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Inhibition of Drug Glucuronidation by Extracts and Constituents of St. John's wort (*Hypericum perforatum*) and Thyme (*Thymus vulgaris*)

DIPLOMA THESIS

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Würzburg and Hradec Králové 2011

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„Hereby I declare that this thesis is my original author's work. All literature and other sources, which I used for the elaboration of this thesis, are stated in the references and properly cited in the text. The thesis has not been used to obtain different or the same degree."

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ACKNOWLEDGEMENTS

I would like to thank the Faculty of Pharmacy for the chance to study abroad and PD Dr. Unger and his team, especially Michael Völker, for their help during my stay in Germany. A very special thanks to PharmDr. Iva Boušová, Ph.D., whose understanding, support and help made this thesis possible.

ABSTRACT

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Title of diploma thesis: Inhibition of Drug Glucuronidation by Extracts and Constituents of St. John's wort (*Hypericum perforatum*) and Thyme (*Thymus vulgaris*)

The issue of herb-drug interactions is discussed in last years more than ever before due to all over the world increasing popularity of herbal drugs. Whereas the awareness of herbal drugs interactions with cytochrome P450 is quite broad, the issue of their interaction with UDP-glucuronosyltransferase (UGT) enzymes, one of the main participants of the second phase metabolism in humans, is still full of questions.

This work is focused on medicinal herbs St. John's wort (*Hypericum perforatum*) and thyme (*Thymus vulgaris*) and their impact on glucuronidation in the *in vitro* studies. The HPLC method for fractionation of herbal extracts was developed. The inhibitory activity of herbal extracts, individual fractions, and selected constituents on UGT was studied in human liver and/or human intestinal microsomes using model substrate TFMU (4-trifluoromethylumbelliferone). Products of these inhibition assays (TFMU glucuronides) were analyzed by RP-HPLC with fluorescence and UV detection as well as by LC/MS/MS. The residual UGT activity was calculated.

St John's wort (SJW) and thyme extracts, as well as biflavones of SJW and flavonoid isorhamnetin exerted significant concentration-dependent UGT inhibitory activity, which was in all performed assays more pronounced in human intestinal than in human liver microsomes. The inhibitory activity of some fractions was also high. Based on the data obtained, it seems likely that mainly naphthodianthrones and phloroglucinols of SJW and flavonoids of thyme may contribute to UGT inhibition. This work showed that SJW, thyme, and some of their constituents are able to significantly inhibit glucuronidation reactions. Further studies are necessary to evaluate the clinical importance of this observation.

ABSTRAKT

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Název diplomové práce: Inhibice glukuronidace léčiv extrakty a obsahovými látkami třezalky tečkované (*Hypericum perforatum*) a tymiánu obecného (*Thymus vulgaris*)

O problematice interakcí rostlinných látek s léčivy se v posledních letech díky celosvětově se zvyšující oblibě přírodních léčiv hovoří více, než kdykoli předtím. Zatímco povědomí o interakcích rostlinných léčiv s cytochromem P450 je poměrně široké, problematika jejich interakcí s enzymy UDP-glukuronosyltransferasami (UGT), jedněmi z hlavních účastníků druhé fáze metabolismu u člověka, je stále plná otázek.

Tato práce je zaměřena na léčivé rostliny třezalku tečkovanou (*Hypericum perforatum*) a tymián obecný (*Thymus vulgaris*) a jejich dopad na glukuronidaci v *in vitro* studiích. Rostlinné extrakty byly frakcionovány pomocí vyvinuté HPLC metody. Inhibiční aktivita rostlinných extraktů, jednotlivých frakcí a vybraných obsahových látek na UGT byla studována v lidských jaterních a/nebo lidských intestinálních mikrosomech za použití modelového substrátu TFMU (4-trifluoromethylumbeliferonu). Produkty těchto inhibičních testů (glukuronidy TFMU) byly analyzovány pomocí HPLC na reverzních fázích s fluorescenční a UV detekcí i pomocí LC/MS. Byla vypočítána zbytková aktivita UGT.

Extrakty z třezalky a tymiánu, stejně jako biflavony z třezalky a flavonoid isorhamnetin z tymiánu vykázaly významnou, na koncentraci závislou UGT inhibiční aktivitu, která byla ve všech prováděných testech výraznější v lidských intestinálních než v lidských jaterních mikrosomech. Inhibiční aktivita některých frakcí byla také vysoká. Na základě získaných dat se zdá pravděpodobné, že zejména naftodianthrony a floroglucinoly třezalky a flavonoidy tymiánu mohou k inhibici glukuronidace přispívat. Tato práce ukázala, že třezalka tečkovaná, tymián obecný a některé z jejich obsahových látek jsou schopny významně inhibovat glukuronidační reakce. Další studie k vyhodnocení klinického významu tohoto pozorování jsou nezbytné.

CONTENTS

1. INTRODUCTION

This work was carried out at the Department of Pharmaceutical Chemistry, Institute of Pharmacy and Food Chemistry of Julius-Maximilians University Würzburg under the supervision of PD Dr. Matthias Unger. The long-term research of his team is oriented on drug metabolism in human body and its influence by herbal extracts. Currently, herb-drug interactions at the level of glucuronidation are one of the main focuses.

Glucuronidation is one of the most important biotransformation pathways in humans for both endogenous compounds and xenobiotics. Inhibition of UDP-glucuronosyltransferases (UGT), enzymes participating in this reaction, may significantly modify metabolism of some substances. With the increasing popularity of herbal supplements, the issue of herb-drug interactions is discussed more and more. The wide use of herbal supplements is in contrast with poor understanding of their pharmacology. Based on previous clinical studies, evaluation of pharmacokinetic drug interactions between herbal and chemical drugs has been shown actual and important.

This work deals with the inhibitory effect of extracts of St. John's wort (*Hypericum perforatum*) and thyme (*Thymus vulgaris*) on the activity of UGT. The extract, extract fractions, as well as active constituents, which have not been involved in previous studies of the team of Dr. Unger yet, were tested in inhibition assays with a prototypic glucuronidation substrate in human liver and intestinal microsomes. Products were analyzed by HPLC and LC/MS analysis. The aim of this work was to evaluate the inhibitory activity of tested samples and try to determine particular compounds that are responsible for the observed inhibition.

2. GENERAL PART

In the general part of this diploma thesis, an overview on chemical composition and pharmacology of St. John's Wort (*Hypericum perforatum*) and thyme (*Thymus vulgaris*) as well as herb-drug interactions including basic information on the biotransformation of xenobiotics with emphasis on glucuronidation (i.e. conjugation of xenobiotics with UDP-glucuronic acid) is provided.

2.1. HERB-DRUG INTERACTIONS

Herb-drug interactions have received more attention in last years due to the all over the world increasing popularity of herbal medicine. Although herbal drugs are generally considered natural, and thus less harmful than chemicals, the widespread use of herbal supplements is contrary to a low knowledge of their pharmacology.

Unlike chemical drugs, herbal supplements are usually not required to get through an often demanding pharmacological testing in a pre-registration process, nor is the post-marketing monitoring needed. Whereas herbal drugs have likely a safe therapeutical profile as a monotherapy, co-medication with nonprescription and commonly prescribed drugs may lead to various interactions - activation or inhibition of the metabolism of concomitantly taken drugs, under- or over-exposure to drugs, and consequently, treatment failure or adverse effects and toxicity.

There are many studies reporting about herb-drug interactions. More is known about interactions with cytochrome P450 enzyme system (CYP), as the main system of the phase I of drug metabolism, the data regarding effect of herbs on glucuronidation in humans are limited (Mohamed & Frye 2011).

2.1.1 Introduction to drug metabolism

Metabolism of xenobiotics, including drugs and herbal constituents, in the organism comprises transport processes and biotransformation reactions, which are generally divided into phase I and phase II. Xenobiotics are converted by enzymatic transformation using the metabolic pathways for endogenous substances.

2.1.1.1 Phase I metabolism

Phase I reactions involves hydrolysis, reduction, oxidation, hydration or isomerization. These reactions lead to an exposition or introduction of a functional

group (–OH, –NH_2, –SH or –COOH) into the original compound. This usually results in only a small increase in hydrophilicity (Parkinson 2001).

The most important role in phase I drug metabolism plays the cytochrome P450 system. It is a family of heme-containing microsomal enzymes particularly concentrated in the liver and intestinal mucosa, but also found in the kidneys, skin, lung, and other tissues (Cott 2001). Six cytochrome P450 enzymes mediate the oxidative metabolism of most of the drugs in common use: CYP1A2, CYP2C8, CYP2C9, CYP2C19, CYP2D6, and CYP3A4 (Caraco 1998).

2.1.1.2 Phase II metabolism

Phase II of biotransformation reactions include glucuronidation, sulfation, acetylation, methylation, and conjugation with glutathione or amino acids. Most of the phase II reactions result in a large increase in hydrophilicity of the compounds and therefore enhance their biliary or renal excretion. Phase II reactions may or may not be preceded by phase I reactions (Turkey et al. 2000).

2.1.1.2.1 Glucuronidation

Glucuronidation is one of the conjugation phase II reactions, which has evolved as a highly selective process in higher organisms (Turkey et al. 2000). The reaction is carried out by UDP-glucuronosyltransferases (UGTs).

UGT superfamily, which includes about 80 families, is further divided into families and subfamilies according to the amino acid sequence. In humans, four UGT families have been described to date, termed UGT1, UGT2, UGT3, and UGT8. Isoforms from UGT1 and UGT2 family are of the main importance. Physiological function of UGT3 family is unknown; UGT8 family uses UDP-galactose to galactosidate ceramide in the synthesis of glycosphingolipids and cerebrosides (Mackenzie et al. 2005).

Enzymes of UGT1 and UGT2 family (divided in subfamilies 2A and 2B) catalyze the transfer of a glucuronosyl residue from UDP-glucuronic acid (UDP-GA), which acts as a co-substrate, to the functional groups of available substrates (e.g. aglycones, small molecular weight lipophilic chemicals) to form ß-D-glucuronides. During the reaction, the α-bond between UDP and the hexose moiety is converted into a β-bond between the acceptor and the sugar (Mackenzie et al. 2008). Synthesis of UDP-GA and progress of the glucuronidation reaction are shown in Fig. 1 and Fig. 2, respectively.

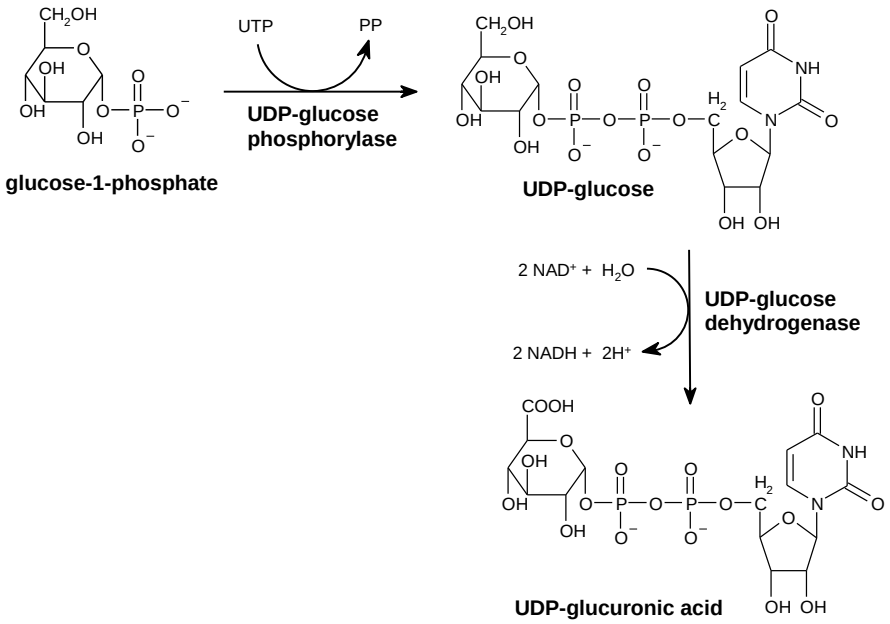


Figure 1 Synthesis of UDP-glucuronic acid (Testa & Krämer 2008).

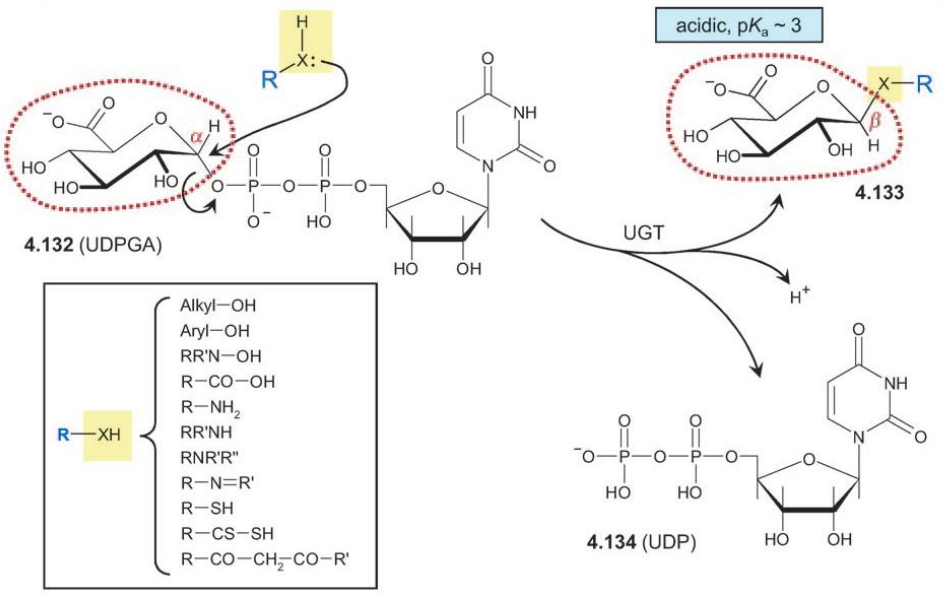


Figure 2 General scheme of glucuronidation reaction including a summary of the functional groups being potential substrates for UDP-glucuronosyltransferases (Testa & Krämer 2008).

The β-D-glucuronides can be formed through hydroxyl (alcoholic, phenolic), carboxyl, sulfuryl, or amino (primary, secondary, or tertiary) linkages. This type of structural diversity in substrate specificity allows thousands of agents, including both endogenous substances (bilirubin, steroid hormones, thyroid hormones, bile acids, and fat-soluble vitamins) and xenobiotics (drugs, chemical carcinogens, environmental

pollutants or dietary constituents) to be targeted by glucuronidation (Turkey et al. 2000).

The main focus is on hepatic glucuronidation since the liver is considered the primary clearance organ (Miners et al. 2010). Evidences indicate that UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A9, UGT2B7, and UGT2B15 are the enzymes of the greatest importance in hepatic drugs and xenobiotics metabolism (Turkey et al. 2000). However, multiple studies have indicated that some extrahepatic tissues, especially those of gastrointestinal tract but also kidney, prostate, mammary tissue, brain and placenta, has the same activity as well. Some of the enzymes, such as UGT1A7, UGT1A8, UGT1A10, and UGT2A1 are expressed exclusively in extrahepatic tissues. Distribution of UGT isoforms differs from tissue to tissue (Turkey et al. 2000, Kiang et al. 2005). Distribution of UGT isoforms in human body is shown in Table 1.

Table 1 Expression of human UDP-glucuronosyltransferase mRNA in the human body (Turkey et al. 2000, Kiang et al. 2005).

	Liver	Bile ducts	Esophagus	Stomach	Small intestine	Colon	Kidney	Brain	Pancreas	Mammary gland	Prostate
UGT1A1	X	X		X	X	X					
UGT1A3	X	X		X	X	X					
UGT1A4	X	X			X	X					
UGT1A6	X	X		X	X	X		X			
UGT1A7[a]			X	X							
UGT1A8			X		X	X	X				
UGT1A9	X					X	X				
UGT1A10		X	X	X	X	X	X				
UGT2A1[b]											X
UGT2B4	X				X						
UGT2B7	X		X		X	X	X	X	X		
UGT2B10	X		X		X					X	X
UGT2B11[c]	X										X
UGT2B15	X		X		X						X
UGT2B17	X										X
UGT2B28	X									X	

[a]Expressed also in lungs, [b]expressed also in nasal epithelium and fetal lungs, [c]expressed also in adrenal glands, skin, adipose tissue and lungs.

UGTs are membrane-bound enzymes with the active side facing lumen of the endoplasmic reticulum (ER) (Fisher et al. 2000). The membrane of ER creates a diffusion barrier for substrates, the co-substrate UDPGA, and resultant glucuronide products. Thus, a “latency” of activity occurs (Meech & Mackenzie 1997). By a disruption of this barrier, the maximal enzyme activity of UGT enzymes is attained. This can be reached by an use of detergents, but the more suitable agent is alamethicin, the antibiotic fungal peptide, which is known to insert into the membranes and form well defined pores (Fisher et al. 2000, Miners et al. 2010).

