CHARLES UNIVERSITY
FACULTY OF PHARMACY IN HRADEC KRÁLOVÉ
DEPARTMENT OF BIOCHEMICAL SCIENCES

Mgr. Tomáš Zárybnický

POTENTIAL TOXICITY OF TERPENES AND THEIR EFFECTS IN LIVER CELLS

Doctoral Thesis

Supervisor: assoc. prof. PharmDr. Iva Boušová, Ph.D.

Consultant: assoc. prof. Ing. Petra Matoušková, Ph.D.

Hradec Králové 2020

I hereby declare that this thesis is my original work which I solely composed by myself under the supervision of assoc. prof. PharmDr. Iva Boušová, Ph.D. All used literature and other sources are summarized in the list of references and properly cited. This work has not been submitted for any different or equal degree.

Prohlašuji, že tato práce je mým původním autorským dílem, které jsem vypracoval samostatně pod vedením svého školitele doc. PharmDr. Ivy Boušové, Ph.D. Veškerá literatura a další zdroje, z nichž jsem při zpracování čerpal, jsou uvedeny v seznamu použité literatury a v práci řádně citovány. Práce nebyla využita k získání jiného nebo stejného titulu.

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Mgr. Tomáš Zárybnický

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ABSTRACT

Charles University
Faculty of Pharmacy in Hradec Králové
Department of Biochemical Sciences

Candidate: Mgr. Tomáš Zárybnický
Supervisor: assoc. prof. PharmDr. Iva Boušová, Ph.D.
Consultant: assoc. prof. Ing. Petra Matoušková, Ph.D.
Title of Doctoral Thesis: Potential toxicity of terpenes and their effects in liver cells

The public interest in herbal products, supplements, extracts or isolated active compounds has been increasing during last decades. The aim of this doctoral thesis was to study selected compounds from the group of monoterpenes and sesquiterpenes and their interactions with liver cells: interactions with drug metabolising enzymes, their potential liver toxicity and looking for novel mechanisms of action.

The literature about hepatotoxic properties of several monoterpenes and sesquiterpenes, that showed liver toxicity, was summarized first. Then, the study was aimed toward a known hepatotoxicant (*R*)-pulegone and its presumed metabolite (*R*)-menthofuran. The toxicity results in precision-cut human liver slices have shown that (*R*)-menthofuran was less toxic to human liver and that the reason may be inter-species differences between human and mice.

Several sesquiterpenes (farnesol, cis-nerolidol, trans-nerolidol, α-humulene, β-caryophyllene, and caryophyllene oxide) have previously inhibited the activity of several cytochrome P450 (CYP) isoforms, especially CYP3A4. These compounds significantly induced CYP3A4 expression via pregnane X receptor interaction in transfected HepG2 cells. The intention was to verify this effect in precision-cut human liver slices, using reverse transcription-quantitative polymerase chain reaction (RT-qPCR). For this reason, a validation study was first performed to check the stability of reference genes in human liver slices, required for normalisation of RT-qPCR data. In the end, no significant modulatory effect on the expression of studied drug metabolising enzymes in liver slices was observed under the effect of studied sesquiterpenes.

The toxicity of potential anticancer agents alantolactone (ALA) and germacrone (GER) was studied against a hepatocyte-like model, differentiated HepaRG cells. While

alantolactone has shown lesser toxicity towards HepaRG cells than highly proliferative cancer cell lines, both compounds have shown production of reactive oxygen species in considerably low concentrations. Using target prediction tool BATMANT-TCM, novel targets 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR) and aromatase (CYP19A1) were predicted for ALA and GER. While both compounds indeed targeted HMGCR, the effect was the most significant in toxic concentrations. They also influenced the aromatase mRNA expression, but each compound differently, showing that the mechanism will not be the same for both. The obtained results of this doctoral thesis extend the knowledge about acting of natural compounds on the human organism further.

ABSTRAKT

Univerzita Karlova
Farmaceutická fakulta v Hradci Králové
Katedra biochemických věd

Kandidát: Mgr. Tomáš Zárybnický
Školitel: doc. PharmDr. Iva Boušová, Ph.D.
Konzultant: doc. Ing. Petra Matoušková, Ph.D.
Název disertační práce: Potenciální toxicita terpenů a její účinky v jaterních buňkách

Veřejný zájem o rostlinné přípravky, potravní doplňky, extrakty a izolované aktivní látky v posledních desetiletích vzrůstá. Cílem této doktorské disertační práce bylo studovat vybrané látky ze skupiny monoterpenů a seskviterpenů a jejich interakce s jaterními buňkami: interakce s enzymy metabolizujícími léčiva, potenciální jaterní toxicitu a nové mechanismy účinku.

Literatura o známá o jaterní toxicitě několika monoterpenů a seskviterpenů byla nejprve shrnuta. Dále jsme se zaměřili na látku (*R*)-pulegon, známou svou jaterní toxicitou, a jeho předpokládaný metabolit (*R*)-menthofuran. Výsledky jejich toxicity v precizních tkáňových řezech z lidských jater ukázaly, že (*R*)-menthofuran byl méně toxický vůči lidským játrům a že pravděpodobným důvodem mohou být mezidruhové rozdíly mezi člověkem a myší.

