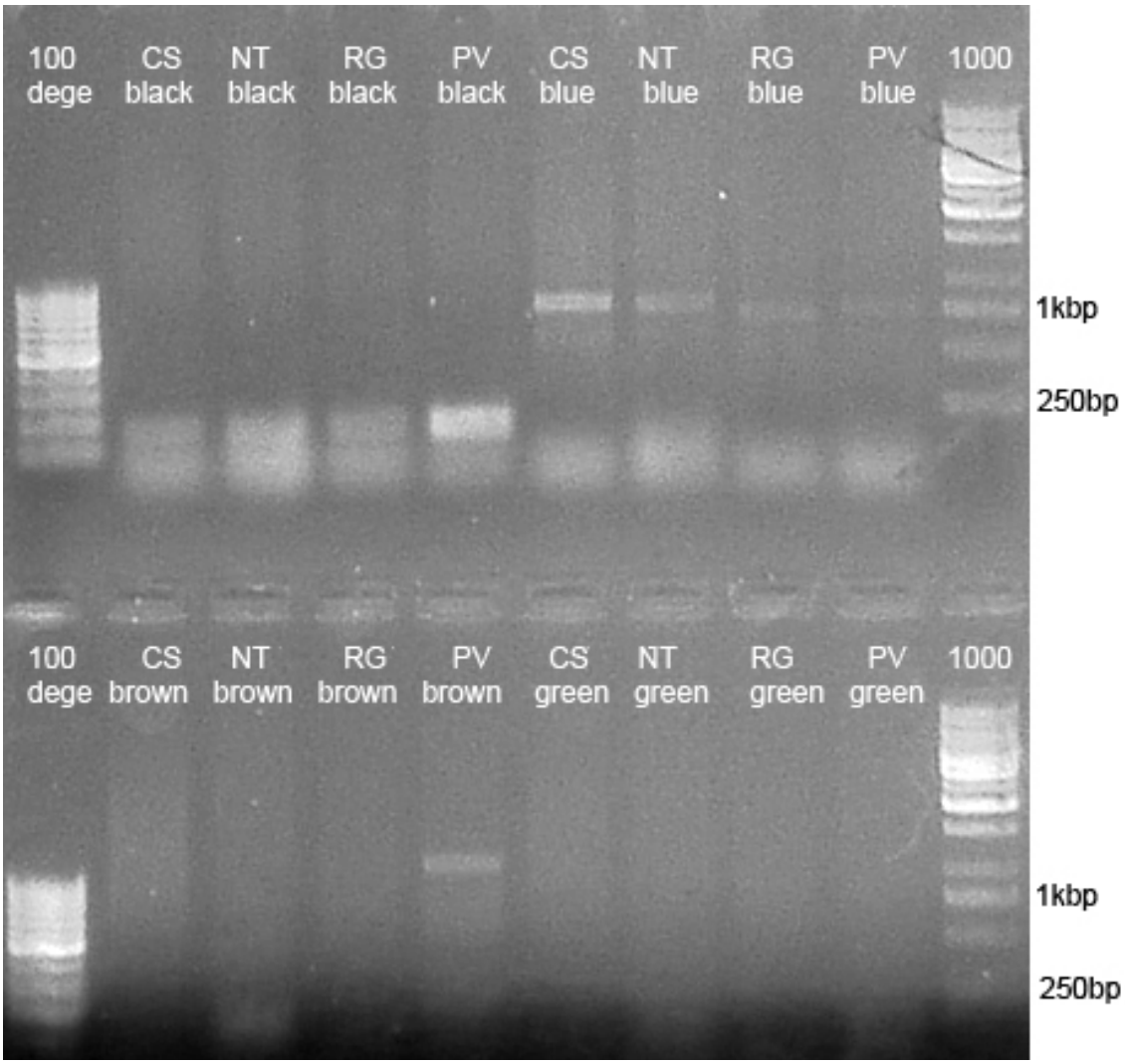


Fig. 13: Electrophoresis of PCR products with use of degenerate primers.

CS-*Cannabis sativa*, NT-*Nicotiana tabacum*, RG-*Ruta graveolens*, PV-*Phaseolus vulgaris*. Line 1, 100 bp ladder; line 10, 1000 bp ladder.