In recent years, there has been an interest in development of „probes“-compounds that permit *in vitro* testing of drug metabolism (Miners et al. 2010). Derivates of umbelliferone (4-methylumbelliferone and 4-trifluoromethylumbelliferone) have been proven as useful substrates for *in vitro* glucuronidation testing. The compounds are glucuronidated by various human UGT isozymes, including UGT1A1, UGT1A3, UGT1A6, UGT1A7, UGT1A8, UGT1A9, UGT1A10, UGT2B7, UGT2B15, and UGT2B17 (Baranczewski et al. 2004, Uchaipichat et al. 2004). TFMU (4-trifluoromethylumbelliferone) is considered to be an ideal substrate for testing of a large number of compounds during drug discovery (Baranczewski et al. 2004). Glucuronidation of TFMU is shown in Fig. 3.

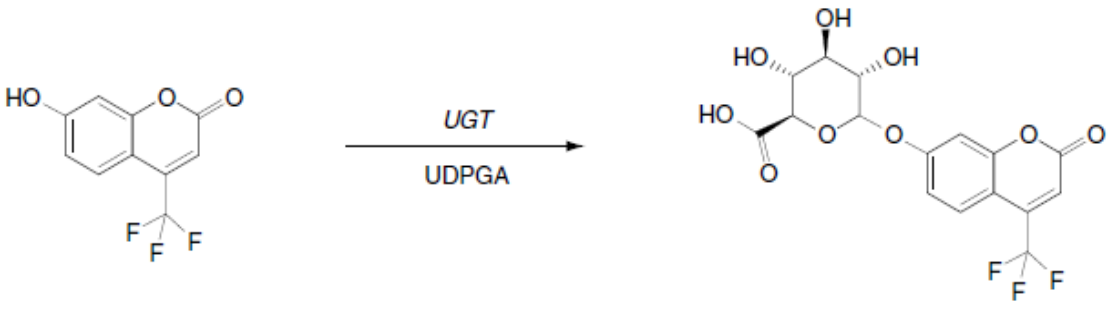


Figure 3 Chemical structures of TFMU and its respective glucuronide (Baranczewski et al. 2004).

2.1.1.3 Drug transporter P-glycoprotein

Beside the phase I and phase II reactions of biotransformation, P-glycoprotein (P-gp) plays an important role in bioavailability of some drugs. P-gp is a multidrug transporter, an adenosine triphosphate (ATP) dependent efflux pump, which moves substrates out of cells. It serves as the barrier in many tissues, such as renal, intestinal,

biliary, and cerebral endothelium or choroid plexus, thereby prevents xenobiotic accumulation in important organs of the body (Tanigawara 2000).

Life-threatening interactions have been reported particularly with drugs that are substrates of cytochrome P450 and/or P-glycoprotein (such as indinavir and cyclosporin). In metabolism of drugs eliminated mainly via glucuronidation (e.g. morphine and mycophenolic acid), the interactions at the level of UGT enzymes are the most important (Mohamed & Frye 2011)..

2.2 ST. JOHN'S WORT (*Hypericum perforatum*)

Hypericum perforatum (Hypericaceae), commonly known as St. John's wort (SJW), is the flowering herbaceous perennial plant widely distributed all over the world (Patočka 2003). It has been, due to its sedative properties, used for centuries as the natural remedy in the treatment of mild psychical disorders, such as insomnia or anxiety. Nowadays, it is one of the top-selling herbal dietary supplements for the treatment of depression, available as the over-the-counter drug or prescribed by physicians (Gaster & Holroyd 2000, Barnes et al. 2001).

The crude drug, Herba Hyperici, is collected just before or during the flowering period of this herb. Although the majority of its therapeutically active constituents are exclusively or predominantly accumulated in flowers, leaves and stems of *H. perforatum* are combined in the crude drug as well (Nahrstedt & Butterweck 1997). Extracts from the crude drug are available in several formulations (e.g. capsules, tablets, tinctures, topical preparations) in herbal products as well as homoeopathic preparations (Turkey & Strassburg 2000, Ang et al. 2002).

2.2.1 Chemistry of St. John's wort

Chemical composition (the content of various constituents) can vary depending on ecological factors, time of harvest, and processing of the harvested plant material. Variation in the content of secondary metabolites is influenced by physiological and genetic factors even within species (Košuth et al. 2003). The main derivates present in the dry crude drug of SJW are naphthodianthrones, phloroglucinols, flavonol derivates, biflavonoids, phenylpropanes, and in smaller amounts also other substances (Nahrstedt & Butterweck 1997).

2.2.1.1 Naphthodianthrones

Naphthodianthrones are typical substances of the genus *Hypericum*, present in flowers and leaves of the crude drug material (Hegnauer 1989). The main representatives of *H. perforatum* are hypericin and pseudohypericin (Fig. 4). The protoderivates protohypericin and pseudoprotohypericin, biosynthetic precursors that lack the 4,4'-bond (Fig. 4), have also been isolated from the plant, as well as an oxidation product of pseudohypericin, cyclopseudohypericin (Haeberlein et al. 1992). All hypericins are unstable towards light. Depending on the developmental stage of the plant, naphthodianthrones occur in concentrations of 0.03 to 0.3% (Čellárová et al. 1994). The amount of pseudohypericin is usually 2 to 4-fold higher than the concentration of hypericin (Košuth et al. 2003). Naphthodianthrones have an intense red color and phototoxic properties (Hölzl 1991). The major photosensitizing constituent of SJW is hypericin. It is believed to cause hypericism (sensitivity to sunlight following the ingestion of SJW) (Durán & Song 1986). Although hypericin was declared to be the compound with antidepressant effect in the past, currently hyperforin (phloroglucinol) was reported to have this activity. Nevertheless, many SJW extracts are still standardized by their content of hypericin (Gaster & Holroyd 2000, Barnes et al. 2001).

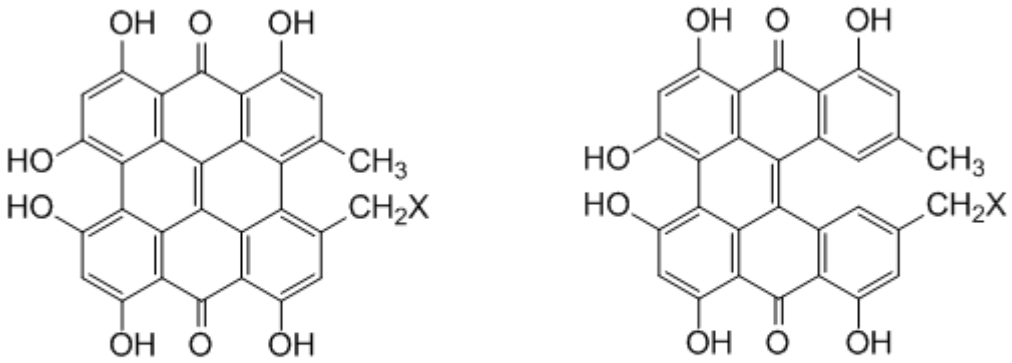


Figure 4 Chemical structures of the naphtodianthrone constituents of *Hypericum perforatum.*

2.2.1.2 Phloroglucinols

The main representative of the phloroglucinols in SJW is hyperforin (Fig. 5). Together with its derivative adhyperforin that contains one additional methyl group (Fig. 5), hyperforin occurs exclusively in the generative parts of the plant. The total

amount of hyperforin/adhyperforin increases from 2.0%/0.2% in flowers to 4.5%/1.6% in unripe and 4.4%/1.8% in ripe fruits. Consequently, plant material collected at the beginning of the fruit ripening period contains more hyperforin than material collected during the flowering time (Maisenbacher & Kovar 1992). Hyperforin and adhyperforin are extremely sensitive to oxidation and unstable in solution when exposed to the air (Tolonen et al. 2003). As mentioned above, hyperforin is believed to cause the antidepressant effect of the herb. Other phloroglucinol derivates of *H. perforatum* are hyperfirin and adhyperfirin (Fig.5). Their structures lack the hydrocarbon chain in the position 7 (Tatsis et al. 2007).

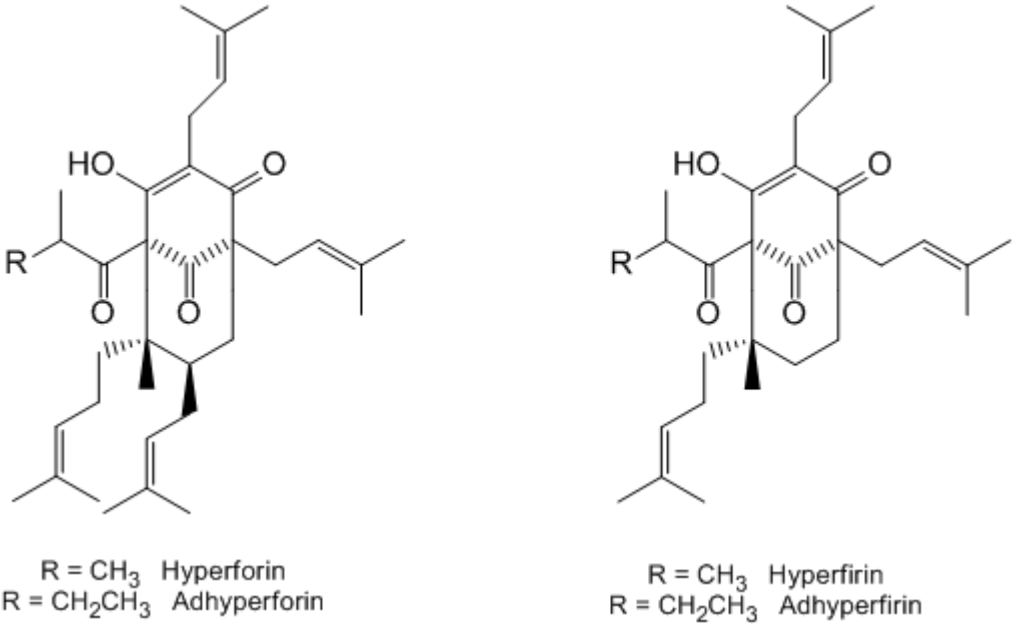


Figure 5 Chemical structures of the phloroglucinoles of *Hypericum perforatum.*

2.2.1.3 Flavonol glycosides

Flavonol glycosides represent with an amount of up to 4% the largest group of biologically active compounds in *H. perforatum*. Most of the flavonoids that are present in SJW are glycosides of quercetin. The free aglycone, which is present in the crude drug in a small amount, arises probably from hydrolysis of flavonoids during herb drying and processing. The dominate flavonoid components are rutin (quercetin-3-rutinoside) and hyperoside (quercetin-3-galactoside), followed by isoquercitrin (quercetin-3-glycoside), quercitrin (quercetin-3-rhamnoside), its derivate dihydroquercitrin (dihydroquercetin-3-rhamnosid), miquelianin (quercetin-3-glucuronide) and the free aglycone, quercetin (Nahrstedt & Butterweck 1997, Patočka 2003, Butterweck & Schmidt 2007). Their structures are shown in Fig. 6.

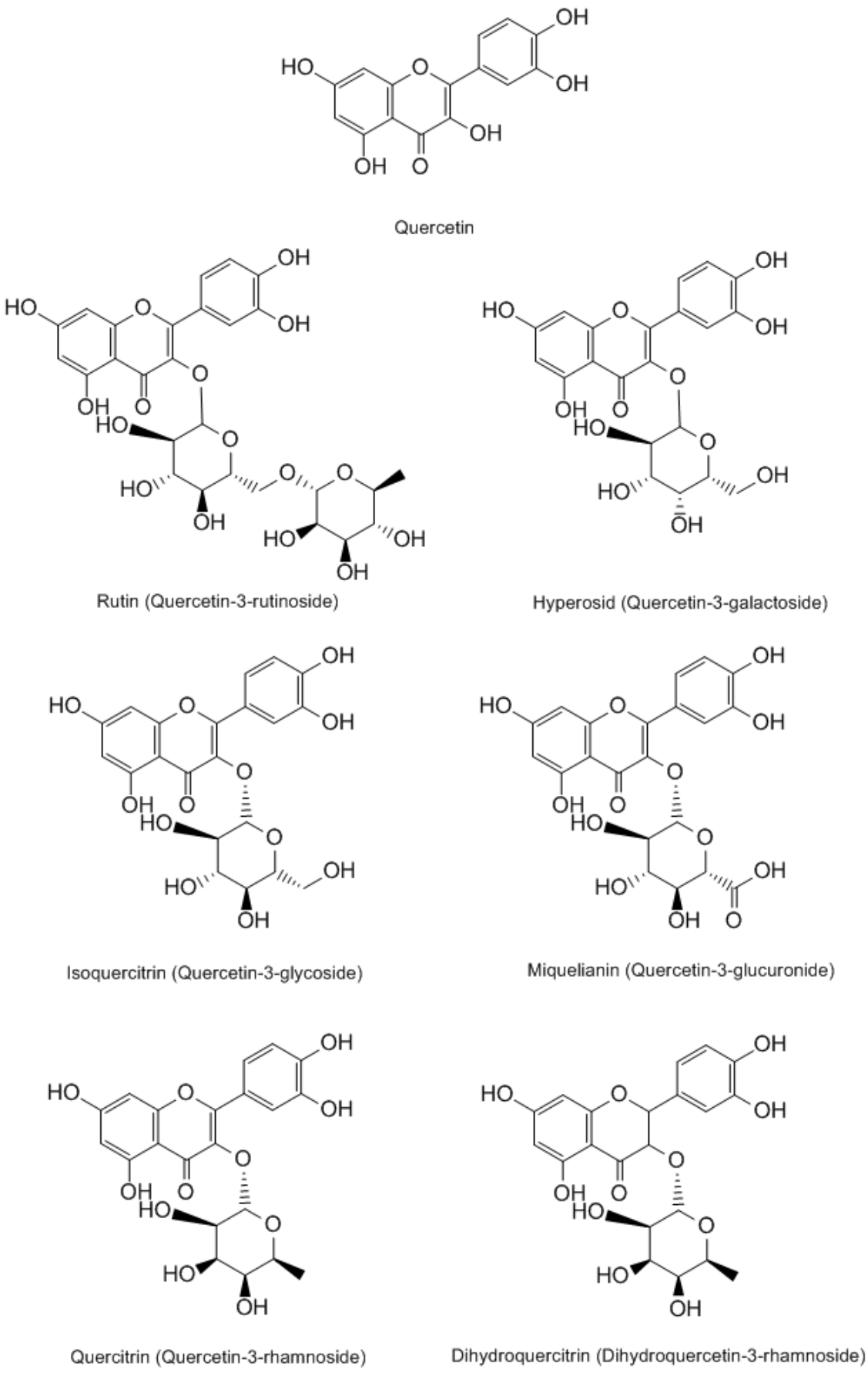


Figure 6 Chemical structures of the flavonol glycosides of *Hypericum perforatum.*

2.2.1.4 Biflavones

Dimeric flavones are represented by I3,II8-biapigenin and amentoflavone (I3',II8-biapigenin). These constituents occur exclusively in the flowering part of SJW in concentrations of 0.1 to 0.5% for I3,II8-biapigenin and 0.01 to 0.05% for amentoflavone, respectively (Berghöfer & Hölzl 1986). Their structures are shown in Fig. 7.

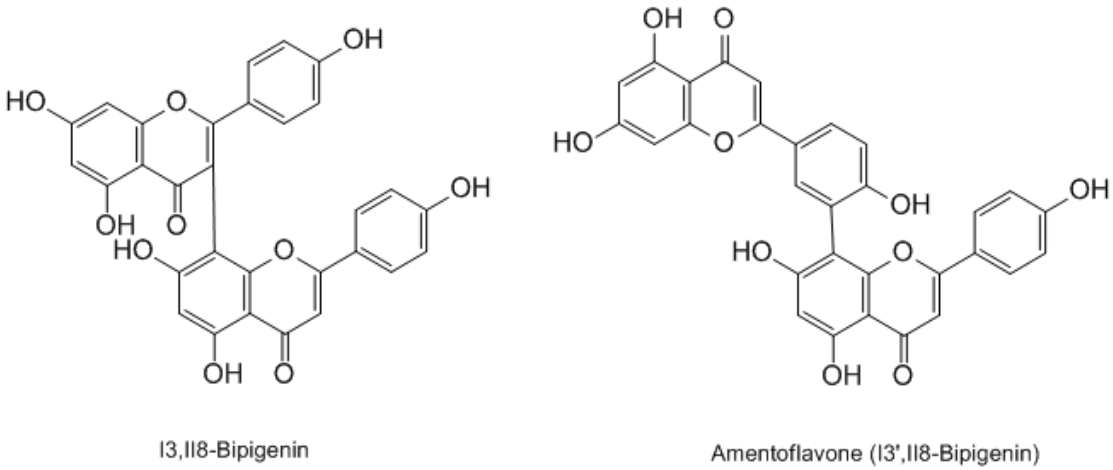


Figure 7 Chemical structures of the biflavones of *Hypericum perforatum.*

2.2.1.5 Phenylpropanes

Mainly esters of hydroxycinnamic acids, such as caffeic acid or *p*-coumaric acid, represent the group of phenylpropanes in SJW (Patočka 2003). Beside esters, chlorogenic acid and 3-*O*-coumaroylquinic acid have been described (Tatsis et al. 2007). Their structures are shown in Fig. 8.