Několik seskviterpenů (farnesol, *cis*-nerolidol, *trans*-nerolidol, α-humulene, β-karyofylen a karyofylenoxid) v předešlých studiích inhibovalo aktivitu několika isoforem cytochromu P450 (CYP), zejména pak CYP3A4. Tyto látky signifikantně indukovaly expresi CYP3A4 v transfekovaných HepG2 buňkách prostřednictvím pregnanového X receptoru. Cílem bylo ověřit tyto účinky v precizních lidských jaterních tkáňových řezech metodou reverzní transkripce-kvantitativní polymerázové řetězové reakce (RT-qPCR). Z tohoto důvodu byla nejdříve ověřena stabilita referenčních genů v lidských jaterních řezech, které jsou nutné pro validaci výsledků RT-qPCR. Nicméně, žádný signifikantní modulační účinek na expresi studovaných enzymů metabolizujících léčiva nebyl pozorován.

Toxicita potenciálních látek s protinádorovým účinkem, alantolaktonu (ALA) a germakronu (GER), byla studována na diferencovaných HepaRG buňkách, modelu podobném lidským hepatocytům. Zatímco ALA vykázal menší toxicitu vůči HepaRG buňkám než vůči vysoce proliferujícím nádorovým buněčným liniím, obě studované látky způsobily produkci reaktivních forem kyslíku v relativně nízkých koncentracích. S využitím nástroje pro predikci cílů BATMAN-TCM byly predikovány nové cíle v podobě 3-hydroxy-3-methylglutaryl-koenzym A reduktasy (HMGCR) a aromatasy (CYP19A1). Zatímco obě látky opravdu cílily na HMGCR, účinek byl nejvýznamnější v toxických koncentracích. Rovněž byla ovlivněna mRNA exprese aromatasy, avšak každá z látek působila jiným způsobem, což naznačuje že mechanismy jejich působení nebudou stejné. Získané výsledky této disertační práce posouvají znalosti o účincích přírodních látek na lidský organismus dále.

Content

1 Introduction

The use of herbs in treating maladies has a long history in both western and eastern countries. The rich libraries of natural compounds contained in herbs are also one of the most important sources for designing new drug candidates. However, just like synthetic drugs, herbs and their extracts also possess adverse effects. In recent years, more and more clinical cases and laboratory data have demonstrated that some herbal products may cause varying degrees of liver damage. In contrast to synthetic drugs, herbs are commonly thought to be safe and harmless by patients, as they come from nature [1-3].

Liver is a major organ of xenobiotic metabolism and is more likely to suffer from drug- or herb-induced injury than other organs. The adverse events include hepatitis, liver fibrosis, liver failure and even death [4, 5]. Nevertheless, these events are the worst case scenario, usually related with various criteria such as the gender, age, organism state and genetic background of patients, the dose and course of herbal treatment, misuse, and abuse of herbs and the quality of herbs (including adulterated products, bacterial contamination, the presence of heavy metals, pesticides, or solvents) [2, 3, 6, 7].

Another factor frequently overlooked by patients is the interactions between the compounds absorbed from plants or herb products, and drugs used by the patients. Concurrent use of herbs or herbal products may mimic, magnify, or oppose the effect of administered drugs. There are known many combinations of natural compounds and drugs that can endanger the life of patient. Among the most well-known and plausible herb-drug interactions are bleeding when warfarin is combined with ginkgo (*Ginkgo biloba*, Ginkgoaceae) or mild serotonin syndrome in patients who combine St John's wort (*Hypericum perforatum,* Hypericaceae) with serotonin-reuptake inhibitors [8].

Because of our interest in natural compounds and herb-drug interactions, this thesis focused on selected molecules from the group of mono- and sesquiterpenes. The intention was to study their liver toxicity, interactions with selected biotransformation enzymes and studying new mechanisms of their biological activity using liver *in vitro* models.

2 Theoretical part

2.1 Terpenes

Plants produce various types of secondary metabolites, many of which have been subsequently exploited for their beneficial effects [9]. Among these secondary plant metabolites, terpenes form an immensely large group of compounds that consist of repeated isoprene (C_5H_8) units. This group can be further classified based on the number of isoprene units (Table 1) or the functional chemical groups (Table 2) [10]. The role of terpenes can be seen in almost all basic plant processes. These include the growth, development, acting as attractants for animals that disperse pollen or seeds, being inhibitors of germination and growth of neighbouring plants and defence against herbivores or pathogens [11, 12].

Table 1. Classification of terpenes based on the number of isoprene units

C_5	Hemiterpene
C_{10}	Monoterpene
C_{15}	Sesquiterpene
C_{20}	Diterpene
C_{25}	Sesterterpene
C_{30}	Triterpene
C_{40}	Tetraterpene

Table 2. Classification of terpenes based on their chemical composition

Hydrocarbons	Limonene, Humulene
Alcohols	Linalool, geraniol, alantolactone
Esters	Linalyl acetate, menthyl acetate
Aldehydes	Cinnamaldehyde, geranial
Ketones	Carvone, thujone
Phenols	Eugenol, thymol
Ethers	Anethole, cineole
Peroxides	Ascaridole, artemisinin

Two routes for the formation of the C_5 building block of the terpene biosynthesis exist in plants. It is done either via the reactions of the mevalonate pathway or through the methylerythritol phosphate pathway. The mevalonate pathway involves the stepwise condensation of three molecules of acetyl coenzyme A (CoA) to form 3-hydroxy-3-methylglutaryl CoA, that is further reduced to mevalonic acid. After two successive phosphorylations and a decarboxylation, isopentenyl diphosphate is formed. On the other hand, the distinct methylerythritol phosphate pathway starts from glyceraldehyde phosphate and pyruvate to form 1-deoxy-D-xylulose 5-phosphate, a molecule that is further intramolecularly reorganized to form 2-methyl-D-erythritol 4-phosphate, which can be further transformed into isopentenyl diphosphate by successive dehydration and reduction steps and at least one phosphorylation (Figure 1) [12].