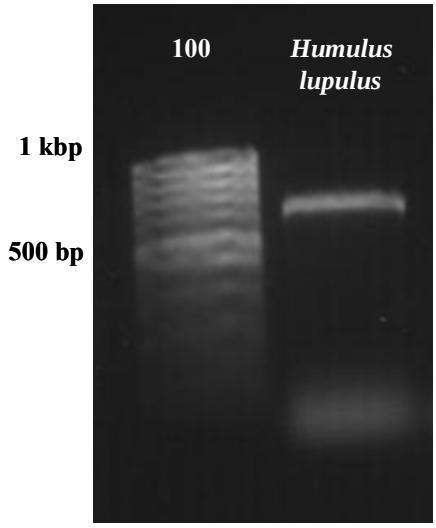


Fig. 14: PCR product with DNA of *Humulus lupulus* and degenerate F, R_{blue} primers.

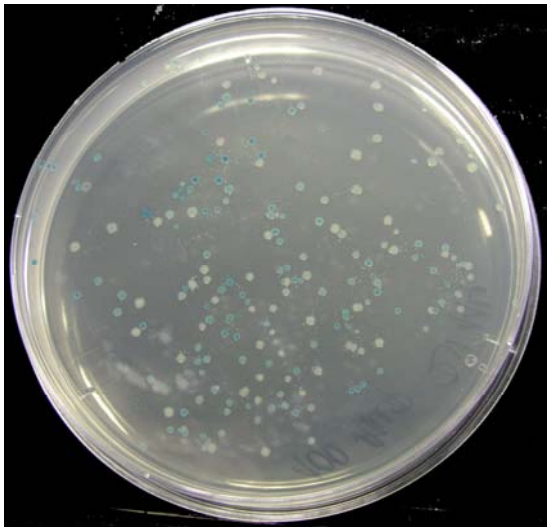


Fig. 15: Blue and white bacterial recombinant colonies on LB agar plate with X-Gal and IPTG.

7.5.5 *Colony PCR*

From 2 to 5 single white bacterial colonies were inoculated with a 25 μL aliquot of a PCR mixture for the control of correct ligation and transformation process. The standard Touchdown PCR reactions were set up with specific pGEM-T Easy Vector primers. Photograph in **Fig. 16** shows the colony PCR electrophoresis of *Ruta graveolens* and *Ruta montana*. PCR products from colony PCR are longer than original PCR products with blue degenerate primers. These fragments include the parts of the pGEM-T Easy Vector defining the pGEM-T Easy Vector primers (~900bp).

White colonies were used to inoculate 5 mL of liquid LB medium containing the appropriate selective antibiotic (ampicilin). They have grown over night in orbital shaker at 37°C. Viable bacterial cultures were pelleted by centrifugation. Plasmids were isolated with use of QIAprep Spin Miniprep Kit and then verified on the electrophoresis gel (**Fig. 17**). All cloned DNAs were sequenced.