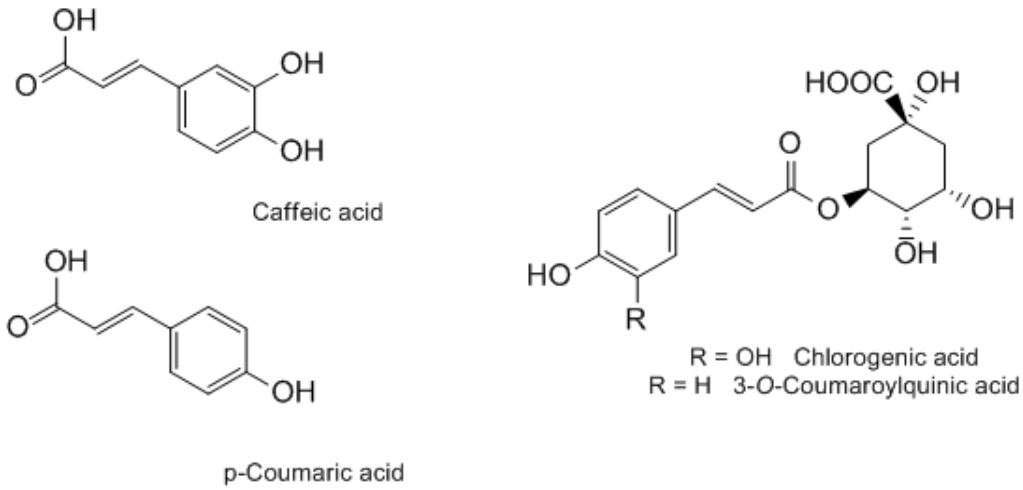


Figure 8 Chemical structures of the hydroxycinnamic acids of *Hypericum perforatum.*

Other constituents present in SJW extracts include tannins, proanthocyanidins (condensed tannins), xanthones, volatile oils, and amino acids (Patočka 2003).

2.2.2 Pharmacology of St. John's wort

Approximately 50–70% of the known phytochemical constituents of SJW exert their own biological activity. The best investigated group of constituents consists of the naphthodianthrones (hypericins) and phloroglucinols (hyperforin, adhyperforin). The group of flavonoids is pharmacologically also interesting. It seems likely that flavonoids do not have just their own activity, but also act as co-effectors to improve the biopharmaceutical properties of hypericin. In general, the clinical efficacy of SJW extracts is probably resulting from the combined contribution of several mechanisms, which themselves seem to have no significant effect (Butterweck & Schmidt 2007).

As mentioned previously, SJW is nowadays the best known for its use in the treatment of depression. Although many randomized controlled trials dealing with the antidepressant efficacy of St. John's wort have been carried out, its benefits are still ambiguous. Generally, it is believed that extracts of *Hypericum perforatum* improve symptoms of mild to moderately severe depressive disorders in adults more than placebo and similarly to standard antidepressants (Linde et al. 2005). The antidepressant mechanism of SJW has not been fully clarified yet (Johne et al. 1999). Evidences show that substances of SJW extracts bind to neuroreceptors in the brain, and/or inhibit the re-uptake of various neurotransmitters that are thought to be involved in depression. It was also considered that *Hypericum* extracts might have the same monoamine oxidase inhibitory effect as other monoamine oxidase inhibitors (MAOi) commonly used for the treatment of depression. Several recent studies have shown that the inhibitory potential of SJW on monoamine oxidase system is very small and insignificant if compared to the effect of commonly used drugs from MAOi group (Gaster & Holroyd 2000, Butterweck & Schmidt 2007).

Clinical trials with extracts of St John's wort have focused mainly on its effects in patients with depression, although there have been several studies exploring its use in other conditions including seasonal affective disorder, chronic fatigue, and premenstrual syndrome (Barnes et al. 2001). Beside the antidepressant efficacy, SJW has been proven in a few *in vitro* studies to have anxiolytic, antibacterial, antiviral and anti-inflammatory activity as well as cytotoxic and apoptosis-including effects in neoplastic cell lines (Schempp et al. 2002).

Safety and tolerability studies have revealed that SJW preparations are well tolerated (Gaster & Holroyd 2000), having even fewer adverse effects than

conventional antidepressants (Barnes et al. 2001, Linde et al. 2005). The incidence of adverse reactions associated with *Hypericum* is in placebo-controlled trials similar to that reported for placebo (Ernst et al. 1998) and generally mild. The most common reported adverse effects are gastrointestinal symptoms, nausea, allergic rash, confusion, and tiredness (sedation) (Gaster & Holroyd 2000, Barnes et al. 2001). Photosensitivity appears to be extremely rare adverse effect with recommended dosages of *Hypericum perforatum*.

2.2.3 Drug interactions with St. John's wort

Studies have confirmed that St. John's wort influences metabolism of drugs by affecting all of the previously mentioned systems: UGT and CYP enzymes as well as P-gp (Tarirai et al. 2010).

The best investigated interactions so far are those of the CYP enzymes. Crude extracts of *H. perforatum* have demonstrated inhibition of CYP1A2, CYP2C19, CYP2C9, CYP2D6, and CYP3A4, with a higher inhibitory activity on the last three mentioned isoforms (Obach 2000). Some studies have shown that SJW is capable of inhibiting CYP3A4 acutely and inducing it after repeated doses (Cott 2001).

To name active substances of SJW extract that are capable of enzyme inhibition, flavonoid quercetin has been shown to inhibit CYP3A4 (Li et al. 1994), and CYP1A2 (Obach 2000) *in vitro.*, as well as UGT1A and UGT1A9 hepatic isoforms of UGT. (Williams et al. 2002, Mohamed & Frye 2010). The phloroglucinol derivative hyperforin non-competitively inhibits CYP2D6 activity and competitively inhibits activity of CYP2C9 and CYP3A4. A competitive inhibition of CYP3A4, CYP2C9, and CYP1A2 activity was shown for the biflavonoids of SJW, e.g. I3,II8-biapigenin. Also hypericin has shown to be a potent inhibitor of some CYP isoforms (Obach 2000) and UGT1A6 in human colon cells (Volak 2010).

Clinical studies revealed the possibility of interaction between some commonly prescribed drugs and concomitantly taken SJW (Table 2). These interactions are pharmacodynamic (SSRIs, Triptans) but mostly pharmacokinetic. Thus, using SSRIs, selective serotonin reuptake inhibitors (citalopram, fluoxetine, fluvoxamine, paroxetine, sertraline), or triptans (sumatriptan, naratriptan, rizatriptan, zolmitriptan) together with *Hypericum perforatum* extract leads to increased serotonergic effect. Combination of SJW with HIV protease inhibitors (indinavir) and HIV non-nucleoside reverse transcriptase inhibitors (efavirenz, nevirapine) brings loss of HIV suppression. Unintended pregnancy or intermenstrual bleeding have been reported as a consequence of SJW and oral contraceptives co-medication. Interactions with warfarin,

cyclosporin, digoxin, or anticonvulsants (carbamazepine, phenobarbitone, phenytoin) also lead to a risk of reduced therapeutic effect (changes in blood clotting, internal bleeding, transplant rejection, seizures etc.) Patients taking these medicines should be advised not to start taking St. John's wort or to discuss its use with healthcare professionals (Barnes et al. 2001).

Table 2 Drug interactions with St. John's wort.

Drug	**Mechanism**	**Consequence**
SSRIs triptans	potentiating of serotonin concentrations[1]	increase in serotonergic effect
HIV protease inhibitors HIV non-nucleoside reverse transcriptase inhibitors	induction of CYP 3A4[1,2], P-gp[2] induction of CYP3A4[1,2]	reduction in antiretroviral efficacy
oral contraceptives	Induction of CYP1A2[1], CYP3A4[2]	unintended pregnancy, intermenstrual bleeding
warfarin	induction of CYP3A4, CYP2C9[1,3]	changes in INR[a] blood clotting, internal bleeding
cyclosporine	induction of CYP3A4 and P-gp[1,2]	transplant rejections
digoxin	induction of P-gp[1,2,4]	uncompensated heart rate, fibrillations
anticonvulsants	induction of CYP3A4[1,5], CYP2C9,CYP2C19[5]	seizures

[a]International normalized ratio is used for reporting results of blood coagulation tests
[1]Henderson 2002, [2]Borelli & Izzo 2009, [3]Tachjian et al. 2010, [4]Johne et al. 1999, [5]Johannessen & Landmark 2010.

As long as SJW has been reported not to inhibit the activity of monoamine oxidase, there is no need of warning against interactions with drugs from the MAOi group. The avoidance of foodstuffs containing tyramine (e.g. cheese or wine) and medicines containing sympathomimetic agents, which are groups known to interact with MAOi drugs, is also not necessary. Topical, homoeopathic preparations and

renally excreted non-psychotropic medicines are not likely to interact with SJW as well (Barnes et al. 2001).

2.3 THYME (*Thymus vulgaris*)

Thymus vulgaris, commonly thyme, is the herb of the Lamiaceae family, well known for its medicinal as well as non-medicinal use. The plant is reported to have a wide range of biological activities such as carminative, antispasmodic, antitussive, expectorant, bactericidal, anthelmintic or astringent activity (Sasaki et al. 2005). It is traditionally used as a treatment of upper respiratory tract illnesses, such as dry coughs, bronchitis, or laryngitis or for digestive disorders such as dyspepsia or gastritis (Rustaiyan et al. 1999, Sasaki et al. 2005).

For the content of flavoring compounds and essential oils, it is used in the food industry as an ingredient of savory formulations, sauces, and liqueurs (Guillén & Manzanos 1998), or in production of cosmetics. Dry or fresh, it is used as a culinary herb (Piccaglia & Marotti 1991).

2.3.1 Chemistry of thyme

The content of particular compounds present in the essential oil of *Thymus vulgaris* differs quantitatively as well as qualitatively within species or even chemotype. This is significantly influenced by environmental conditions (Guillén & Manzanos 1998, Sasaki et al. 2005).

Phenols constitute the major and the most analyzed group of thyme metabolites. Based on the chemical structure, substances can be further subdivided into four main categories- derivates of hydroxycinnamic acid, hydroxybenzoic acid derivates, flavonoids, and phenolic terpenes (Hossain et al. 2010). Natural polyphenols occur primarily in conjugated form, with one or more sugar residues. Detected can be both, unhydrolyzed structures as well as aglycones (Bravo 1998).

2.3.1.1 Hydroxycinnamic acid derivates

Derivates of hydroxycinnamic acid (Fig. 9) are represented by caffeic acid, chlorogenic acid, *p*-coumaric acid, rosmarinic acid, dicaffeoylquinic acid and ferulic acid (Hossain et al 2010).

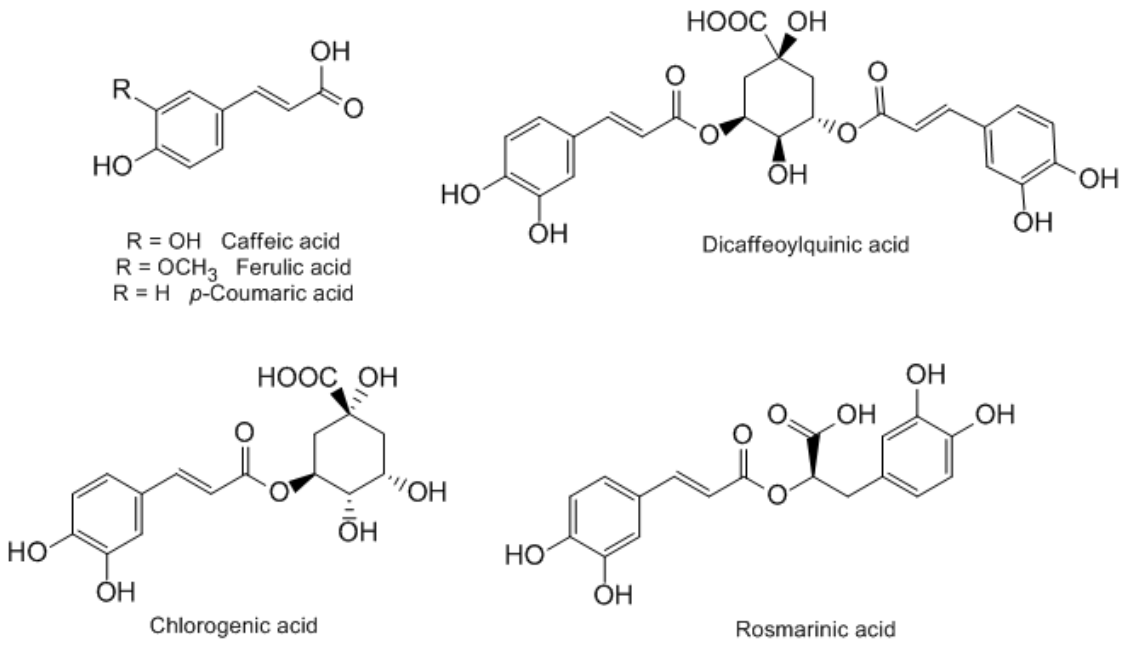


Figure 9 Hydroxycinnamic acid derivates of *Thymus vulgaris.*

2.3.1.2 Hydroxybenzoic acid derivatives

Structures of 4-hydroxybenzoic acid and its derivatives gallic acid, syringic acid, vanillic acid, and pyrocatechuic acid have been identified in thyme (Hossain 2010). Gentisic acid (2,4-dihydroxybenzoic acid) was detected as well (Proestos 2005). Their structures are shown in Fig. 10.

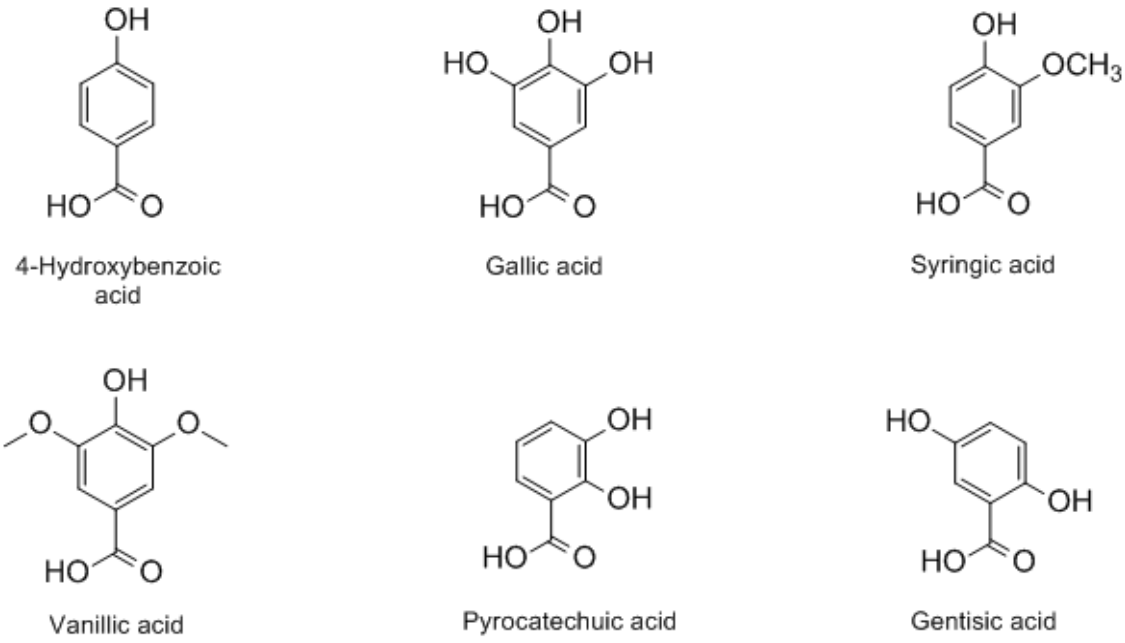


Figure 10 4-Hydroxybenzoic acid derivates of *Thymus vulgaris.*

2.3.1.3 Flavonol derivatives

Flavonoids represent the biggest group of secondary metabolites in thyme. The non-sugar-based flavonoids of thyme are quercetin, apigenin, luteolin, isorhamnetin, cirsimaritin, and gallocatechin. Flavonoids in thyme are most commonly found as glycoside derivates, from which the conjugates with hexose (e.g. glucose, galactose, fructose or rhamnose) and rutinose are the most usual (Hossain 2010, Rice-Evans et al. 1996). The best known glycoside is rutin (quercetin-3-rutinoside). Structures of the most common flavonoid derivatives found in thyme are shown in Fig. 11.