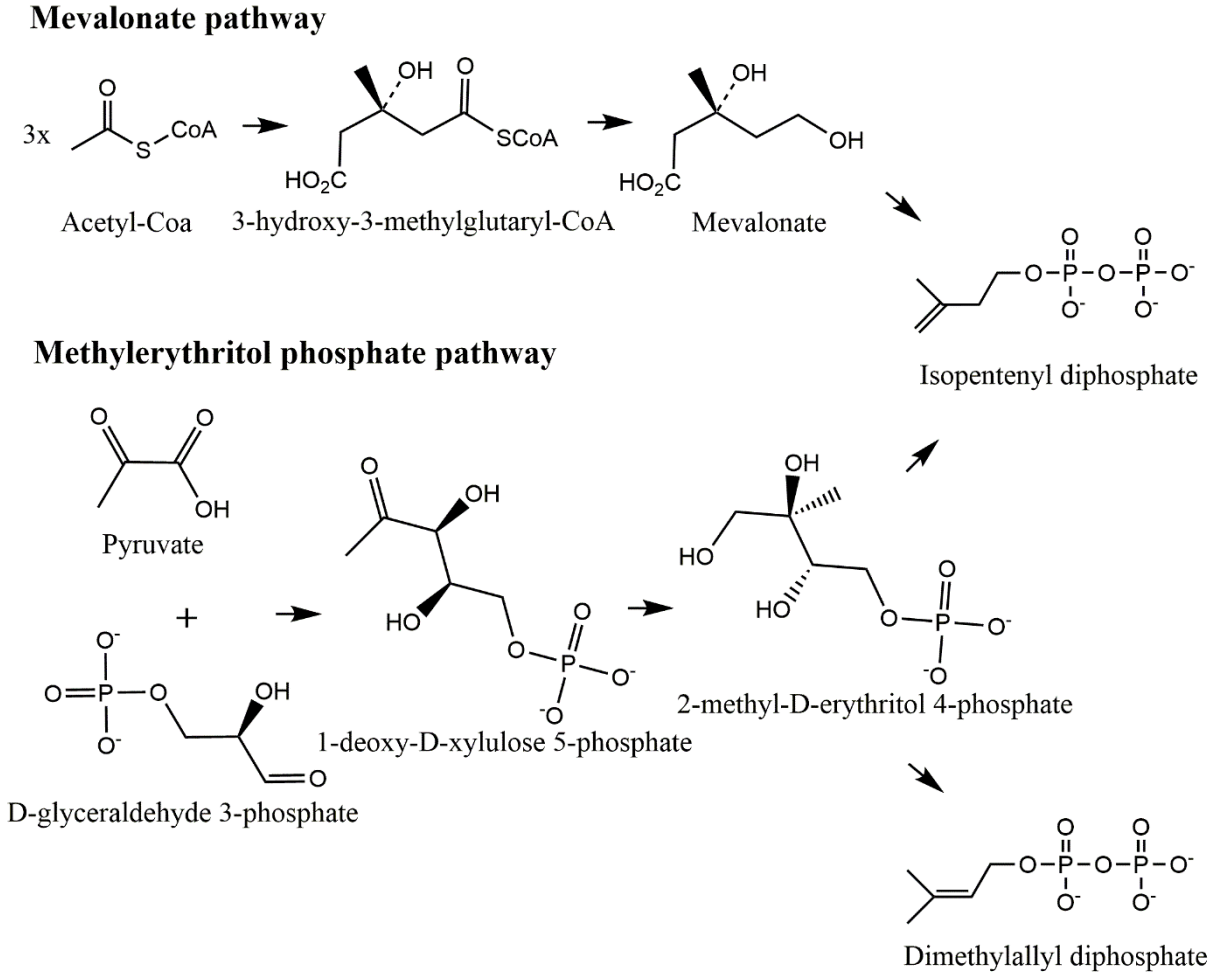


Figure 1. Mevalonate and methylerythritol phosphate pathways, leading isopentenyl diphosphate synthesis

The use of terpenes by humans dates as far as ancient Egypt and they hold a special place in both chemical and world history. The application of terpenes range from flavour and fragrance, for which they are added to foods, beverages, perfumes, soaps, toothpaste, tobacco and other products; to hormones or medicine [13]. Some terpenes also find use in industry as raw materials in the manufacture of adhesives, coatings, emulsifiers and speciality chemicals [12]. Monoterpenes and sesquiterpenes were the main target compounds of this thesis and will be therefore the only groups of terpenes further elaborated.

2.1.1 Monoterpenes

Terpenes with a basic structure consisting of two linked isoprene units are known as monoterpenes. They are described as the most representative molecules among terpenes, constituting 90% of the essential oils, with a great variety of structures [14]. Because of monoterpenes being inexpensive, attractive smell and high volatility, they have been used in flavourings and fragrances since the beginning of the 19th century [15]. Showing promising biological activity, such as antimicrobial, hypotensive, anti-inflammatory and antipruritic effects, they are also of medical interest [16-18]. Nevertheless, monoterpenes pulegone and menthofuran are very famous for their ability to induce liver damage in case of misuse or abuse of essential oils containing them and they became of interest for our research.