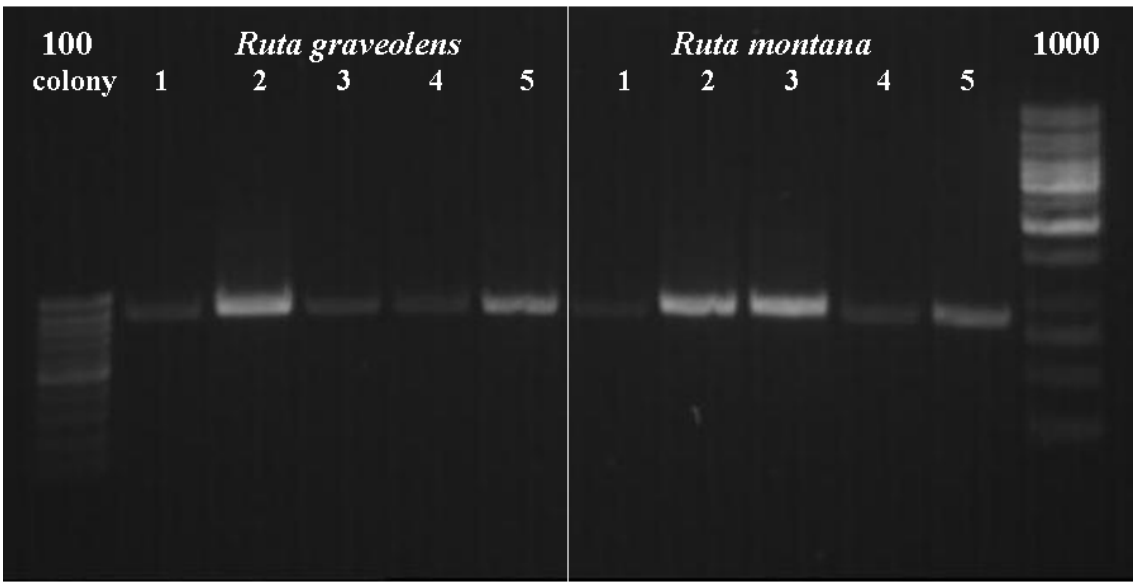


Fig. 16: Electrophoresis of PCR products from Colony PCR.

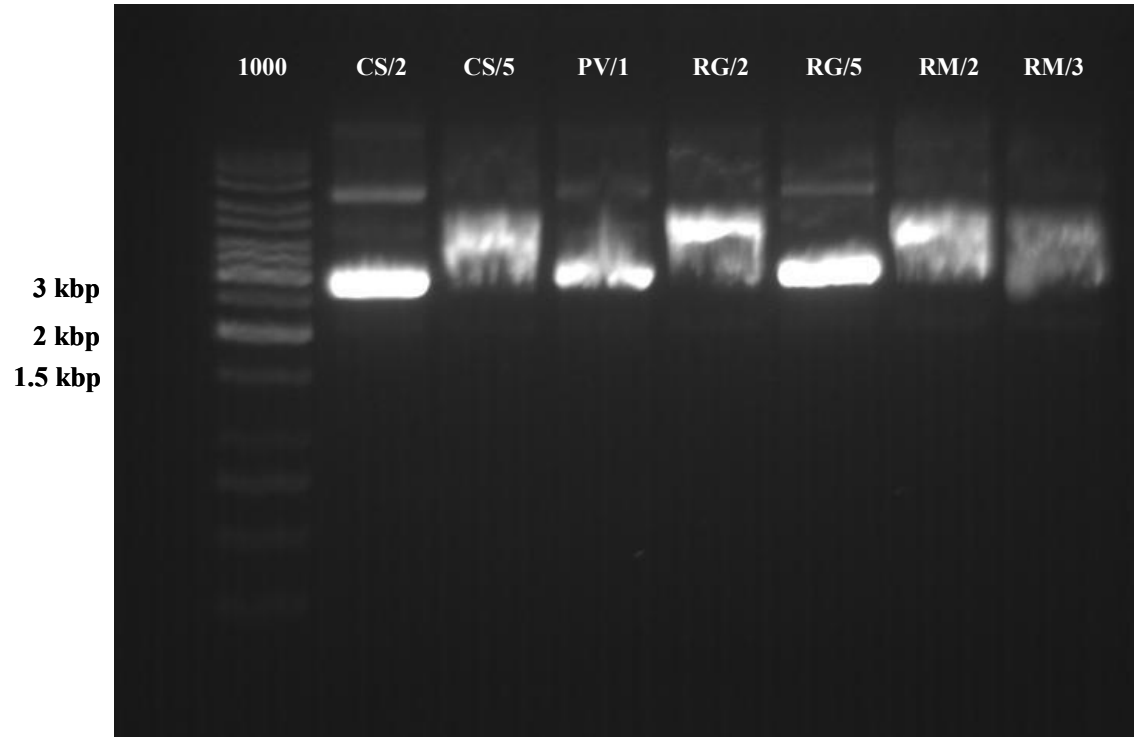


Fig. 17: Verifying electrophoresis of minipreparations of cloned DNA. CS *Cannabis sativa*, PV *Phaseolus vulgaris*, RG *Ruta graveolens*, RM *Ruta montana*.

7.5.6 *Assessment of new sequences*

New amino acids sequences obtained in ABI format were treated by software DNA BASER v. 2.11.0[148] in fully functional trial version. This software automatically detects and assembles sequences belonging to the same contig and suggests alterations to correct discrepancies and mismatches in the low quality ends. Fragments of DNA of *Ruta graveolens*, *Cannabis sativa* and *Nicotiana tabacum* were succesfully corrected, but sequences of *Humulus lupulus* and *Ruta montana* were not reliable. These fragments included many inreparable mistakes. They were excluded from the next alignment.