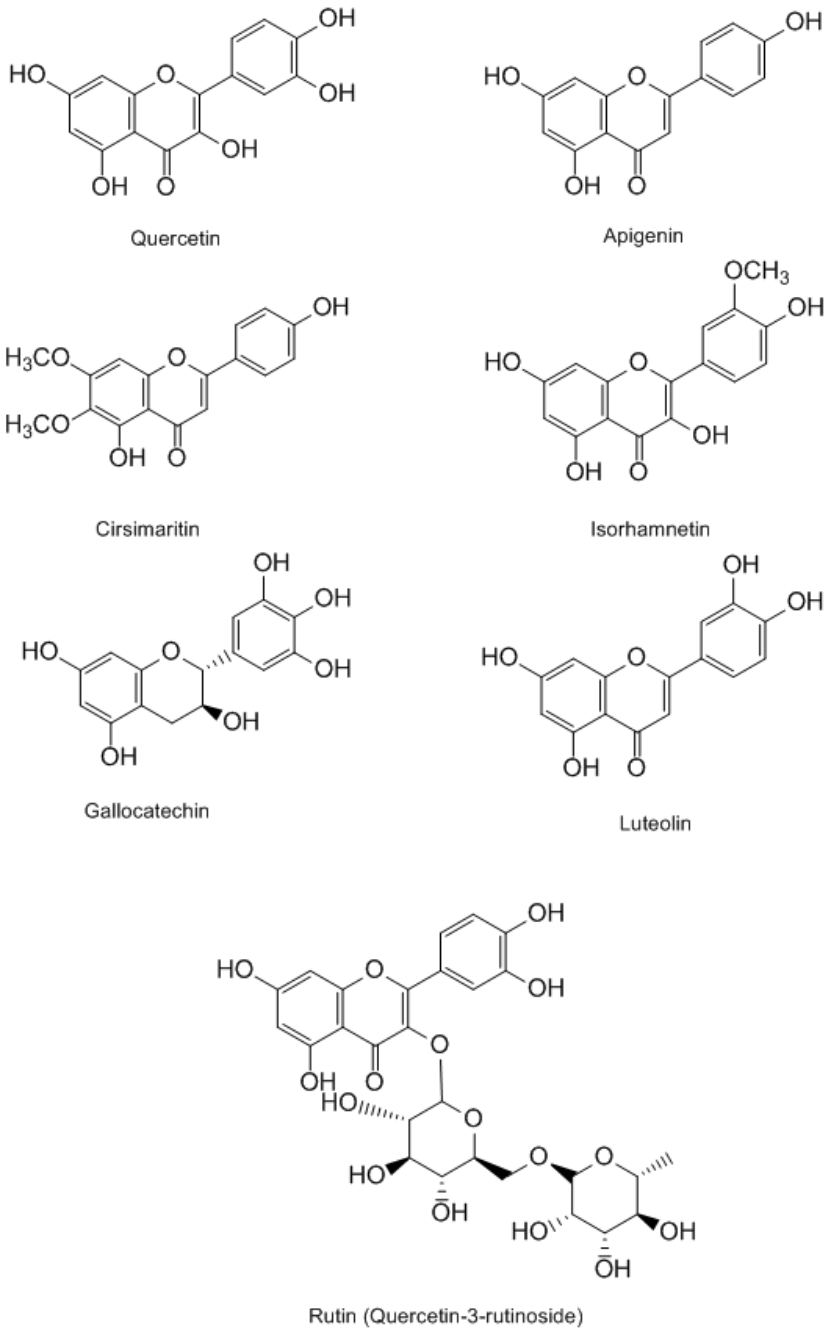


Figure 11 Flavonoids of *Thymus vulgaris.*

2.3.1.4 Phenolic terpene derivates

Phenolic terpenes involve the most widely known compounds of thyme, thymol and carvacrol (Fig. 11). While thymol occurs uniquely in thyme, carvacrol has been identified in most species of the Lamiaceae family. Derivatives of carnosic acid (Fig. 12), such as methylcarnosate, methoxycarnosol or epirosmanol, belong to this group as well (Hossain et al. 2010).

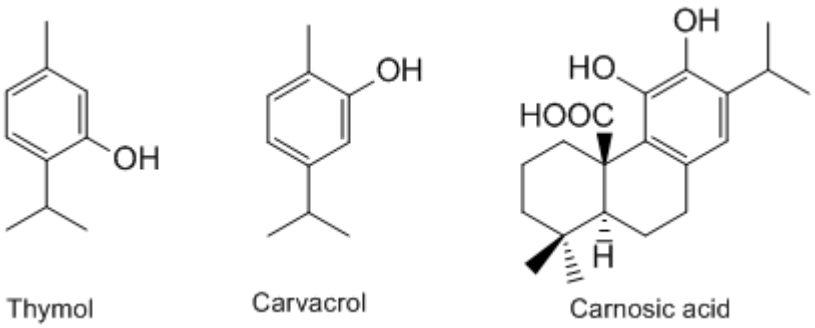


Figure 11 Phenolic terpene derivates of *Thymus vulgaris.*

2.3.2 Pharmacology of thyme

It is generally known that Lamiaceae species have potent antioxidant properties, mostly due to the contained polyphenolic compounds (Adam et al. 1998, Hossain et al. 2008, Soković et al. 2010). Polyphenolic substances are a class of higher plant secondary metabolites with reducing properties, which allow them to act as reducing agents, hydrogen donators, and singlet oxygen quenchers (Zheng & Wang 2001). They have also metal-chelating activity (Rice-Evans et al. 1997). The potency of phenols to act as antioxidants, antimutagens and scavengers of free radicals results from these features. They have been referred to have possible benefits in the prevention of cancer or cardiovascular diseases (Bravo 1998, Kähkönen 1999 et al.)

Essential oil of thyme is reported to have a high and broad antibacterial activity, better against Gram-positive than Gram-negative bacteria (Soković et al. 2010). This antimicrobial effect is believed to be caused by the high percentage of phenolic compounds (mainly thymol and carvacrol) in its essential oil (Adam et al. 1998, Soković 2010). Antifungal activity of thyme extract has been also reported (Centeno et al. 2010). It seems likely that carvacrol interferes with the activity of fungi cell wall enzymes like chitinase (chitin synthetase) as well as α- and β-glucanases (Adam et al. 1998).

2.3.3 Drug interactions with thyme

Interactions of thyme with other substances have not been properly studied so far. Some published studies have reported about interactions of thyme and its constituent thymol and carvacrol with enzymes of phase I and phase II metabolism in mice. These compounds caused an increase in the activity of biotransformation enzymes (Sasaki et al. 2005). Thyme extract has also shown the ability to activate PXR and thereby influence CYP3A4 (Kluth et al. 2006).

3. AIM OF THE WORK

The aim of this work was:

- to determine potential inhibitory activity of St. John's wort (*Hypericum perforatum*) and thyme (*Thymus vulgaris*) extracts on glucuronidation of a model substrate in human liver microsomes and human intestinal microsomes

- to develop a suitable HPLC method for analysis and fractionation of St. John's wort extract and to adjust it for an analysis and fractionation of thyme extract

- to determine the inhibitory effect of individual fractions of the St. John's wort and thyme extracts on glucuronidation of a model substrate in human liver microsomes

- to identify compounds with UGT inhibitory activity present in particular fractions of the St. John's wort and thyme extracts

- to study the inhibitory activity of biflavones present in St. John's wort extract and of flavonoid isorhamnetin present in thyme on glucuronidation of model substrate in human liver microsomes and human intestinal microsomes

4. MATERIAL AND METHODS

4.1 Chemicals

Herba Hyperici containing 0.16% (min. 0.08%) of hypericin was obtained from Heinrich Klenk (Schwebheim, Germany). Herba Thymi was purchased from Caesar & Loretz (Hilden, Germany). HPLC grade methanol (LiChrosolv®), rutin, and hyperosid were purchased from Merck (Darmstadt, Germany). HPLC grade acetonitrile was obtained from Fisher Scientific (Schwerte, Germany). Acetic acid of HPLC grade, formic acid solution (50%), dimethyl sulfoxide, uridine 5′-diphosphoglucuronic acid trisodium salt, 4-trifluoromethylumbelliferone, magnesium chloride hexahydrate, potassium dihydrogen phosphate, potassium phosphate dibasic, zinc sulfate heptahydrate, quercetin, isorhamnetin and apigenin were purchased from Sigma-Aldrich (Taufkirchen, Germany). The 4-methyl-umbelliferyl-β-D-glucuronide dihydrate was purchased from Fisher Scientific (Nidderau, Germany). I3,II8-biapigenin and amentoflavone were purchased from PhytoLab (Vestenbergsgreuth, Germany). Luteolin, isoquercitrin and apigenin-7-glucoside were purchased from Carl Roth (Karlsruhe, Germany). Human liver microsomes (HLM, protein content 20 mg/mL) and human intestinal microsomes (HIM, protein content 10 mg/mL) were obtained from BD Biosciences Gentest (Heidelberg, Germany). Laboratory water was purified with the Milli-Q water purification system (Millipore, Schwalbach, Germany).

4.2 Preparation of herbal extracts

4.2.1 *Hypericum perforatum*

The crude drug of SJW was grinded to a fine powder with a mill. Methanol extract (theoretically 50 mg/mL) was obtained in a two-step extraction with 80% methanol (15 min with the first half of the final volume + 30 min with the second half) in an ultrasonic bath. Both, the crude drug and the extract were stored in a freezer under –20 °C. Later, the real concentration (20 mg/mL) of the SJW extract was adjusted according to the weight of the residue after evaporation. For LC/MS (liquid chromatography tandem mass spectrometry) analysis, a solution was prepared by evaporating the methanol and dissolving the residue in dimethyl sulfoxide.

4.2.2 *Thymus vulgaris*

The processing of the crude drug and the extraction were performed as described in *4.2.1*. The exact concentration of 50 mg/mL was guaranteed by evaporating the extraction solvent and re-dissolving the residue in methanol.

4.3 Glucuronosyltransferase assay

4.3.1 Incubation procedure

Reaction mixtures contained 0.1 M potassium phosphate buffer (pH 7.4) enriched with 3 mM $MgCl_2$, 25 µL of human liver microsomes or human intestinal microsomes stock solution (0.2 mg/mL protein), 2 µL of inhibitor and 4 µL of a mixture of 2.5 mM TFMU and 62.5 µg/mL alamethicin (final 50 µg/mL of microsomal protein) For a control reaction, 2 µL of DMSO were added instead of an inhibitor. Samples were pre-incubated on ice for 15 min and after 5 min at 37 ºC reaction was initiated by an addition of 50 µL of 8 mM UDP-GA. Total volume of reaction mixture was 200 µL. Samples were incubated for 30 min at 37 ºC. After 15 min of incubation, samples were gently mixed. Reaction termination was done by adding of 300 µL of a cold MeOH/$ZnSO_4$ solution with 1.25 µg/mL 4-methylumbelliferylglucuronide (MUG) as internal standard. After 10 min at 4 ºC, the products were centrifuged (12 000 rpm, 6 min) and supernatants were analyzed by HPLC and LC/MS/MS.

4.3.2 Sample analysis

HPLC was carried out on an Agilent Technologies 1100/1200 Series system with a G1311A quaternary pump for pre-extraction and a G1312A binary pump for separation, using a G1314A ultraviolet and a G1321A fluorescence detector. For data processing the ChemStation for LC 3D Systems version B.02.01 software was used.

Electrospray mass spectrometric analysis was performed on an Agilent Technologies 1200 Series HPLC Instrument with a G1315C Diode Array detector coupled to an Agilent Technologies 6460 triple quadrupole mass spectometer with an electrospray ionization source using jet stream technology.

Both analytical systems were obtained from Agilent Technologies (Waldbronn, Germany). The Agilent Mass Hunter workstation software versions B.02.01 (acquisition) and B.04.00 (data analysis) was used for system control and data processing.

4.3.2.1 HPLC analysis with UV or fluorescence detection

One hundred µL of each sample was injected on a reverse phase Synergi MAX-RP column (4.6 x 75 mm, particle size 4 µm; Phenomenex, Aschaffenburg, Germany). The mobile phases consisted of (A) 0.1% formic acid in deionized water and (B) acetonitrile. Gradient elution at a flow rate of 1 mL/min was used with a gradient from 15% B to 45% B in 6 min. For pre-extraction (loading step) water with 0.1% of formic acid was used for 2 min, with a flow rate of 3 mL/min. The eluate was recorded at 335 nm (UV detection, excitation) and 410 nm (emission).

4.3.2.2 LC/MS/MS analysis

LC/MS/MS analysis was performed using a Synergi MAX-RP column (2.0 x 50 mm, particle size 4 µm) with a C12 precolumn (4 x 3 mm), both purchased from Phenomenex (Aschaffenburg, Germany). Chromatographic elution was carried out at a flow rate of 0.6 mL/min using 0.4% acetic acid as solvent A and acetonitrile as solvent B with a linear gradient from 5% B to 55% B in 4 min, keeping at 100% for 2.5 min and re-equilibration back to 5% for 4.5 min. The injection volume was 10 µL for SJW and 5 µL for thyme.

The MS experiments were performed in the negative ion electrospray ionization (ESI) mode using multiple reaction monitoring (MRM) with a fragmentor voltage of 75 V and capillary voltage of 3500 V. Nitrogen was used for the jet stream (350°C; 10L/min) and as the nebulizing gas (45 psi). The flow rate and the temperature of the drying gas were 8L/min and 350°C, respectively. The monitored transitions were 405→229 for TFMUG and 351→175 for MUG (internal standard).

4.4 HPLC analysis of herbal extracts

4.4.1 *Hypericum perforatum*

Reversed-phase HPLC was performed using an Agilent 1100 Series HPLC system equipped with a G1312A binary pump (Agilent Technologies, Waldbronn, Germany) and a G1315B Agilent 1100 Diode Array Detector. The ChemStation Software for LC systems (version B.03.02; Agilent Technologies) was used for data processing.

The analytical method was performed using a ZORBAX SB-C18 (4.6 x 150 mm, particle size 5 µm) column with a 4.6 x 12.5 mm pre-column (Agilent Technologies). The flow rate was 1.0 mL/min. The solvent system consisted of 0.1% formic acid in

water (phase A) and 0.1% formic acid in methanol (phase B). Elution was performed using a linear gradient from 15% to 100% B in 12 min, 100% B for 5 min (12-17 min), and 15% B for next 3 min (17-20 min). The column temperature was set to 30 °C. Ten µL of SJW extract was injected and the signal was recorded at 320 and 590 nm.

4.4.2 *Thymus vulgaris*

To analyze thyme extract, the HPLC method used for St. John's wort (*4.4.1)* was adapted. The gradient was from 15% to 100% B in 17 min, and 100% B was hold for next 5 min. Re-equilibration was achieved in 3 min. Signals were recorded at 254 and 320 nm. Other parameters of HPLC analysis were the same as in *4.4.1*.

4.5 Semipreparative HPLC fractionation of herbal extracts

4.5.1 *Hypericum perforatum*

For preparation of extract fractions the same HPLC system and all parameters were used as described in *4.4.1* except for the column and the corresponding flow rate. The separation was performed using a semipreparative ZORBAX SB-C18 (9.4 x 150 mm, 5 µm; Agilent) column with a SB-C8 pre-column (9.4 x 15 mm, 7 µm; Agilent). The flow rate was set to 4.0 mL/min.

Samples for further glucuronosyltrasferase-inhibition assays were collected using a capillary tube connected to the detector outlet. Fifty µl of the extract (20 mg/mL) was injected. The eluent was collected into glass tubes from 2nd till 17th min. The tube was changed every minute, thus 15 samples were taken. The whole procedure was carried out twice using the same test tubes to get the more concentrated samples. With the flow rate of 4 mL/min, every test tube was filled with 8 mL of eluent. Collected fractions were evaporated with pressurized air (40 ºC). After evaporation of the solvent, the residues were dissolved in 100 µL of DMSO using vortex shaking and short stay in ultrasonic bath to enhance the dissolution process.

4.5.2 *Thymus vulgaris*

The extract of *Thymus vulgaris* was fractionated using the same method as described in *4.4.2,* with a column and a flow rate as described in *4.5.1*. Fifty µL of thyme extract was injected. Samples were collected every two minutes from 2nd till 22nd min of analysis. Thus, 10 fractions were obtained. The whole procedure was carried out four times to get the final volume of 200 µL extract injected. After

evaporation of the collected fractions (32 mL each), the residues were dissolved in 100 µL of DMSO.

4.6 Identification of selected constituents in SJW and thyme

To identify compounds in SJW and thyme extracts, RP-HPLC and LC/MS/MS were performed. For analytical systems specification see *4.3.2*, the parameters of liquid chromatography are described in *4.4.1* (SJW) and *4.4.2* (thyme). MS analysis was performed in single ion monitoring (SIM) mode with a fragmentor voltage of 75 V and m/z values regarding to searched constituents (Table 5 and Table 12). Other parameters of MS analysis are described in *4.3.2.2*.

For identification of particular substances in the obtained chromatogram, spiking experiments were carried out. A sufficient amount of a standard (stock solutions 0.1 mg/mL in MeOH or DMSO) was added into the herbal extract (10 mg/mL in MeOH) and the extract was analyzed using the methods described in *4.4.1* (SJW) and *4.4.2* (thyme). Following compounds, which are commercially available, were tested: amentoflavone, I3,II8-biapigenin, hyperosid, and isoquercitrin for SJW and apigenin, apigenin-7-glucoside, isorhamnetin, and luteolin for thyme. Furthermore, quercetin and rutin were used for spiking experiments with both extracts.