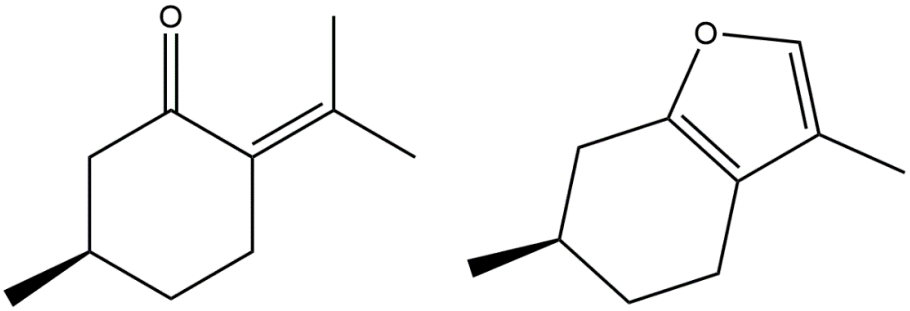
2.1.1.1 Pulegone

The monoterpene (*R*)-(+)-pulegone (PUL) was determined to be the major constituent of pennyroyal oil, comprising about 80–90% of this oil. Pennyroyal oil is a mint oil obtained from the leaves of *Mentha pulegium* (Lamiaceae) and *Hedeoma pulegoides* (Lamiaceae). Tea or oil prepared from the leaves of these plants has been used for decades as an aromatic stimulant, carminative, headache remedy or abused abortifacient [19]. Misuse or abuse of pennyroyal oil has led to many cases of intoxication, resulting in severe liver injury and even death, mostly in adult women using the oil to induce abortion. In those cases of patients who ingested a large amount of pennyroyal oil and resulted in hepatic toxicity that, upon further investigation, was classified as hepatic centrilobular necrosis [20-23]. During *in vivo* studies, PUL has been determined as the major hepatotoxic component of pennyroyal oil. The only component

showing higher toxicity than PUL was monoterpene menthofuran. However, menthofuran forms only 0.2% of the pennyroyal oil [24].

2.1.1.2 Menthofuran

The (*R*)-(+)-menthofuran (MF) is not only a minor component of pennyroyal oil, it is also described as a mammalian metabolite of PUL. MF is similarly present in other mint species [25, 26]. Like PUL, the *R*-isomer of menthofuran is more toxic isomer. Cytochrome P450 (CYP) plays a significant role in the biotransformation of PUL to MF and also in PUL and MF toxicity. Using recombinant enzymes, it was determined that CYP2E1 is the major metabolising enzyme for both PUL and MF. The CYP1A2 and CYP2C19 also play a small role. However, the significance of CYP1A2 is higher in MF metabolism [27].



(*R*)-(+)-Pulegone (*R*)-(+)-Menthofuran

Figure 2. Structure of monoterpenes (*R*)-(+)-Pulegone and (*R*)-(+)-Menthofuran

2.1.2 Sesquiterpenes

Sesquiterpenes are terpenes composed of 3 isoprene units. They have drawn the attention of the research community owing to their considerable anti-inflammatory, antitumorigenic, antioxidant, and antiparasitic activities [28]. They are also of large interest in pharmacy and cosmetology, since many sesquiterpenes were found to be percutaneous permeation enhancers [29]. Several linear (nerolidol and farnesol) and cyclic (α-humulene, β-caryophyllene, caryophyllene-oxide, alantolactone and germacrone) sesquiterpenes became a target of our studies (Figure 3). Although these compounds and herbal remedies containing them have demonstrated multiple beneficial

and health-promoting activities, information concerning possible herb–drug interactions and safety are limited.