Repaired sequences were compared with all of 15 complete leguminous IFS genes. The alignment analyses of the sequences were performed with use of the program Vector NTI 9 and on-line on the web site http://www.ebi.ac.uk/emboss/align/ (downloaded 11.4.2008). The alignment algorithm either inserts a gap or allows a mismatch to remain in alignment and in order to maximize similarity, according to the evolutionary model, what is summarized in the substitution matrix. The mismatch may be the result of a mutation or sequencing error.

Some parameters and statistics of the alignment are in the following **Tables VIII-X**. **Identity** (e.g. 614/769 (79.8%)) represents a count of the number of positions over the length of the alignment where all of the residues or bases at that position are identical. It is followed by '/769', the length of the alignment, and '(79.8%)', the percentage of positions in the alignment, where there are identities. **Similarity** represents a count of the number of positions over the length of the alignment where ≥ 51% of the residues or bases at that position are similar. Any two residues or bases are defined as *similar* when they have positive comparisons (as defined by the comparison with matrix being used in the alignment algorithm). It is followed by '/769, the length of the alignment, and '(79.8%)', the percentage of positions in the alignment, where there are similarities. Identity and Similarity values are the same, in the case of the alignment of two sequences. **Gaps** (e.g. 91/769 (11.8%)) represents a count of the number of positions over the length of the alignment where there are one or more sequences with a gap. It is followed by '/769', the length of the alignment, and '(11.8 %)', the percentage of positions in the alignment, where there are gaps. The columns (**Tables XI-XIII**) “START” and “END” specify to which position of the whole legume IFS gene, the first and the last nucleotide of the PCR product align to. The Identity, Similarity and Gaps values refer to the alignment of the appropriate part of the legume IFS gene (**Fig. 18**).

Identities between all IFS genes and new sequences were more than 65% in all cases at the nucleotide level; in the case of the *Ruta graveolens* and IFS gene of *Pueraria montana* even more than 79%. Alignement of this pair is demonstrated in **Fig 18**. The position of fragments of *Ruta graveolens*, *Cannabis sativa* and *Nicotiana tabacum* is approximately identical. The most of the length of the cloned fragments was aligned to the cDNA of selected legume IFS genes. However, one part of the cloned fragments (position 176-260) did not match. The reason for this could be that the section represents an intron or that the IFS sequence in analysed plants differs from the legume IFS. The possibility that the cloned fragments are not a part of a gene encoding IFS protein but are part of a different gene also has to be taken into account.

Table XI.: Alignment of cDNA of legume IFS genes and the cloned DNA fragment of *Ruta graveolens*, L-lenght of alignment product, I-identity, G-gaps

***Ruta graveolens* (767 bp) vs.**		**L**	**I**	**G**	**Position (bp)**	
		bp	**%**	**%**	**START**	**END**
Glycine max	AF 195798	775	77.7	12.0	720	1409
	AF 195799	767	76.8	11.3	720	1400
	AF 195819	767	76.8	11.3	720	1400
	AY 552613	767	76.9	11.3	735	1414
	DQ 835285	775	77.5	12.0	720	1409
Medicago truncatula	AY 167424	776	70.9	12.8	720	1405
	AY 939826	770	70.5	12.1	720	1400
Pisum sativum	AF 532999	776	70.1	12.8	720	1405
Pueraria montana	AF 462633	769	**79.8**	11.8	720	1399
Trifolium pratense	AF 195810	767	76.3	11.3	720	1399
	AF 195811	767	76.5	11.3	720	1399
	AY 253284	783	70.9	14.4	723	1408
Vigna radiata	AF 195807	767	76.8	11.3	720	1399
	AF 195808	767	76.5	11.3	720	1399
	AF 195809	767	76.5	11.3	720	1399

Table XII.: Alignment of legumes IFS genes and DNA fragment of *Cannabis sativa.*