4.7 Calculation of residual enzyme activity

All results of inhibition assays are based on the data from HPLC and/or LC/MS/MS analysis. The peak area ratios (AR) of the product 4-triflouromethylumbelliferylglucuronide (TFMUG) and the internal standard 4-methylumbelliferylglucuronide (MUG) of each sample were calculated as follows:

$$\text{Area ratio (AR)} = A_{TFMUG}/A_{MUG}$$

The arithmetic mean of two incubation samples was used for the calculation of the UGT residual activity. Results are expressed as percentage of residual activity, i.e. the activity in the presence of an inhibitor. Because the control incubation without an inhibitor can be considered as 100 % activity, results of the other samples are related to this value. The obtained values were rounded to integral numbers.

$$\text{Residual activity [\%]} = AR_{sample}/AR_{control} * 100$$

5. RESULTS

5.1 ST. JOHN'S WORT (*Hypericum perforatum*)

5.1.1 Inhibition of TFMU glucuronidation by St. John's wort extract

Hypericum perforatum extract was tested in three different concentrations (10 µg/mL, 50 µg/mL, and 250 µg/mL) on both, human liver (HLM) and human intestinal (HIM) microsomes. *H. perforatum* exerted significant inhibitory effect at all tested concentrations. This effect was concentration-dependent. Ten µg/mL extract decreased the initial activity of HLM and HIM by 19% and 57%, respectively. The inhibitory activity of 50 µg/mL extract was higher (the residual activity in HLM was 26% and 4% in HIM). The highest concentration of SJW extract (i.e. 250 µg/mL) decreased the glucuronidation activity of HLM and HIM by 97% and 99%, respectively. Results are summarized in Table 3 and in Fig. 12. The inhibition of glucuronidation in HIM was notably higher.

Table 3 Inhibition of glucuronidation by *Hypericum perforatum* extract.

Concentration of St. John's wort extract	**Residual activity [%] ± SD [%]**	
	HLM	**HIM**
0 µg/mL	100 ± 0.9	100 ± 1.5
10 µg/mL	81 ± 9.0	43 ± 0.4
50 µg/mL	26 ± 1.7	4 ± 0.3
250 µg/mL	3 ± 0.0	1 ± 0.1

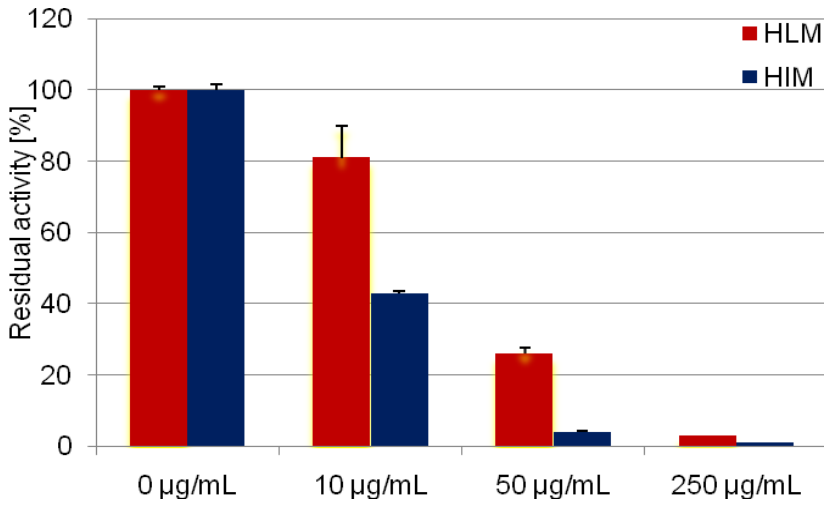


Figure 12 Inhibitory effect of three different concentrations of *H. perforatum* extract on TFMU glucuronidation in human liver microsomes and human intestinal microsomes.

5.1.2 Fractionation of St. John's wort extract

The method for fractionation of SJW extract was developed. The semipreparative analysis run, during which fifteen fractions were collected into glass tubes, is shown in Fig. 13. Vertical lines illustrate the process of collection and divide peaks of the chromatogram into corresponding fractions.

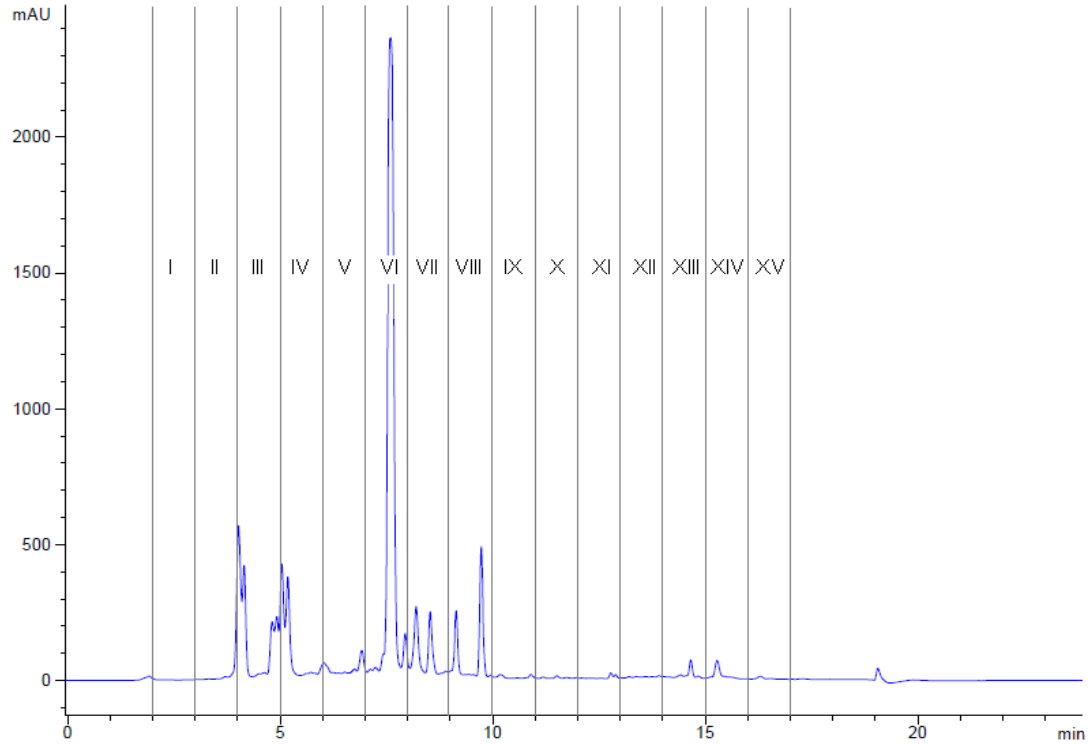


Figure 13 HPLC chromatogram of the fractionation of St. John's wort (*H. perforatum* in 80% methanol, 20 mg/mL, injected volume 100 µL, for analysis details see *4.5.1*).

5.1.3 Inhibition of TFMU glucuronidation by fractions of St. John's wort extract

Collected fractions were tested for inhibitory activity on glucuronidation in human liver microsomes. The obtained results, which are summarized in Table 4 and Fig. 14, have proven a significant inhibitory effect of fractions No. III, IV, V, VI, VIII, XI, XII, XIII, XIV, and XV of SJW extract.

Table 4 Inhibitory effect of SJW fractions on the glucuronidation of TFMU in HLM.

	Residual activity [%] ± SD [%]		
	LC/MS/MS	**Fluorescent**	**UV**
Control	100 ± 1.4	100 ± 1.1	100 ± 0.6
SJW extract (200 µg/mL)	3 ± 0.1	4 ± 0.0	2 ± 0.0
Fraction I	95 ± 2.1	92 ± 0.9	91 ± 0.9
Fraction II	94 ±1.3	93 ± 0.5	93 ± 1.3
Fraction III	74 ± 0.1	78 ± 0.9	74 ± 0.6
Fraction IV	64 ± 0.3	65 ±0.2	63 ± 0.3
Fraction V	84 ± 0.3	82 ± 2.2	66 ± 0.9
Fraction VI	83 ± 0.3	82 ± 0.1	77 ± 0.3
Fraction VII	85 ± 0.4	84 ± 0.7	83 ± 0.9
Fraction VIII	78 ± 1.0	76 ± 0.4	75 ± 0.2
Fraction IX	89 ± 0.1	93 ± 1.0	90 ± 0.5
Fraction X	88 ± 3.3	89 ± 1.6	88 ± 2.0
Fraction XI	79 ± 1.6	78 ± 0.4	77 ± 0.9
Fraction XII	70 ± 0.1	70 ± 1.3	69 ± 0.8
Fraction XIII	57 ± 1.5	54 ± 1.4	52 ± 1.3
Fraction XIV	62 ± 0.1	61 ± 0.3	60 ± 0.2
Fraction XV	76 ± 1.0	76 ± 1.3	75 ± 1.1

To compare the selectivity of individual analytical techniques, analysis was performed using the same method with different analytical detectors. With respect to the fact that LC/MS/MS using multiple reaction monitoring is more sensitive and selective than fluorescence or ultraviolet detection, data from LC/MS/MS analysis are shown in Fig. 14.

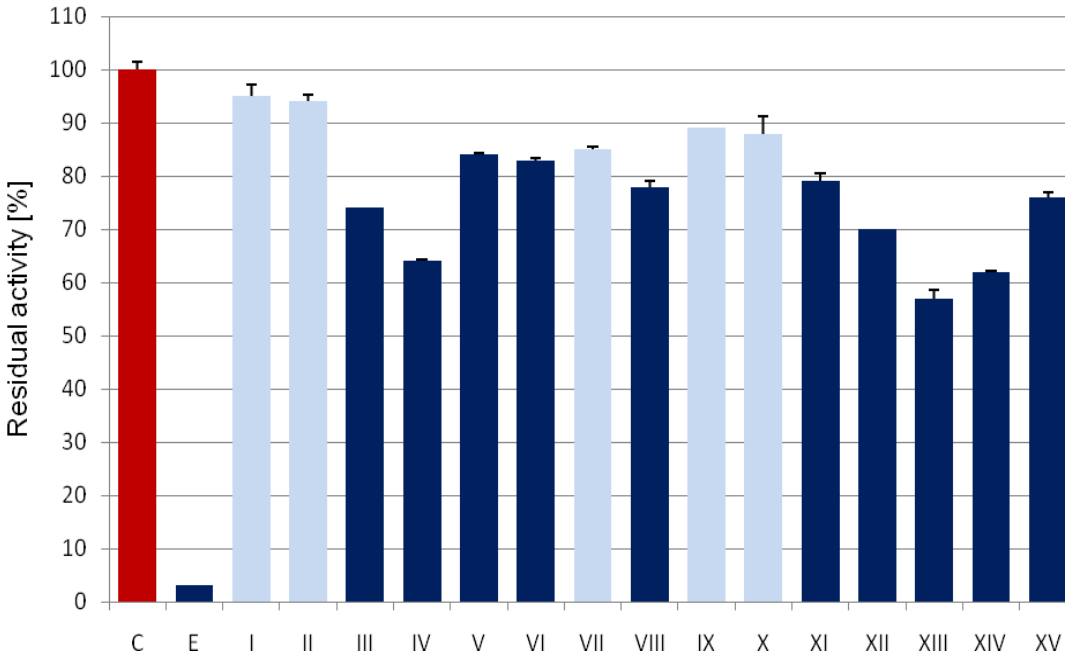


Figure 14 Inhibitory effect of SJW fractions on the glucuronidation of TFMU in HLM (results of LC/MS analysis). Letter C indicates control and letter E stands for whole SJW extract (200 µg/mL).

Residual activities of UGT (expressed in %) obtained with SJW extract as well as with individual fractions are compared to the activity of control sample (no inhibitor was present; marked by letter C in Fig. 14), which represents 100%. Column E shows inhibitory activity of the whole SJW extract. Fractions with residual activity higher than 85% were excluded from further studies (depicted in light blue color in Fig. 14), i.e. fractions I, II, VII, IX, and X. Remaining 10 samples were analyzed in order to identify present substances.

5.1.4 Identification of compounds present in individual fractions of St. John's wort extract

Seventeen different m/z (mass-to-charge ratio) values (matching 19 compounds of SJW extract) were tested using single ion monitoring (SIM) mode of LC/MS analysis. The list of tested compounds and their m/z values is shown in Table 5.

Table 5 Compounds of *Hypericum perforatum* tested in SIM mode of LC/MS analysis.

Compound	Mr	Negative mode	Positive mode
		m/z	m/z
Rutin	610.52	609	611
Adhyperforin	550.80	549	551
I3,II8-Biapigenin	538.47	537	539
Hyperforin	536.78	535	537
Protopseudohypericin	522.47	521	523
Pseudohypericin	520.46	519	521
Protohypericin	506.47	505	507
Hypericin/Isohypericin	504.46	503	505
Adhyperfirin	482.71	481	483
Miquelianin	478.37	477	479
Hyperfirin	468.68	467	469
Hyperosid/Isoquercitrin	464.39	463	465
Dihydroquercitrin	450.40	449	451
Quercitrin	448.39	447	449
Chlorogenic acid	354.31	353	355
Coumarylquinic acid	338.10	337	339
Quercetin	302.24	301	303

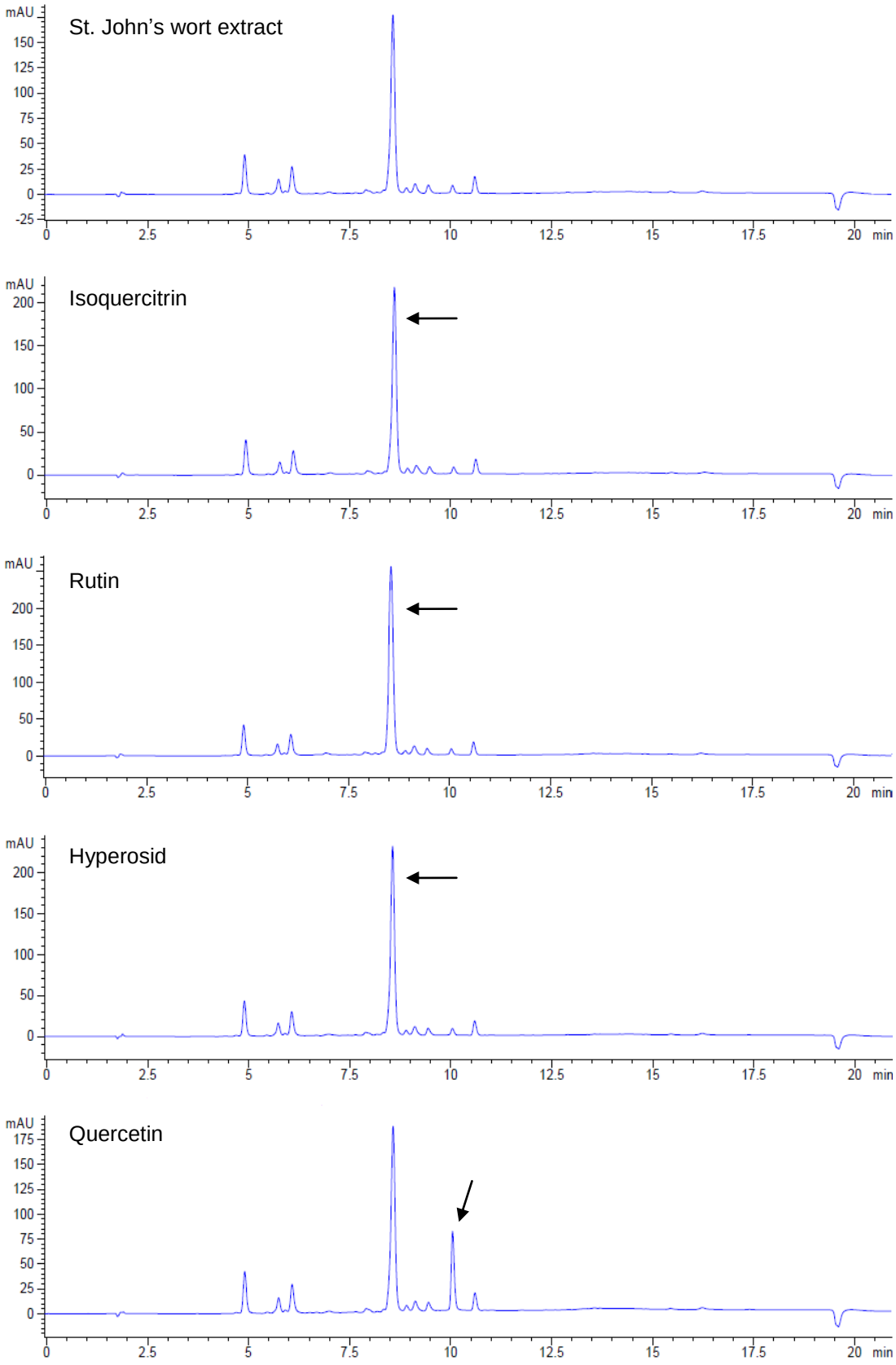
Beside LC/MS/MS analysis, fractions were tested also using HPLC analysis with fluorescence detection. Data obtained from both analyses are presented in Table 6.

Table 6 LC/MS/MS and HPLC retention times of compounds found in particular fractions of SJW extract.

Fraction	Retention time [min]		Possible compound*
	HPLC- UV	**LC/MS/MS**	
III	6.685; 7.225;	4.4	Chlorogenic acid
	7.491; 7.764	5.2	Coumaroylquinic acid
IV	7.763; 8.178;		
	8.370; 8.601;		
	8.682		
V	9.013; 9.115;		
	9.330; 9.554		
VI	9.684; 9.824;	7.8	Dihydroquercitrin
	10.188; 10.536	7.9	Miquelianin
		8.0	**Rutin**
		8.1	**Hyperosid/ Isoquercitrin**
VIII	11.695; 12.253;	9.5	**Quercetin**
	12.687	10.1	**I3,II8-Biapigenin**
XI	14.640; 14.840;		
	15.116; 15.311;		
	15.449		
XII	15.685; 15.862;		
	16.213; 16.385;		
	16.538		
XIII	16.539; 16.773;	14.4	Hyperfirin
	17.066	14.7	Adhyperfirin
		15.1	Hyperforin
XIV	17.883	15.3	Adhyperforin
XV	---		

*Compounds from Table 5 that could theoretically belong to the signal (corresponding m/z values). Written in bold are compounds whose retention times have been proven by the spiking experiment (presented below).