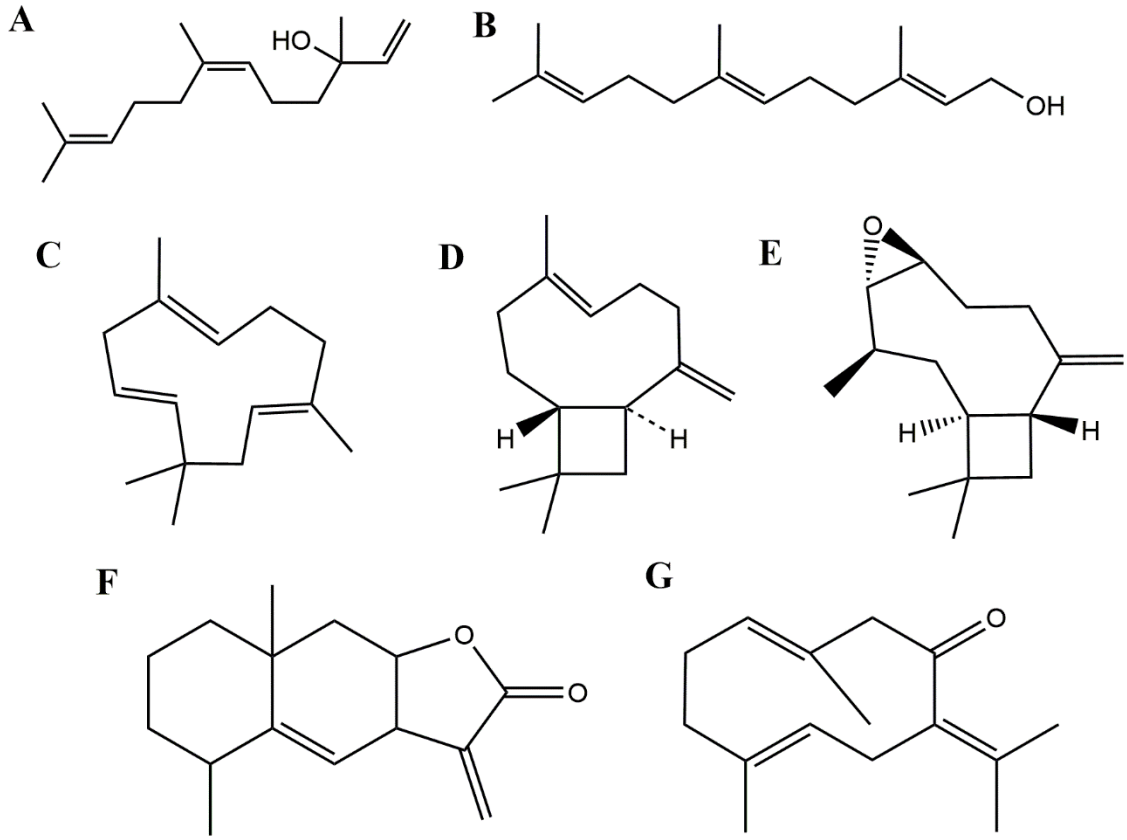


Figure 3. Structures of studied sesquiterpenes: A) cis-Nerolidol, B) Farnesol, C) α Humulen, D) β-Caryophyllen, E) Caryophyllen oxide, F) Alantolactone and G) Germacrone

2.1.2.1 Nerolidol

Linear alcohol nerolidol has two stereoisomers - *cis*-nerolidol (cNER) and *trans*-nerolidol (tNER) - and it is a highly potent enhancer of percutaneous permeation [30]. Both cNER and tNER were previously described to substantially inhibit CYP1A, 2B and 3A activities in rat and human subcellular fractions, but no significant changes to other biotransformation enzymes were detected [31]. However, tNER after a single dose application to mice increased CYP2B, 3A and 2C activities and mRNA expression in liver and small intestine. It also elevated, aldo-ketoreductase 1C and carbonyl reductase activities in liver. On the other hand, tNER decreased the NAD(P)H:quinone oxidoreductase 1 activity in small intestine of the tested mice [32].

2.1.2.2 Farnesol

Similarly to nerolidol, farnesol (FAR) is a highly potent enhancer of percutaneous permeation [30]. Just like nerolidol, FAR showed marked inhibition of CYP1A, CYP2B and CYP3A activities in rat and human subcellular fractions, but no significant changes to other biotransformation enzymes were found [31].

2.1.2.3 α-Humulene

Sesquiterpenes α-humulene (HUM), β-caryophyllene and caryophyllene-oxide are major components of the essential oil isolated from *Myrica rubra* (Myricaceae) leaves. This essential oil has shown anti-proliferative and pro-apoptotic effect toward several cancer cell lines (e.g. HCT8, SW620, SW480, HT29 and Caco2), but induced no change in viability of primary rat hepatocytes [33]. Moreover, HUM inhibited CYP3A4 activity in rat and human microsomes. Activity of this subfamily of CYP is very important for drug biotransformation and potential interaction could be harmful for patients [34].

2.1.2.4 β-Caryophyllene

β-Caryophyllene (CAR), a flavouring agent since the 1930s, showed and antioxidant effect and potential protective effect on liver fibrosis and its inhibitory capacity on hepatic stellate cell (HSC) activation in liver from carbon tetrachloride-induced fibrosis rat model. Testing the scavenging activity of free radicals and inhibition of 5-lipoxygenase, CAR has shown more potent effect than HUM or α-tocopherol [35]. However, CAR has also shown inhibitory effect toward CYP3A4 in rat and human liver fractions [34].

2.1.2.5 Caryophyllene-oxide

A single dose application of caryophyllene-oxide (CAO) to mice increased CYP2B, 3A and 2C activities and mRNA expression in liver and small intestine. CAO also elevated aldo-ketoreductase 1C and carbonyl reductase activities in liver and decreased NAD(P)H:quinone oxidoreductase 1 activity in small intestine [29]. In rat and human liver fractions, CAO has shown the highest inhibitory effect toward CYP3A4 activity among the studied sesquiterpenes from *Myrica rubra* essential oil, HUM and CAR [33, 34].

2.1.2.6 Alantolactone

Alantolactone (ALA) is a sesquiterpene lactone and one of the major sesquiterpene lactone compounds isolated from the roots of *Inula helenium* (Asteraceae). These roots have been used as a medicine historically against various ailments such as asthma, cough, bronchitis and it is studied as a potential anticancer agent [36, 37]. It is a compound of interest for its ability to inhibit the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and signal transducer and activator of transcription 3 (STAT3) [38, 39]. Both transcription factors regulate many common genes, including those regulating apoptosis, which makes ALA a potential candidate for anticancer research. However, practically no information about its toxicity towards healthy cells is known.