***Cannabis sativa* (783 bp) vs.**		**L**	**I**	**G**	**Position (bp)**	
		bp	**%**	**%**	**START**	**END**
Glycine max	AF 195798	787	76.1	13.6	718	1402
	AF 195799	785	74.4	13.1	667	1401
	AF 195819	787	74.5	13.7	718	1402
	AY 552613	785	74.5	13.1	732	1417
	DQ 835285	785	75.9	13.1	718	1402
Medicago truncatula	AY 167424	791	68.4	13.8	717	1408
	AY 939826	785	68.0	13.1	717	1402
Pisum sativum	AF 532999	797	68.4	15.1	717	1408
Pueraria montana	AF 462633	788	**77.7**	13.8	718	1402
Trifolium pratense	AF 195810	788	74.1	13.7	718	1402
	AF 195811	787	74.2	13.6	718	1402
	AY 253284	808	68.3	17.1	716	1402
Vigna radiata	AF 195807	787	74.5	13.6	718	1402
	AF 195808	787	74.2	13.6	718	1402
	AF 195809	787	74.2	13.6	718	1402

Table XIII: Alignment of legumes IFS genes and DNA fragment of *Nicotiana tabacum.*

***Nicotiana tabacum* (763 bp) vs.**		**L**	**I**	**G**	**Position (bp)**	
		bp	**%**	**%**	**START**	**END**
Glycine max	AF 195798	763	76.9	12.3	720	1388
	AF 195799	763	74.7	12.3	720	1388
	AF 195819	763	74.7	12.3	720	1388
	AY 552613	763	74.8	12.3	745	1404
	DQ 835285	763	76.8	12.3	720	1388
Medicago truncatula	AY 167424	779	69.4	14.9	720	1388
	AY 939826	774	69.3	14.5	720	1383
Pisum sativum	AF 532999	769	69.6	13.1	720	1394
Pueraria montana	AF 462633	763	**78.9**	12.3	720	1388
Trifolium pratense	AF 195810	763	74.4	12.3	720	1388
	AF 195811	763	74.6	12.3	720	1388
	AY 253284	779	69.4	15.4	723	1397
Vigna radiata	AF 195807	763	74.7	12.3	720	1388
	AF 195808	763	74.4	12.3	720	1388
	AF 195809	763	74.4	12.3	720	1388