Spiking experiments were performed to verify some of the results of HPLC and LC/MS/MS analysis, which are presented in Table 6. Chromatograms of spiking experiments are shown in Fig. 15, retention times of identified compounds in Table 7.


St. John’s wort extract
Isoquercitrin
Rutin
Hyperosid
Quercetin
mAU
min

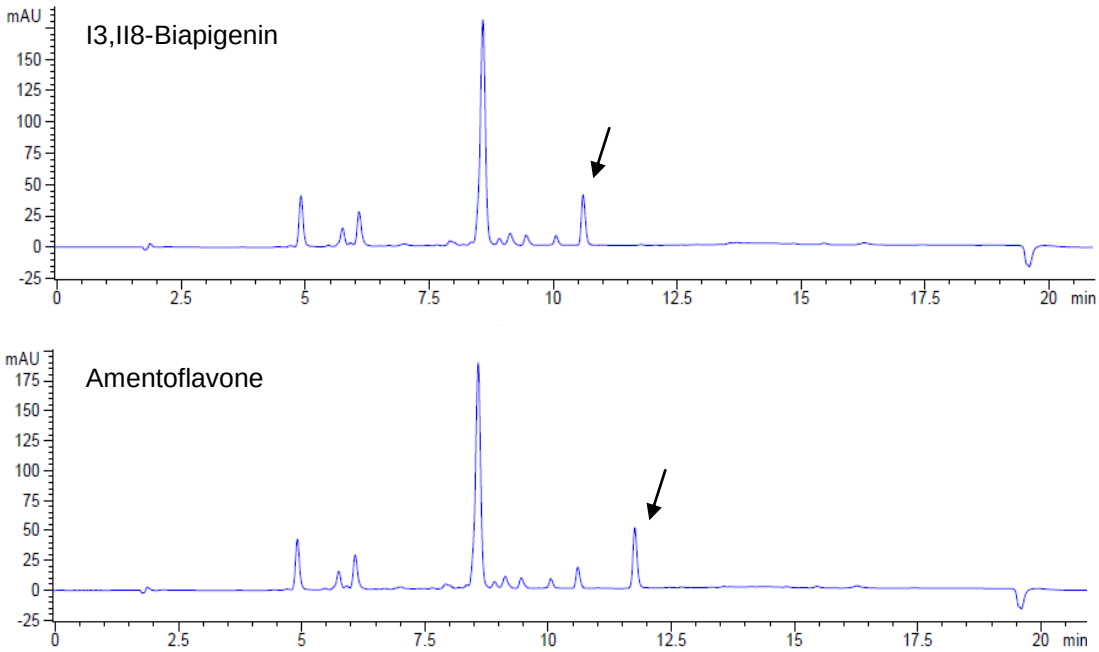


Figure 15 Identification of some substances of *H. perforatum* by spiking experiments.

Table 7 Substances of St. John's wort extract identified by spiking experiments.

Compound	Retention time [min]	Corresponding fraction*
Rutin	8.546	VI
Hyperosid	8.560	VI
Isoquercitrin	8.606	VI
Quercetin	10.048	VIII
I3,II8-Biapigenin	10.604	VIII
Amentoflavone	11.760	IX

*according to *4.5.1*

5.1.5 Determination of the shift in retention times for different used analytical systems

With respect to the use of different analytical systems (LC/MS/MS; HPLC system with UV detection; HPLC system with DAD), retention times differ. To make the data comparable, it was necessary to determine the shift of retention times in chromatograms obtained by particular systems. Three dominant peaks in the chromatograms were selected and their retention times compared (Table 8).

Table 8 Comparison of the retention times for three dominant peaks in chromatogram of the analysis of SJW extract *(4.4.1)*, the semipreparative analysis *(4.5.1),* and the HPLC and LC/MS/MS analysis *(4.6.1)*.

Fraction	HPLC *(4.4.1)*	HPLC *(4.5.1)*	HPLC *(4.6.1)*	LC/MS/MS *(4.6.1)*
VI	8.571	7.551	10.188	8.0
VIII	10.047	9.111	11.695	9.5
VIII	10.458	9.707	12.253	10.1

Retention times of the semipreparative analysis (*4.5.1*) and the LC/MS analysis (*4.6.1*) differ about 2.6 minutes (Table 6). Retention times of semipreparative analysis (*4.5.1,* Fig. 13) are about 0.9 minute lower than those in chromatograms of spiking experiments (*4.4.1*), and about 0.4 minute lower than retention times of LC/MS analysis (*4.6.1*). Retention times of LC/MS and HPLC analyses (*4.6.1*) differ in about 2.15 min.

5.1.6 Inhibition of TFMU glucuronidation by biflavonoids of St. John's wort

The biflavones of *H. perforatum*, I3,II8-biapigenin and amentoflavone (I3',II8-biapigenin), were tested to prove their inhibitory activity on TFMU glucuronidation catalyzed by UGT in HLM and HIM. Inhibition assay with three different concentrations of each compound (1 µM, 10 µM, and 100 µM) and both human liver (HLM, 20 mg/mL protein) and human intestinal (HIM, 10 mg/mL protein) microsomes was carried out.

Both biflavones of *H. perforatum* exerted significant inhibitory activity on the glucuronidation with comparable effects. The inhibition of UGT was more pronounced in HIM. The results are shown in Table 9 and Fig. 16.

Table 9 Inhibition of human liver and human intestinal microsomes by I3,II8-biapigenin and amentoflavone at various concentrations (1-100 μM). Activity of UGT in HLM and HIM in the absence of tested biflavones was taken as 100% for corresponding incubations.

	Residual activity [%] ± SD [%]		
	LC/MS/MS	**Fluorescent**	**UV**
HLM			
I3,II8-Biapigenin 0 μM	100 ± 5.6	100 ± 4	100 ± 3.7
I3,II8-Biapigenin 1 μM	93 ± 2.4	88 ± 1.4	89 ± 1.4
I3,II8-Biapigenin 10 μM	54 ± 0.8	50 ± 0.8	50 ± 0.4
I3,II8-Biapigenin 100 μM	0 ± 0.0	1 ± 0.0	0
Amentoflavone 0 μM	100 ± 5.6	100 ± 4	100 ± 3.7
Amentoflavone 1 μM	88 ± 0.4	84 ± 1.1	84 ± 1.2
Amentoflavone 10 μM	39 ± 1.6	36 ± 0.9	36 ± 1.1
Amentoflavone 100 μM	7 ± 0.2	6 ± 0.2	6 ± 0.2
HIM			
I3,II8-Biapigenin 0 μM	100 ± 1.0	100 ± 1.3	100 ± 1.6
I3,II8-Biapigenin 1 μM	41 ± 0.0	43 ± 1.0	40 ± 0.9
I3,II8-Biapigenin 10 μM	8 ± 0.1	12 ± 0.2	8 ± 0.2
I3,II8-Biapigenin 100 μM	0 ± 0.0	5 ± 0.0	7 ± 1.0
Amentoflavone 0 μM	100 ± 1.0	100 ± 1.3	100 ± 1.6
Amentoflavone 1 μM	38 ± 0.0	40 ± 0.1	37 ± 0.0
Amentoflavone 10 μM	6 ± 0.3	10 ± 0.6	7 ± 0.3
Amentoflavone 100 μM	1 ± 0.1	6 ± 0.0	0

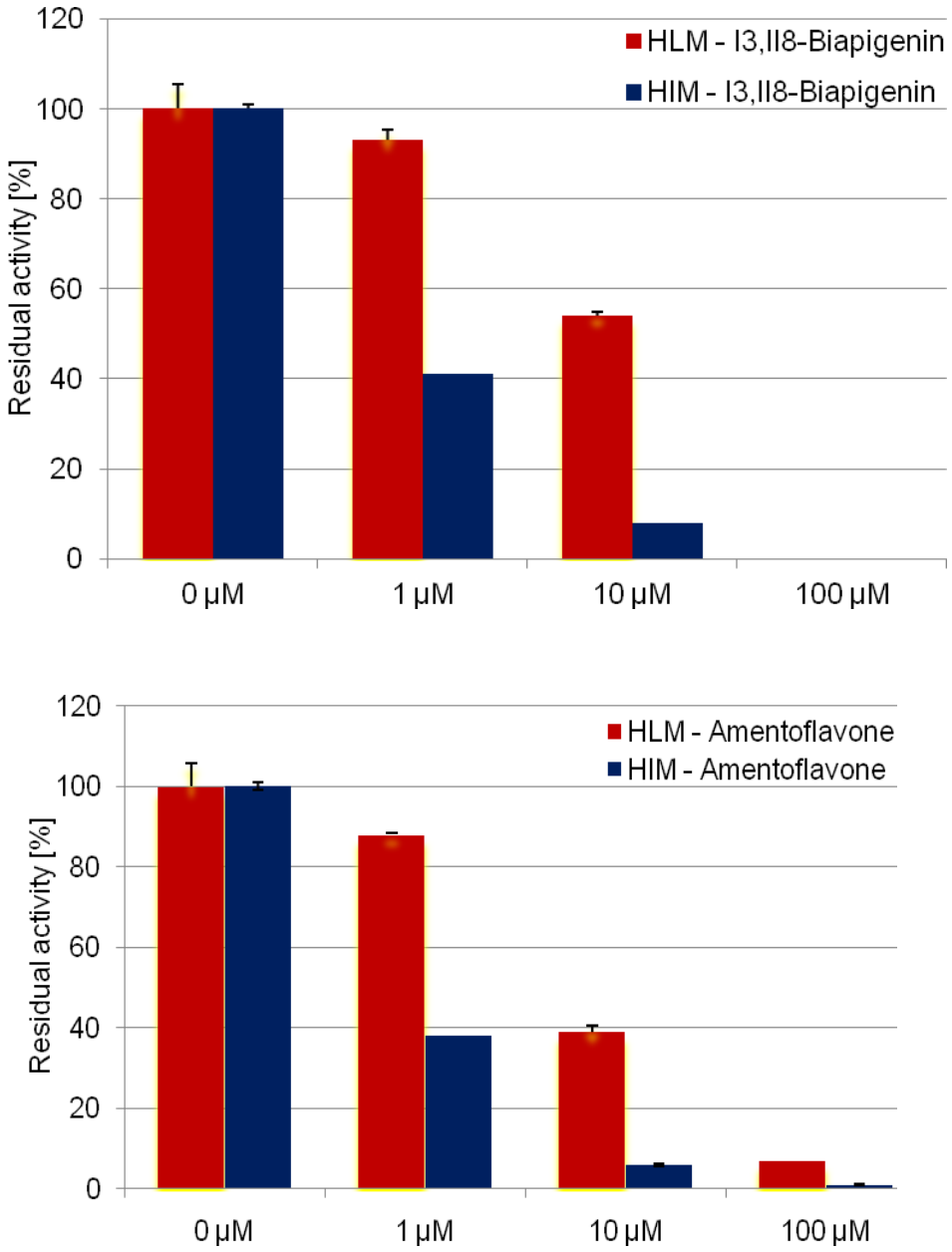


Figure 16 Comparison of the inhibitory activity of 1, 10, and 100 µM solutions of I3,II8-biapigenin and amentoflavone on TFMU glucuronidation by human liver microsomes (HLM), and human intestinal microsomes (HIM).

5.2 THYME (*Thymus vulgaris*)

5.2.1 Inhibition of TFMU glucuronidation by thyme extract

Thyme extract was tested for inhibition of TFMU glucuronidation in concentrations of 10 µg/mL, 50 µg/mL and 250 µg/mL with both human liver (HLM) and human intestinal (HIM) microsomes. Thyme extract exerted concentration-dependent inhibitory activity on the glucuronidation of TFMU, which was considerably lower than that of SJW extract in corresponding concentrations. The observed inhibitory activity was slightly stronger in HIM than in HLM. The results are summarized in Table 10 and shown in Fig. 17.

Table 10 Inhibition of TFMU glucuronidation by *Thymus vulgaris* extract.

Concentration of thyme extract	**Residual activity [%] ± SD [%]**	
	HLM	**HIM**
0 µg/mL	100 ± 0.9	100 ± 1.5
10 µg/mL	94 ± 2.2	76 ± 2.7
50 µg/mL	61 ± 2.0	32 ± 1.1
250 µg/mL	17 ± 0.8	5 ± 0.1

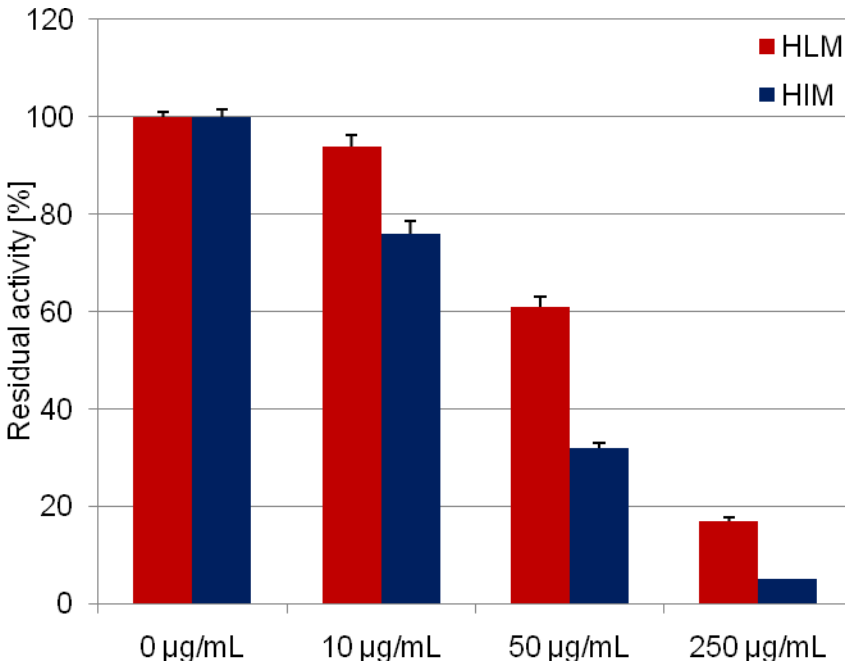


Figure 17 Inhibitory effect of three different concentrations of *Thymus vulgaris* extract on TFMU glucuronidation in human liver microsomes (HLM) and human intestinal microsomes (HIM).

5.2.2 Fractionation of thyme extract

The fractionation method, which was developed for SJW extract, was modified with respect to different spectrum of compounds present in thyme extract. The run during which 10 fractions of thyme extract were collected is shown in Fig. 18. Vertical lines in the chromatogram show fractionation of the extract.

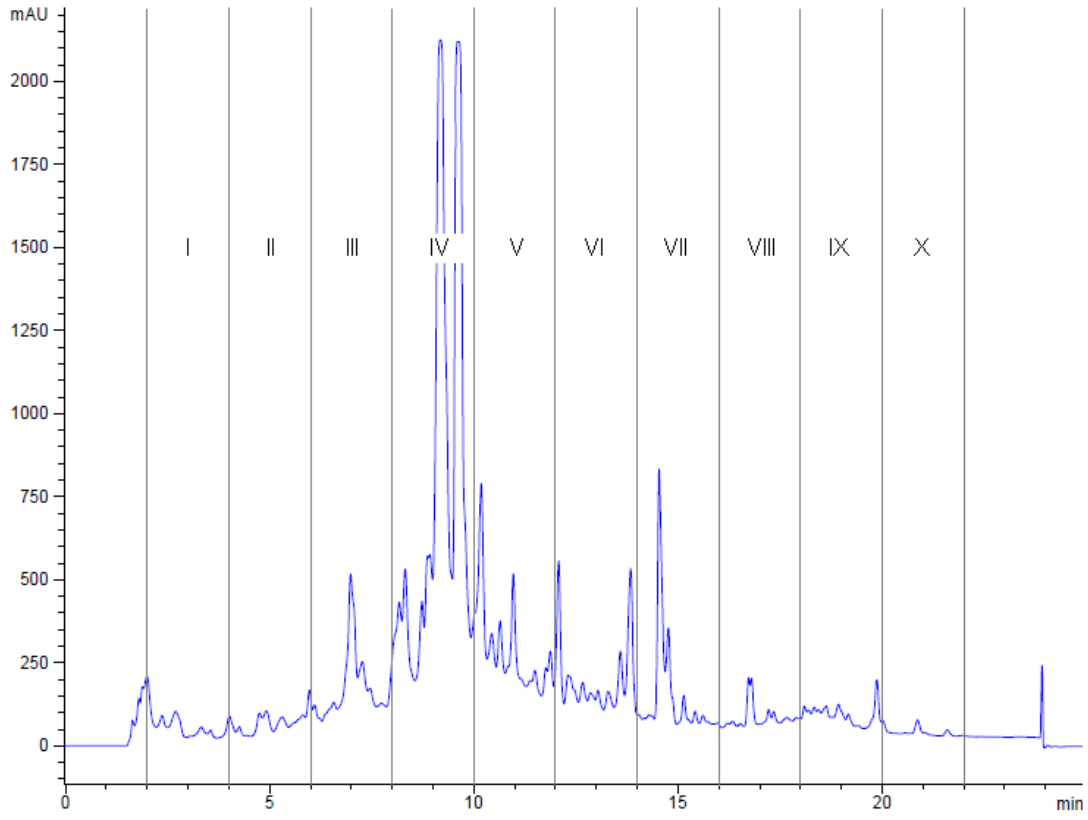


Figure 18 HPLC chromatogram of the fractionation of thyme. (*Thymus vulgaris* in 80% methanol, 50 mg/mL, injected volume 40 µL, for analysis details see *4.5.2*).