2.1.2.7 Germacrone

Another sesquiterpene with biological activity highly similar to ALA is germacrone (GER). GER is the main bioactive constituent found in *Zedoary* oil product, which is extracted from *Curcuma zedoaria* Roscoe (Zingiberaceae). This tropical plant is used as a natural flavour and medicinal herb, traditionally used for the treatment of dyspepsia, menstrual disorders, flatulence, fever, and cough [40, 41]. Although GER also inhibits STAT3 activation, no effect was observed for NF-κB transcription factor in breast cancer cells [42-44]. Due to the half-maximal inhibitory concentration (IC_{50}) of GER towards cancer cells being above 100 μM, its potential is quite limited. GER has therefore become a target of studies that intended to potentiate its biological activity and toxicity toward cancer cell lines [45, 46]. Despite the intention of potentiating GER activity, no studies related to GER safety were performed.

2.2 Liver *in vitro* models

The use of liver *in vitro* models is essential for both clinical and pre-clinical applications. These models are essential for bringing new insights into the pathologies of liver diseases, drug or other xenobiotic toxicity, xenobiotic biotransformation or drug-drug and drug-herb interactions. Liver has a key role in the removal of drugs and toxic substances from the body, it is highly susceptible to drug accumulation and associated liver damage. Extremely rigorous testing is necessary to validate the safety of any xenobiotic to humans. Animal models have been historically the first choice for

toxicity testing, which lead to an astronomical number of animals used in research continuously [47]. With the idea to decrease these numbers, 3R principles (Replacement, Reduction and Refinement) were suggested in 1959. It created a concept for humane use of animals in research and promoting formation and using of alternative models in research [48]. In the end, no model is perfect and either *in vitro* or *in vivo* models have their pros and cons that need to be taken in consideration, depending on the spectrum of our research.

2.2.1 Conventional 2D cell cultures

Cell biology frequently relies on 2D models generated from dissociated cells, cultures that are expanded on plastic surfaces. They are either primary cell cultures derived directly from harvested tissue or immortalized cell lines (i.e. primary cells genetically transformed to produce rapidly proliferating, uniform, easily cultured, artificial phenotypes). The majority of mammalian cells can be expanded into adherent colonies on culture plates, which have been proven to be relatively low cost and easy to manipulate and maintain [49].

2.2.1.1 Primary hepatocytes

Primary hepatocytes are often employed as a "gold standard", because they display many phenotypic functions of the liver when compared to other *in vitro* models [50]. They are frequently used for drug metabolism and hepatotoxicity assessment and can be used either as single cell suspension or plated in a monolayer [51]. Unfortunately, primary cultures of hepatocytes are limited by many issues. They maintain their wild-type characteristics only for a limited time because of de-differentiation and they are cultured in lower densities than in physiological liver. Similarly, they can have a limited availability (human liver), batch-batch variability and decreasing CYP expression during culture, significantly limiting the translatability of this model [52-54]. To overcome the shortages of primary hepatocytes, the method of proliferating genes transduction into primary human hepatocytes was devised to release the hepatocytes temporarily from cell-cycle arrest without immortalization and changing their phenotype [55, 56]. It was found that this effect is also approachable through the use of a proper cocktail of growth factors and small molecules [57, 58].

2.2.1.2 Sandwich-cultured hepatocytes

By seeding primary hepatocytes between two layers of collagen (Figure 4), it results in a modification with a retained cellular polarity, correct localization of basolateral and canalicular transporters as well as formation of functional bile networks [59, 60]. This set up makes them a suitable tool for evaluating hepatobiliary drug transport *in vitro* [61]. However, due to varying CYP activity in sandwich hepatocytes, it may not be considered a suitable tool for metabolic stability assessments with compounds predominantly cleared by certain CYP enzymes [62].