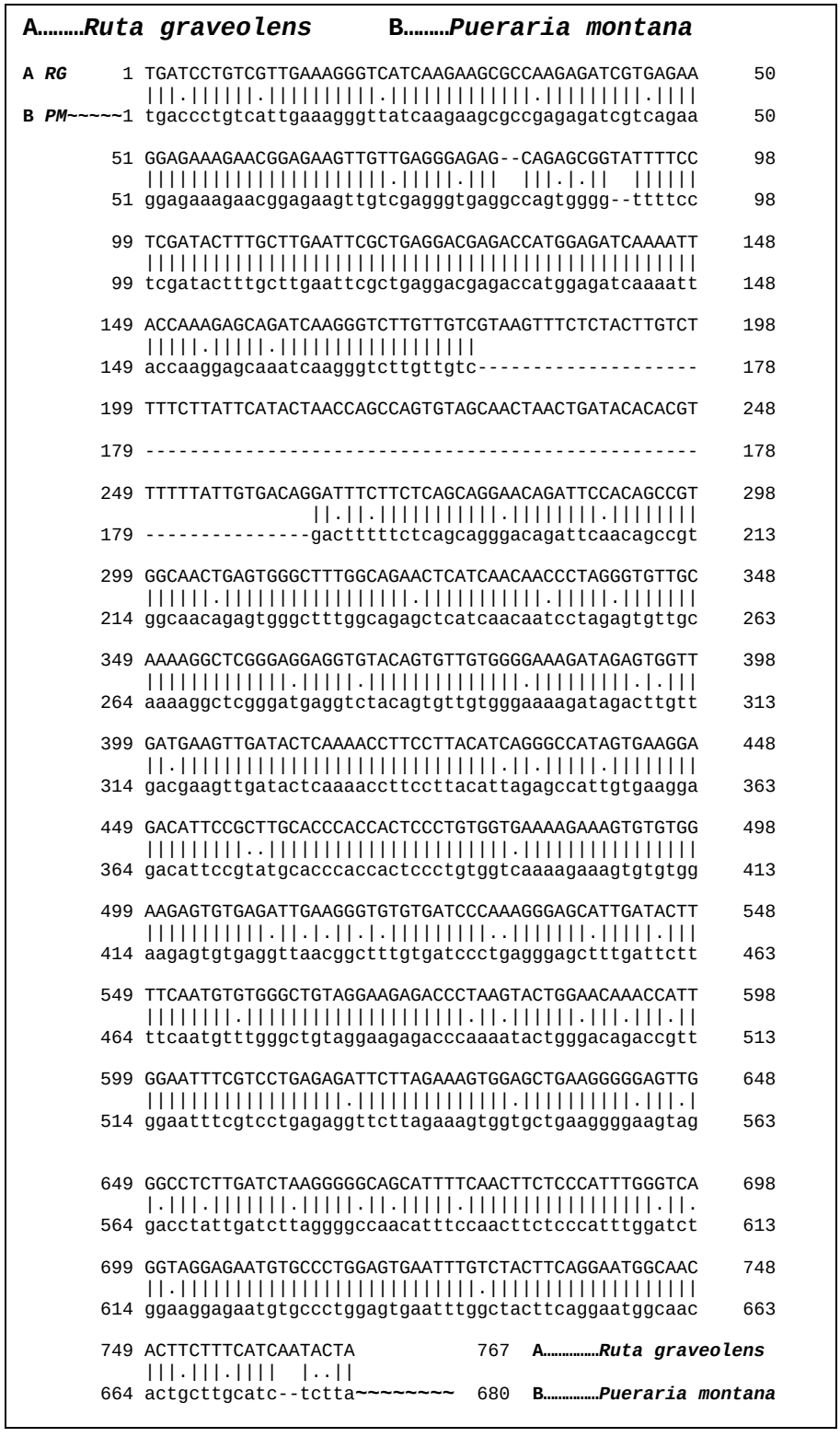


Fig. 18: Alignment fragments of IFS gene *Pueraria montana* and *Ruta graveolens*. Matrix: EDNAFULL, Gap penalty: 10.0, Extend penalty: 0.5, Length: 769, Identity: 614/769 (79.8%), Similarity: 614/769 (79.8%), Gaps: 91/769 (11.8%), Score: 2730.5

However, the NCBI Web BLAST (Basic Local Alignment Search Tool)[149] service provides comparing of nucleotide sequences with the NCBI nucleotide databases. The BLAST nucleotide algorithm finds similar sequences to the one in interest. The alignment of new fragments of *Ruta graveolens*, *Cannabis sativa* and *Nicotiana tabacum* and nucleotide databases gave 80-98% coverage with sequence of IFS gene of Fabaceae family. In the case of the same alignment with IFS gene of *Beta vulgaris*, the only non-legume that has been described was of 88% coverage.

It has shown the utility the CODEHOP PCR primer design strategy for identifying and characterizing previously unknown DNA. While the focus of this study was on the few plant species, other plant families can be easily targeted with use of analogous approaches.

In order to confront these results with IFS from legumes and to obtain information about functional, structural or evolutionary relationships between the sequences, it is necessary to identify the whole gene.

8 CONCLUSIONS

- Five competitive ELISAs in combination with semipreparative HPLC were used for the determination of individual isoflavones in 20 species from Rutaceae family belonging to five genera, i.e.: *Citrus*, *Fortunella*, *Poncirus*, *Ruta* and *Severinia*. The presence of several important isoflavones, i.e. daidzein, genistein, biochanin, formononetin, and their glucosides, was demonstrated. Quantitative, as well as qualitative, data were different between individual species. Certain isoflavonoid immunoreactivities have been detected in all samples under study. The HPLC-MS-SIM analysis confirmed the presence of all immunoreactive isoflavones; moreover, ononin, for which an immunoassay was not available, was detected. The general presence of biosynthesis of isoflavonoids in the Rutaceae family could be proposed.
- The spectrum of isoflavones in wild type *A. thaliana* was observed, despite the absence of a homologue to leguminous IFS in *A. thaliana* genome. It is possible to conclude that in the *A. thaliana* genom must be another gene responsible for isoflavone biosynthesis.
- With the help of molecular biology methods, the study was focused on the identification of gene/genes encoding the IFS apart from Fabaceae family. There were identified new sequences in the genome of *Cannabis sativa, Ruta graveolens* and *Nicotiana tabacum*, highly homologous to IFS described in several Fabaceae species. Now there is working on acquiring the whole sequences of the genes from these plants.
- The system of combination of ELISA methods, semipreparative HPLC and HPLC-MS, is applicable for screening of low levels isoflavones in non-leguminous taxa.