5.2.3 Inhibition of TFMU glucuronidation by fractions of thyme extract

The inhibition assay was carried out to clarify the inhibitory effect of thyme extract fractions. Some of them have shown high inhibitory potency. Results are presented in Table 11 and Fig. 19.

Table 11 Inhibitory effect of individual fractions of thyme extract

	Residual activity [%] ± SD [%]		
	LC/MS/MS	**Fluorescence**	**UV**
Control	100 ± 1.0	100 ± 1.2	100 ± 1.3
Thyme extract 1000 µg/mL	9 ± 0.9	11 ± 0.3	34 ± 16.0
Fraction I	93 ± 1.7	91 ± 0.7	70 ± 22.7
Fraction II	93 ± 2.5	94 ± 2.2	51 ± 42.3
Fraction III	94 ± 1.3	82 ± 1.0	23 ± 3.8
Fraction IV	71 ± 7.2	41 ± 3.8	57 ± 3.6
Fraction V	36 ± 0.9	39 ± 0.3	135 ± 3.6
Fraction VI	6 ± 0.2	6 ± 0.2	8 ± 2.5
Fraction VII	5 ± 0.0	5 ± 0.1	6 ± 0.4
Fraction VIII	20 ± 0.2	18 ± 0.1	20 ± 0.1
Fraction IX	8 ± 0.3	8 ± 0.2	9 ± 0.1
Fraction X	50 ± 0.8	47 ± 0.2	48 ± 0.5

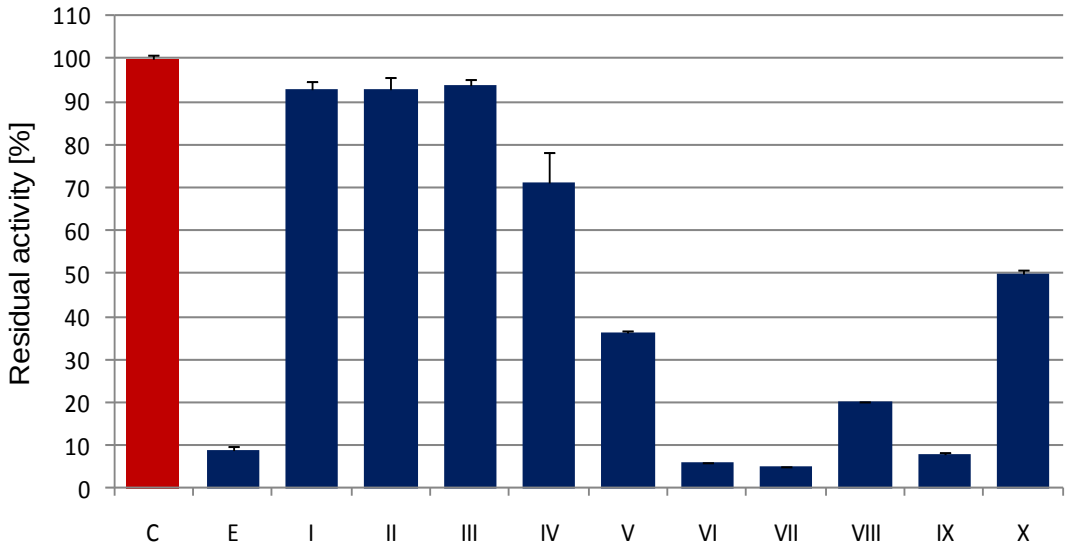


Figure 19 Inhibitory effect of individual fractions of *Thymus vulgaris* extract on the TFMU glucuronidation catalyzed in HLM (results of LC/MS analysis). Column C shows control sample (no inhibitor was present), column E shows effect of whole extract (1 mg/mL).

5.2.4 Identification of compounds present in individual fractions of thyme extract

All isolated fractions were further tested using LC/MS analysis *(4.6.2.)* to identify present substances. The list of analyzed compounds and their m/z values are shown in Table 12.

Table 12 Compounds of *Thymus vulgaris* tested in SIM mode of LC/MS analysis.

Compound	**Mr**	**Negative mode**	**Positive mode**
		m/z	**m/z**
Rutin	610.52	609	611
Isorhamnetin-3-hexoside	478.40	477	479
Luteolin-3'-O-glucuronide	462.08	461	463
Luteolin-7-O-glucuronide	448.10	447	449
Apigenin-7-O-glucoside	432.38	431	433
Rosmarinic acid	360.31	359	361
Chlorogenic acid	354.31	353	355
Isorhamnetin	316.26	315	317
Quercetin	302.24	301	303
Luteolin	286.24	285	287
Apigenin	270.24	269	271
Syringic acid	198.17	197	199
Caffeic acid	180.16	179	181
Gallic acid	170.02	169	171
Vanillic acid	168.04	167	169
p-Coumaric acid	164.16	163	165
Gentisic acid	154.12	153	155
Thymol/Carvacrol	150.22	149	151
4-Hydroxybenzoic acid	138.12	137	139

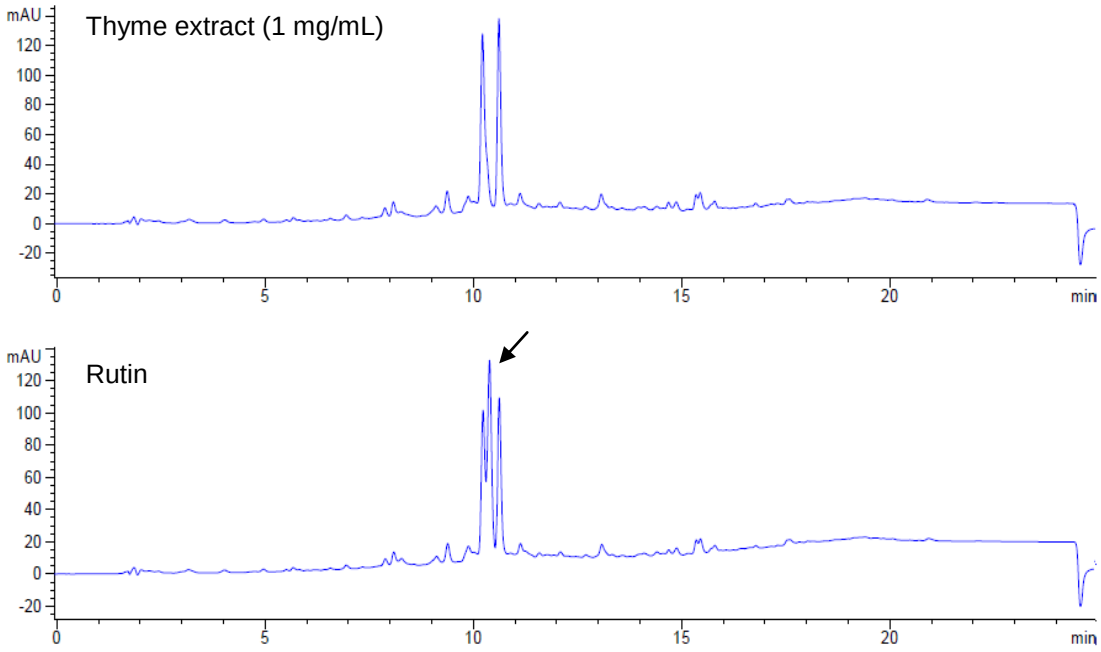
Based on data of the LC/MS/MS analysis, several compounds were identified (Table 13).

Table 13 Identification of several compounds present in *Thymus vulgaris* extract by LC/MS analysis in SIM negative mode.

Fraction	Retention time	Possible compound*
II	4.336	Syringic acid
II	6.051	4-Hydroxybenzoic acid
III	7.269	Caffeic acid
IV	9.182	Luteolin-7-O-glucoside
IV	9.538	Luteolin-3'-O-glucuronide
IV	9.709	Isorhamnetin-3-O-hexoside
IV	10.044	Rosmarinic acid
VI	12.518	**Luteolin**
VI	13.449	**Apigenin**

*Compounds from Table 12 that could theoretically belong to the signal (corresponding m/z values). Written in bold are compounds whose retention times have been proven by a spiking experiment (presented below).

Spiking experiments were carried out to verify some of the results of HPLC and LC/MS/MS analysis (Table 13). Chromatograms are shown in Fig. 20, retention times of identified compounds are included in Table 14.



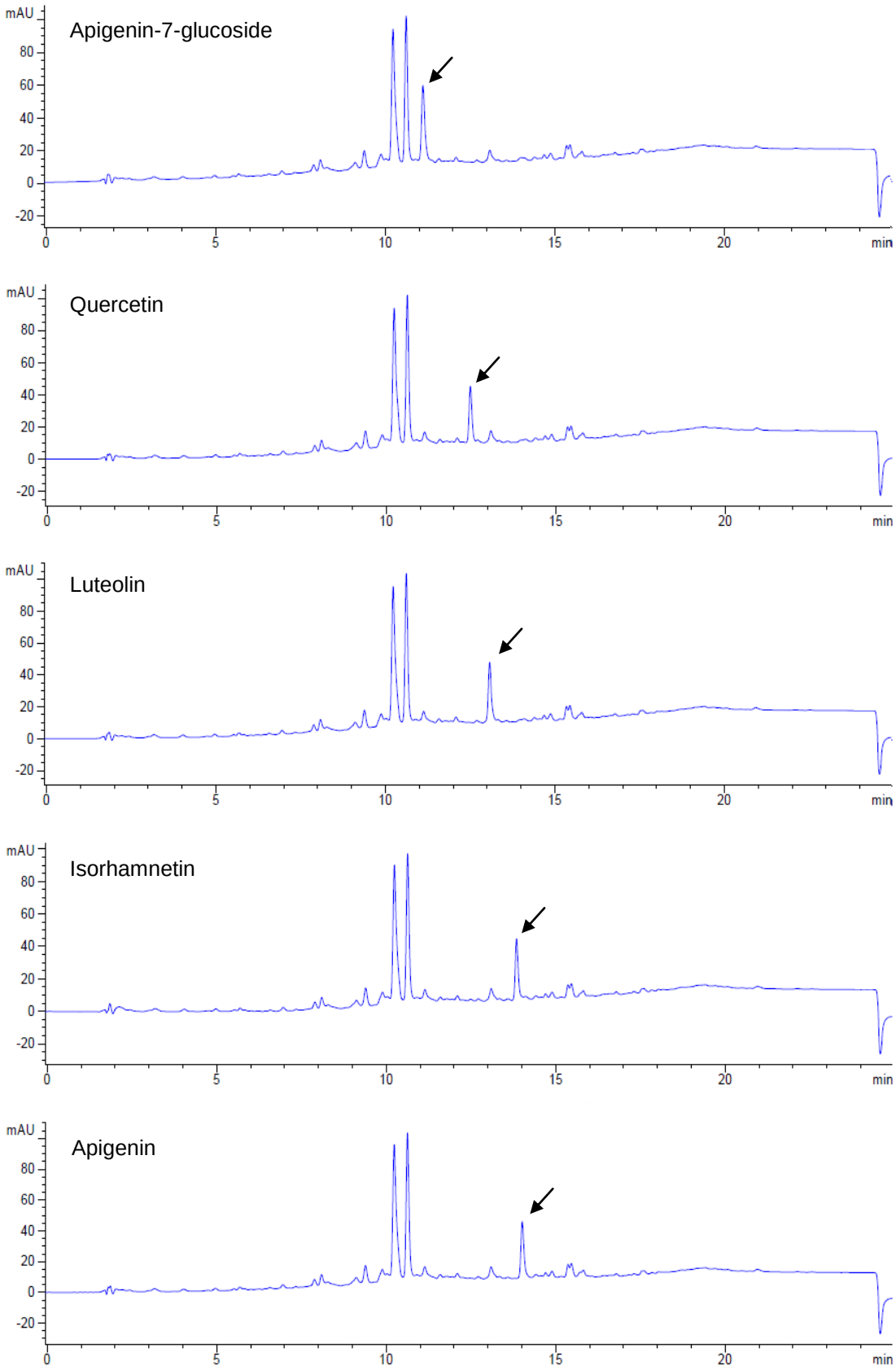


Figure 20 Identification of several substances present in *Thymus vulgaris* extract by spiking experiments.

Table 14 Substances of thyme extract identified by spiking experiments.

Compound	Retention time	Fraction*
Rutin	10.389	IV
Apigenin-7-glucoside	11.112	V
Quercetin	12.481	VI
Luteolin	13.078	VI
Isorhamnetin	13.845	VI
Apigenin	14.013	VI / VII

*Determination of the fraction is based on the *4.5.2* method (Figure 18)

5.2.5 Determination of the shift in retention times for different used analytical systems

Presented retention times differ with respect to the different analytical systems that were used. To determine the shift, retention times of the two highest peaks in chromatograms were compared (Table 15).

Table 15 Comparison of the retention times for three dominant peaks in chromatograms of the extract analysis *(4.4.2)* and the semipreparative analysis *(4.5.2)* of thyme.

Fraction*	HPLC *(4.4.2)*	HPLC *(4.5.2)*
IV	10.241	9.185
IV	10.631	9.624
VII	13.845	14.532

*Determination of the fraction is based on the *II.5.2* method (Figure 18)

The difference in retention times obtained by LC/MS/MS analysis and semipreparative HPLC analysis (Fig. 20) is about 1.5 minute.

5.2.6 Inhibition of TGMU glucuronidation by isorhamnetin

Isorhamnetin, a flavonoid of *Thymus vulgaris*, was tested to prove its inhibitory effect on TFMU glucuronidation. Inhibition was tested with isorhamnetin (1, 10 and 100 μM) on human liver microsomes (HLM) and human intestinal microsomes (HIM).

Isorhamnetin exerted inhibitory activity on UGT in both HLM and HIM. The inhibition of UGT in HIM was significantly stronger when more concentrated solutions of isorhamnetin were used. The results are shown in Table 16 and Fig. 21.

Table 16 Inhibition of TFMU glucuronidation in HLM and HIM by the flavonoid isorhamnetin at various concentrations.

	Residual activity [%] ± SD [%]		
	LC/MS/MS	**Fluorescence**	**UV**
HLM			
Isorhamnetin 0 µM	100 ± 5.6	100 ± 4.0	100 ± 3.7
Isorhamnetin 1 µM	101 ± 2.7	97 ± 3.6	96 ± 3.1
Isorhamnetin 10 µM	85 ± 2.3	81 ± 2.9	81 ± 2.7
Isorhamnetin 100 µM	87 ± 1.7	82 ± 2.0	82 ± 1.7
HIM			
Isorhamnetin 0 µM	100 ± 1.0	100 ± 1.3	100 ± 1.6
Isorhamnetin 1 µM	72 ± 0.0	72 ± 0.8	72 ± 0.4
Isorhamnetin 10 µM	27 ± 0.8	29 ± 0.9	28 ± 0.6
Isorhamnetin 100 µM	30 ± 0.4	33 ± 0.3	30 ± 0.1

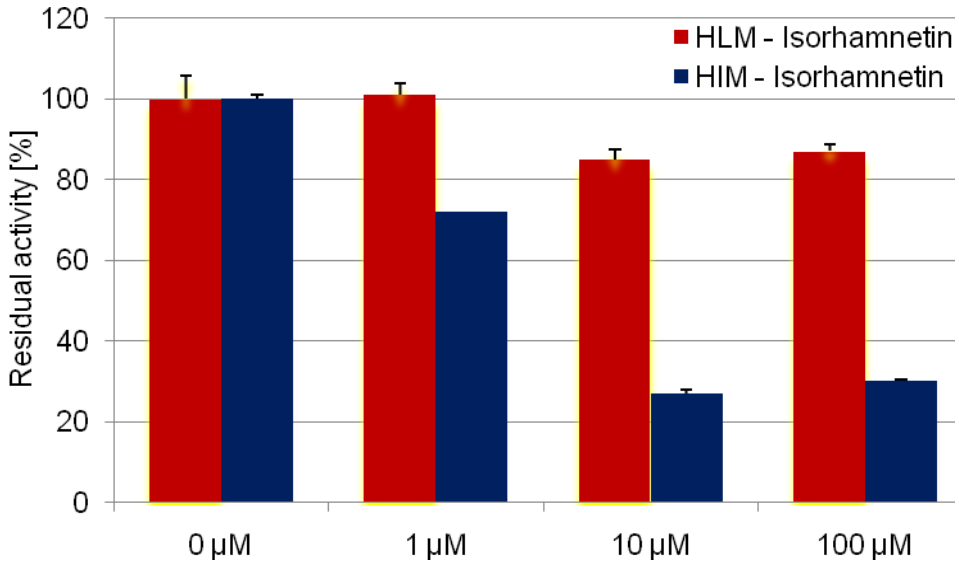


Figure 21 Inhibition of TFMU glucuronidation by isorhamnetin at various concentrations tested on human liver (HLM) and human intestinal (HIM) microsomes.