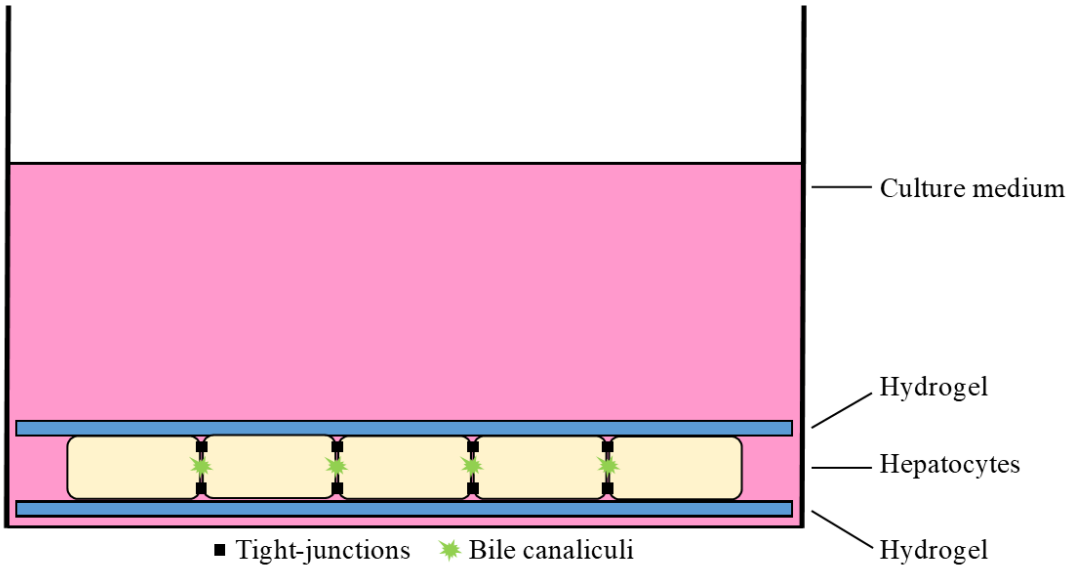


Figure 4. Sandwich culture hepatocytes

2.2.1.3 Immortalized cell lines

For these cells, rapid proliferation, resistance to de-differentiation and improved passaging are typical. It makes them convenient to maintain, expand, and retain phenotypic consistency between experiments. The shortcomings can be an abnormal phenotype, differences in cell morphology in comparison to healthy tissue, changes in barrier permeability and expression of important proteins that might deviate from the real tissue [63]. Several strategies have been employed to solve the issue of human hepatoma cell lines losing liver-specific functions, including co-culture systems with primary human hepatocytes, overexpressing liver-enriched transcription factors, CYP enzymes, or proliferation inhibitors [64]. The two most frequently used liver hepatoma cell lines are HepG2 and HepaRG cells [63].

HepG2 cell line was derived from a hepatocellular carcinoma of a 15-year-old Caucasian male and because of endogenously low expression of CYP (especially CYP3A4), glutathione-S-transferase isoforms GSTA1/2 and GSTM1 and several drug transporters (bile salt export pump, organic anion transporter C, sodium-dependent uptake transporter and organic cation transporter) it is a poor choice for drug metabolism and toxicity testing [65, 66]. On the other hand, the expression of CYP3A7 and CYP1A1 is sufficient, suggesting foetal liver phenotype [65, 67]. So far, HepG2 cells were exploited in numerous kinds of experiments, examining cytoprotective, antioxidative, hepatoprotective, anticancer, antisteatotic, bioenergetic homeostatic and anti-insulin resistant properties of various bioactive compounds of chemical and botanical origin [68].

HepaRG cell line was derived from a hepatoma of a female patient with cirrhosis, following hepatitis C virus infection [69]. This perspective progenitor cell line has a low expression of liver-specific genes during proliferative phase. However, the bipotent progenitor HepaRG cells start to transform into hepatocyte-like cells, forming hepatocyte islands, surrounded by biliary-like epithelial cells [70]. The differentiation of confluent HepaRG cells is supported by exposition to 2% dimethyl sulfoxide (DMSO) in culture medium. However, the existing method of HepaRG differentiation is time-consuming and cannot avoid DMSO at high concentrations, which can interfere with experiments [71]. Therefore, novel approaches to HepaRG differentiation, such as culture medium modifications, are expected [72]. Unlike other human liver cell lines, differentiated HepaRG cells express many drug metabolising enzymes at levels very similar to those in primary human hepatocytes. In particular, HepaRG express various nuclear receptors (pregnane X receptor and constitutive androstane receptor), transporters and specific markers of adult hepatocytes, such as albumin, haptoglobins and aldolase B [66, 73].

2.2.1.4 Hepatocytes derived from stem cells

Substantial progress has been made in generating functional hepatocytes from adult-derived pluripotent cells for toxicity screening, generating disease models, or they can be used even for the treatment of liver failure. With the development of embryonic stem cells (ESC) and, more recently, human induced pluripotent stem cells (iHPSC), novel possibilities opened. These cells can undergo cell replication without losing their ability to differentiate into any type of somatic cell. While some cell types have a

tendency to develop more spontaneously (cardiomyocytes), hepatocytes and renal tubular cells require significant technical manipulation [74].

2.2.2 3D liver models

To solve the deficiencies of 2D models (e.g. reduction of gene activity and expression of genes involved in drug metabolism, variable drug responsiveness and a limited capacity to predict toxicity), approaches to improve the *in vivo*-like cell density, anatomy of liver lobule, oxygen induced zonation, a circulatory system, cell-cell and cell-matrix interactions, have driven 3D models development [75]. Nevertheless, some 3D models are limited by laborious preparation and are not suitable for high-throughput screening. Despite that, they represent an exciting opportunity for functionally more relevant clinical modelling. The microenvironment of the hepatocyte *in vivo* is very important to the maintenance of normal function, including its response to endogenous and exogenous substrates [76]. Hepatocytes possess multiple apical surfaces (bile canalicular surfaces) and two basolateral surfaces, which makes re-establishing their cell polarity *in vitro* more challenging, than with other cell types [51, 77]. Depending on the position within hepatic lobule (functional liver unit), hepatocytes assume distinct biochemical programs. Zonation of various biochemical pathways in liver lobule is presented in Figure 5 [78].