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10 LIST OF ABBREVIATIONS

4CL	4-coumaroyl-CoA-ligase
Ab	antibody
AcOH	acetic acid
AG	antigen
ASCR	Academy of Science of the Czech Republic
bp	base pairs
BSA	bovine serum albumin
C4H	cinnamate-4-hydrolase
cDNA	***genomic deoxyribonucleic acid***
CE	capillary electrophoresis
CODEHOP	consensus-degenerate hybrid oligonucleotide primer
CTAB	cetyltriethylammonium bromide
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
dGTP	2'-deoxyguanosine 5'-triphosphate
DMSO	dimethyl sulfoxide
dNTP	deoxyribonucleotide triphosphate
dTTP	2'-deoxythymidine 5'-triphosphate
ED	electrochemical detector
EDTA	ethylendiamintetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ESI	electrospray ionization
G	gaps
GC	gas chromatography
HN-APCI	heated nebuliser-atmospheric pressure chemical ionization
HP	Hewlett Packard
HPLC	high performance liquid chromatography
HRT	hormone replacing therapy
CHD	coronary heart desease
CHI	chalcone isomerase
CHR	chalcone reductase
CHS	chalcone synthase

I	identity
ICT	Institute of Chemical Technology
IFD	isoflavone dehydrogenase
IFS	isoflavone synthase
IOMT	isoflavone-*O*-methyltransferase
IPTG	isopropyl-1-thio-ß-D-galactoside
IR	infrared
Kbp	kilo base pairs
L	lenght
LB media	Luria Bertani media
LDL cholesterol	low-density lipoprotein cholesterol
MALDI-TOF-MS	matrix-assisted laser desorption/ionization-time of flight-mass spectroscopy
MEKC	micellar electrophoretic capillary chromatography
MeOH	methanol
MS detector	mass spectrometric detector
MUAF, AF	Mendel University of Agriculture and Forestry in Brno, Faculty of Agronomy
NADHP	nicotinamide adenine dinucleotide phosphate
NCBI	National Center for Biotechnology Information
OPD	*o*-phenylendiammine
PAL	phenylalaninammonia lyase
PBS-TW	phosphate buffered saline-Tween
PCR	polymerase chain reaction
PTFE	polytetrafluoroethylen
PTKs	protein tyroxine kinases
RIA	radioimmunoassay
RPM	revolutions per minute
SERMs	selective estrogen receptor modulators
SFE	supercritical fluid extraction
SIM	single ion monitoring
SPE	solid phase extraction
TAE buffer	mixture of Tris BASE, sodium acetate and EDTA
Taq DNA polymerase	thermostable DNA polymerase named after the thermophilic bacterium *Thermus aquaticus*

TLC	thin-layer chromatography
T_m	melting temperature
TR-FIA	time-resolved fluoroimmunoassay
Tris BASE	2-amino-2-(hydroxymethyl)-1, 3-propanediol
UV-Vis-DAD detector	ultraviolet-visible diode array detector
X-Gal	5-bromo-4-chloro-3-indoly-galactoside

11 PUBLICATION ACTIVITIES

Original articles

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12 ATTACHMENTS

Attachment 1: Distribution of Isoflavonoids in Non-Leguminous Taxa - an Update, Macková Z., Koblovská R., Lapčík O.: *Phytochemistry* **2006**, *67*, 849.

Attachment 2: Isoflavonoids in the Rutaceae Family: Twenty Selected Representatives of the Genera *Citrus*, *Fortunella*, *Poncirus*, *Ruta* and *Severinia*, Koblovská R., Macková Z., Vítková M., Kokoška L., Klejdus B., Lapčík O.: *Phytochemical analysis* **2008**, *19*, 64.

Attachment 3: Isoflavonoids are Present in *Arabidospis thaliana* Despite the Absence of any Homologue to Known Isoflavonoids Synthases, Lapčík O., Honys D., Koblovská R., Macková Z., Vítková M., Klejdus B.: *Plant Physiology and Biochemistry* **2006**, *44*, 106.