6. DISCUSSION

The issue of herb-drug interactions is discussed more and more in the last years due to increasing consumption of herbal supplements. There are some commonly used medicinal drugs, such as *Echinacea*, *Ginkgo biloba*, garlic, ginseng, and many others which have been proven to have a high potency to interact with prescription and other concurrently taken drugs (Fugh-Berman 2000, Hu et al. 2005, Tachjian et al. 2010, Tarirai et al. 2010). Although also St. John's wort and thyme are generally considered the safe natural drugs, there are many evidences that St. John's wort belongs to these „problematic" herbs. So far, studies have generally focused mainly on pharmacokinetic interactions of these herbs with cytochrome P450, as one of the most important biotransformation systems in human body. However, recent studies have shown that the role of glucuronidation and its potential inhibition is equally important.

Several *in vitro* studies dealing with herbal extracts and their inhibition of glucuronidation have been published to date (reviewed in Mohamed & Frye 2011). These are focused on *Kampo*, a traditional Japanese mixture of several medicinal herbs (Katoh et al. 2009; Nakagawa et al. 2009), *Andrographis paniculata* and *Orthosiphon stamineus* (Ismail et al. 2010), and *Ginkgo biloba* (Mohamed & Frye 2010). A study of Mohamed et al. (2010) tested a few herbs (e.g. ginseng, *Echinacea*, black cohosh, garlic, milk thistle, valerian, and saw palmetto) for inhibition of UGT1A1. Based on available data, extracts of St. John's wort or thyme have been investigated in UGT inhibition assays only to a little extent. Two independent studies proved quercetin, a constituent occurring in both extracts, to be the inhibitor of UGT1A and UGT1A9 hepatic isoforms. (Williams et al. 2002, Mohamed & Frye 2010). In recent study of Volak (2010), hypericin is reported to be an inhibitor of UGT1A6. The results obtained during this diploma thesis proved St. John's worth and thyme to be inhibitors of glucuronidation. It can be also suggested that these medicinal herbs may act as inhibitors of this enzymatic process in humans.

Considering liver the main organ involved in glucuronidation, standard inhibition assays deal primarily with human liver microsomes. However, the intestinal glucuronidation may play an important role in bioavailability of some oral drugs as well. According to Mizuma (2009) it seems likely intestinal glucuronidation, and its contribution to the first-pass metabolism, has a greater impact on oral bioavailability than hepatic glucuronidation metabolism in humans. Most of the performed inhibition assays were carried out in both human liver and intestinal microsomes using a

prototypic glucuronidation substrate 4-trifluoromethylumbelliferone. The UGT inhibitory effect of the tested extracts was more pronounced in HIM. Comparing spectra of human UGT isoforms present in HLM and HIM, UGT1A8 and UGT1A10 are generally believed to be the gastrointestinal tract representatives only (Turkey & Strassburg 2000, Kiang et al. 2005). Although Li et al. (2007) provided evidence that their mRNA is expressed at low levels also in primary human hepatocytes, UGT1A8 and UGT1A10 have stayed mainly the important intestinal isoforms. Among substances that are the substrates of UGT1A8 and UGT1A10 belong mycophenolic acid (Mohamed & Frye 2010), niflumic acid (Sakaguchi et al. 2004), and troglitazone (Watanabe Y. et al. 2002). Both isoforms also catalyze 4-glucuronidation of raloxifene (Kemp et al., 2002) and 3-*O*-glucuronidation of morphine (Stone et al. 2003). Glucuronidation of propofol, naloxone, and naltrexone (Cheng et al. 1999) is catalyzed by UGT1A8, as well as 6-glucuronidation of raloxifene (Kemp et al. 2002). These data suggest that metabolism of these important and widely used drugs as well as other compounds, which are substrates of UGT1A8 and UGT1A10, may be significantly influenced by inhibitors of these isoforms. Based on the obtained results, it can be stated that extracts of *Hypericum perforatum* and *Thymus vulgaris* belong among these inhibitors. To support this statement, it would be necessary to carry out assays with pure preparations of these UGT isoforms. Extracts of St. John's wort and thyme were tested in concentrations of 10, 50, and 250 µg/mL and the concentration-dependent inhibition of glucuronidation was observed. Next step in this field may be testing these extracts in concentrations that could be reached in human body (especially in HLM) under normal conditions. Nevertheless, this is more a task for clinical studies and it was not a part of this work.

The identification of substances involved in UGT inhibition followed, so the extracts were first fractionated. The goal was to obtain fractions, which would contain just a narrow range of compounds and thus simplify their identification. A suitable method that would be efficient but reasonably long must have been developed. Many different solvents were tested in order to achieve required separation. Runs with acetonitrile as a solvent B, as well as testing of an ion pair chromatography with tetrabutyl ammonium chloride, did not bring results that would overweight disadvantages of these solvents (e.g. price, complicated preparation, unfavorable effect to the column etc.). Composition of solvents was finally established to 0.1% formic acid in water (solvent A) and 0.1% formic acid in methanol (solvent B). The developed method was tested with an addition of intended substrate (4-trifluormethylumbelliferone) and internal standard (methylumbelliferylglucuronide) to prevent a possible overlapping of their signals with signals of searched substances and

proved to be suitable. In this work generally, analytical methods of HPLC with fluorescent and/or UV detection and LC/MS/MS were performed. Results of all performed analyses are listed in the thesis, although data from LC/MS/MS are, considering this way of detection as the most accurate, presented as valid. The comparable results were reached using fluorescent detection, while UV detection did not prove to be accurate enough to be used for detection of glucuronides in future studies.

From the fifteen collected fractions of SJW, especially those at the beginning and at the end of chromatogram have shown high inhibitory effect on glucuronidation. The inhibitory effect of selected fractions was studied in human liver microsomes only. The highest inhibitory effects were obtained with fraction XIII and XIV, which reduced initial UGT activity by 43% and 38%, respectively. Although the results have not been clarified by testing the standards, compounds identified in the fraction XIII were hyperfirin, adhyperfirin, hyperforin, while adhyperforin was found in the fraction XIV. Although some of the published studies dealing with the analysis of SJW chemical composition were carried out in the absence of light (Tolonen et al. 2002, Tolonen et al. 2003, Tatsis et al. 2007), the extraction of crude drug for our experiments was performed under common laboratory conditions, which could influence results of inhibitory assays due to the decomposition of light-sensitive constituents (e.g. naphthodianthrones and phloroglucinols). The extent to which these conditions distorted obtained results is unknown; nevertheless we could speculate that the inhibitory effect may be even stronger. However, phloroglucinols and naphthodianthrones may have the greatest effect on glucuronidation from all of the SJW chemical constituents and it would be interesting to test this hypothesis. Nevertheless, this experiment was not performed due to the high instability and purchase price of these compounds. Other fraction with high inhibitory activity (36%) was fraction IV, unfortunately its active constituents have not been identified. Their chemical characterization can be just estimated from the position of their signals between phenylpropanes in fraction III and flavonoids in fraction VI.

Flavonoids have received more attention in recent years because of their antioxidant capacity and their impact on human health (Bravo 1998). Within the context of SJW, it is believed that they significantly support the effect of its pharmacologically active compounds, such as phloroglucinols (Butterweck & Schmidt 2007). Although the inhibitory activity of fractions containing flavonoids is not high (17% inhibition by fraction VI), these compounds could support the pharmacological effect of other active constituents by reducing the rate of their biotransformation (inhibition of

biotransformation enzymes), which could be possible due to the relatively high concentration of flavonoids in SJW.

Biflavones amentoflavone and I3,II8-biapigenin were identified in the fraction VIII and IX, respectively. Their contribution to UGT inhibition in HLM was not significant; fraction containing amentoflavone was excluded from further studies for its low inhibitory effect (11%). Interestingly, inhibitory effect exerted by pure biflavones (standards of amentoflavone and I3,II8-biapigenin) was high and even slightly higher in the case of amentoflavone. It is likely that low concentrations of biflavones in SJW extract (0.1-0.5% for I3,II8-biapigenin, ten times less for amentoflavone; Berghöfer & Hölz 1986) are not sufficient for massive inhibition of the drug metabolism. These findings support the need to test all identified compounds at physiological levels that they can reach. In the case of SJW, the contribution of biflavones to the inhibition of glucuronidation is probably not relevant due to their low concentration in herbal supplements.

Thyme assay proved thyme to be a significant inhibitor of glucuronidation. Again, the inhibition was more pronounced in HIM. Thyme has not been investigated for UGT inhibitory activity yet. There is a study that deals with influence of thyme leaves and its phenolic constituents, thymol and carvacrol, on phase I and phase II biotransformation enzymes (Sasaki et al. 2005), however UGT was not included.

To obtain a detectable amount of potential inhibitors in individual fractions, an extract of 1000 μg/mL concentration was prepared for the semipreparative analysis. Likely because of the higher concentration, broader spectrum of chemical constituents of thyme or prolonged period of one fraction collecting (two min in contrast to one min in SJW assay), the inhibition of glucuronidation was stronger in thyme fractions inhibition assays. However, the changes in the method were made advisedly, with respect to individual character of extracts.

Published data regarding effects and pharmacology of thyme are scarce. A bit more is known about its chemical composition, but unlike SJW where almost all secondary metabolites and their structures have been already identified, the spectrum of compounds present in thyme is extensively broad and it is not possible to involve all of them. Polyphenols, especially flavonoids, receive the highest attention in the published literature mainly for their antioxidant activity (Rice-Evans et al. 1997, Kähkönen et al. 1999, Zheng & Wang 2001) and thus the identification screening was focused mainly on these compounds. The list of tested compounds was prepared in accordance with the work of Hossain et al. (2010).

The obtained results have shown that the fraction with the highest inhibitory potency were at the end of the chromatogram, especially the fractions VI, VII and IX (94, 95 and 92% inhibition), respectively. By further examination of these fractions, flavonoids quercetin, luteolin, apigenin and isorhamnetin were identified in fraction VI. Inhibitory effect of these fractions was higher than that of fraction IV (29%), where glycosides of these flavonoids have been identified. Inhibitory properties of glycosides are in general lower than these of their aglycones. The inhibitory activity of pure isorhamnetin on UGT in HIM and HLM was tested in concentrations of 1, 10 and 100 μM. Its inhibitory effect was more pronounced in HIM than in HLM especially in higher concentrations (10 μM and 100 μM) although the difference between inhibition caused by these two concentrations was not significant (e.g. UGT inhibition in HLM was 15% and 13% for 10 μM and 100 μM isorhamnetin, while it was 73% and 70% inhibition of UGT in HIM for 10 μM and 100 μM isorhamnetin). Flavonoids represent the biggest group of secondary metabolites present in thyme and thereby may play a significant role in the inhibition of glucuronidation of some concomitantly taken drugs. Beside flavonoids, monoterpene derivates of thyme, thymol and carvacrol, receive considerable attention in literature. However, performed handling with the samples does not guarantee preservation of volatile compounds in them. Also LC/MS/MS analysis is probably not a suitable method for their determination; gas chromatography (GC) resp. GC/MS would be preferable due to their unstable character.

In this work, inhibition of glucuronidation by extracts of St. John's wort, thyme and their constituents was under investigation. In previous studies, St. John's wort has demonstrated the ability to influence the oral bioavailability of several drugs by a co-induction of the metabolic enzyme CYP3A4 and transporter P-gp *in vitro* as well as *in vivo* (Tarirai et al. 2010). In addition, SJW inhibits CYP1A2, CYP2C9, CYP2C19, and CYP2D6 (Obach 2010). From active substances of SJW extract, flavonoid quercetin was proven to inhibit CYP3A4 (Li et al. 1994) and CYP1A2 (Obach 2000), hyperforin showed inhibition of CYP2C9, CYP2D6 and CYP3A4, and biflavonoids inhibited CYP3A4, CYP2C9 and CYP1A2 isoforms. Also hypericin was shown to be a potent inhibitor of some CYP isoforms (Obach 2000) and UGT1A6 in human colon cells (Volak 2010). Interestingly, the above mentioned compounds exerted the potency to inhibit UGT enzymes in this work.

This work has been carried out as a part of long-term research conducted by PD Dr. Unger's team. In the last few years, they have been trying to elucidate the role of UGT inhibition by various herbal extracts in the biotransformation of commonly taken drugs. Other experiments regarding SJW extract and its active compounds, although

not included in this thesis, were conducted simultaneously with those described above. Thus, only a small part of all experiments, which have been carried out to date, is presented. The experimental part of this work was focused mainly on flavonoids and biflavones. Nevertheless, further research in this field (such as inhibition of glucuronidation by extracts and extract constituents in concentrations that can be reached in plasma, testing the constituents with the purified UGT isoforms in inhibition assays, as well as an improvement of the developed analytical method in sense of involving a next step that would provide a better separation within individual fractions) is necessary to perform.

7. CONCLUSIONS

Following conclusions were made on the basis of the results obtained:

- The inhibitory activity of St. John's wort and thyme extracts was determined using UGT model substrate TFMU in human liver microsomes (HLM) and human intestinal microsomes (HIM). Both extracts were tested in concentrations of 10, 50, and 250 µg/mL. SJW and thyme extracts exerted significant concentration-dependent inhibitory activity, which was in both cases more pronounced in HIM. Inhibitory activity of SJW extract was higher than that of thyme extract in HLM as well as in HIM.

- The suitable HPLC method to analyze the extract of St. John's wort was developed. As solvent A and B, 0.1% formic acid in water and 0.1% formic acid in methanol was used, respectively. The linear gradient from 15% to 100% of B for 12 minutes, 100% of B for 3 minutes and 3 minutes for re-equilibration was used. The method was performed using a ZORBAX SB-18 (4.6 x 150 mm, particle size 5 µm) column with a 4.6 x 12.5 mm precolumn (Agilent Technologies). The extract of St. John's wort was fractionated using the developed analytical method with the semipreparative ZORBAX SB-18 (9.4 x 150 mm, particle size 5 µm) column and a 9.4 x 15 mm precolumn (Agilent Technologies) into 15 fractions. The HPLC method for analysis of thyme extract was adapted from SJW assay. All parameters were kept; just the time of analysis was extended by 5 minutes. The thyme extract was fractionated into 10 fractions using the semipreparative HPLC method, the column and the precolumn as described for SJW.

- All collected fractions of SJW and thyme extracts were tested for UGT inhibitory activity in HLM. Ten fraction of SJW extract (No. III, IV, V, VI, VIII, XI, XII, XIII, XIV, and XV) with residual UGT activity lower than 85% of control were subjected to further testing. All fractions of thyme extract were further tested. Some of them, especially fractions No. VI, VII, VIII, and IX, showed significant UGT inhibitory activity.

- Flavonoids rutin, hyperosid, isoquercitrin, quercetin, and biapigenin were identified in SJW extract by LC/MS/MS analysis in SIM mode, HPLC analysis

with UV detection and by spiking experiments. Flavonoids isorhamnetin, luteolin, and apigenin were discovered in thyme extract using LC/MS/MS analysis in SIM mode and spiking experiments.

- The biflavones of St. John's wort, I3,II8-biapigenin and amentoflavone (I3',II8-biapigenin), inhibited TFMU glucuronidation in HLM and HIM in concentration-dependent manner (e.g. amentoflavone 10 μM decreased UGT activity in HLM and HIM by 61% and 94%, respectively). Flavonoid isorhamnetin also showed concentration-dependent inhibitory effect on UGT activity in HLM and HIM (e.g. it caused 15% and 73% inhibition of glucuronidation activity in HLM and HIM at 10 μM concentration, respectively). Inhibitory effects of all the before mentioned compounds were more pronounced in HIM than in HLM.

8. LIST OF ABBREVIATIONS

ATP	adenosine triphosphate
CYP	cytochrome P450
DAD	diode array detector
DMSO	dimethyl sulfoxide
ESI	electrospray ionization
ER	endoplasmic reticulum
GC	gas chromatography
GC/MS	gas chromatography tandem mass spectrometry
HIM	human intestinal microsomes
HIV	human immunodeficiency virus
HLM	human liver microsomes
HPLC	high performance liquid chromatography
LC/MS	liquid chromatography tandem mass spectrometry
m/z	mass-to-charge ratio in LC/MS
MAOi	monoamine oxidase inhibitor
MeOH	methanol
mRNA	messenger ribonucleic acid
MUG	4-methylumbelliferyl-ß-D-glucuronide
P-gp	P-glycoprotein
PXR	pregnane X receptor
RP-HPLC	reversed-phase high pressure liquid chromatography
SD	standard deviation
SIM	single ion monitoring
SJW	St. John’s wort
SSRIs	selective serotonin reuptake inhibitor
TFMU	4-trifluoromethylumbelliferone
UDP	uridine diphosphate
UDP-GA	uridine diphosphate glucuronic acid
UGT	uridine diphosphate glucuronosyltransferase

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