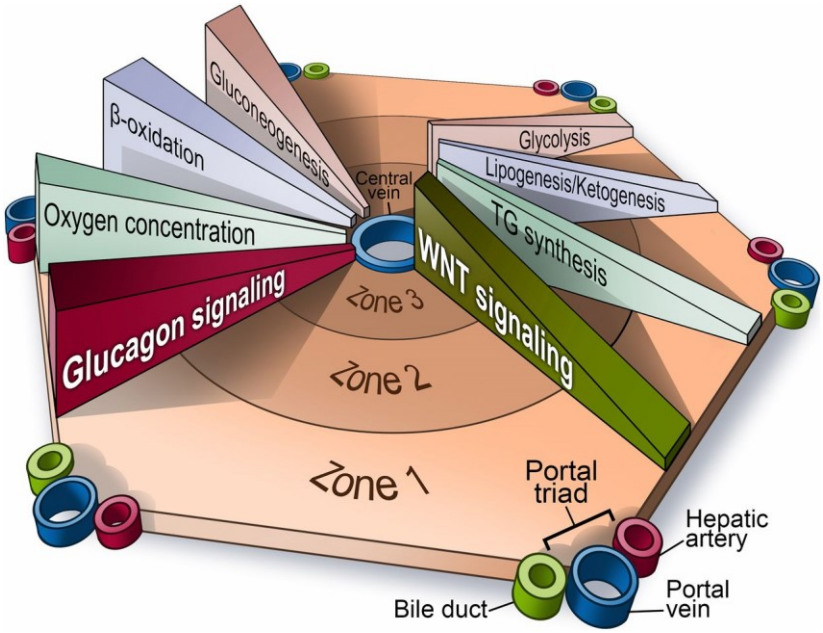


Figure 5. Zonation of different biochemical pathways in the liver

2.2.2.1 Precision-cut liver slices

To mimic the liver pathophysiology is challenging because of the intricacy of cellular composition and cell heterogeneity, the active role of the extracellular matrix and the complex tissue arrangement. This issue can be overcome by using precision-cut liver slices (PCLS), where the multi-cellular architecture is preserved (Figure 6). It was described that in PCLS, the viability can be maintained for up to 15 days [79]. However, despite liver slices containing the whole range of phase I and phase II xenobiotic metabolic enzymes, the decline in their activities in culture is still a major restriction [80-82]. It was also proven, that fibrosis appears during prolonged culture of PCLS, demonstrated by increased expression of heat shock protein 47, pro-collagen 1A1 and increased collagen 1 protein levels. On the other hand, this is a promising model to study antifibrotic effects of drugs [83]. It is also known that liver slices suffer from oxidative stress, which is a result of the slicing procedure and a high oxygen tension, which is necessary for culture of liver slices [84].

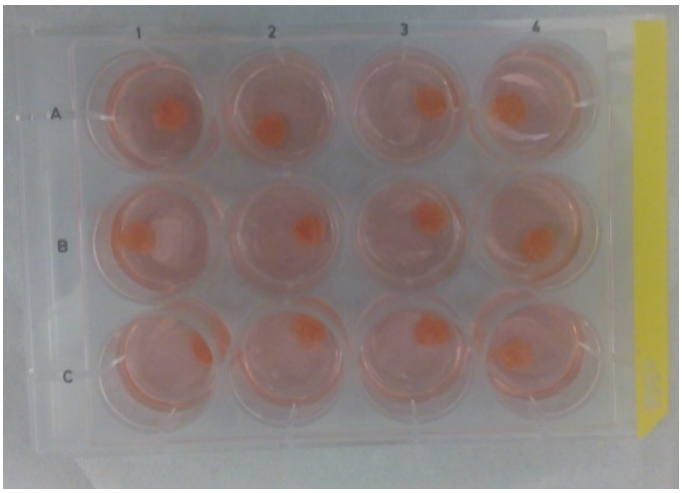


Figure 6. Precision-cut human liver slices

Human tissue for preparation of PCLS is usually obtained from the partial hepatectomy, from surgical waste for discard, explanted tissue or non-transplantable tissue. Liver slices can also be produced from diseased livers, such as patients with severe fibrosis and cirrhosis and resected malignant or metastatic liver tissue can also be used to obtain tumour slices [80].

2.2.2.2 Spheroids

Forming spheroid models is one of many methods to avoid or to ameliorate hepatocyte de-differentiation and to avoid forcing cells to adjust to an artificial and rigid surface [85, 86]. There exist multiple techniques to form spheroids and similarly, spheroids can be formed from primary hepatocytes, hepatoma cell lines or co-cultures [87]. It has been shown previously, that primary human hepatocytes (PHH) can be maintained for longer periods of time and with stable viability and production of essential molecules, such as albumin and urea, in spheroid culture [88, 89]. Also, cellular polarity and formation of functional bile ducts has been described [88]. This system can be used in diverse applications, including drug toxicity screening and investigating the idiosyncratic drug-induced liver injury [90].

The 3D organotypic models of cancer are "multicellular tumour spheroids", which are constructed from homogeneous cancer cells or co-cultures on nonadherent surfaces, where the cell suspension undergoes aggregation and compaction [86, 91]. Liver multicellular tumour spheroids have been used for understanding to microenvironmental chemoresistance of hepatocellular carcinoma. This type of drug resistance is associated with the crosstalk between hepatocellular carcinoma cells, hepatic stellate cells and the other stromal cells [92, 93]. On the other hand, because of missing circulation inside the spheroid and central hypoxia, the multicellular tumour spheroid can be less sensitive to anticancer agents, than monolayer models [94]. The maximum size of spheroids consisting of viable cells was reported to be 100–150 μm because of lack of oxygen [95-97].

2.2.2.3 Co-culture systems

Cells are not single entities but depend on the signals and interactions from other cells and therefore, hepatocyte-only models will not represent actual functionality when testing liver toxicity. Hepatocyte co-cultures usually contain hepatocytes with a single added cell type, such as endothelial cells, fibroblasts, Kupffer cells, stellate cells or other cell lines [98-101]. However, adding multiple cell types is possible as well. Co-culture systems can belong into both 2D and 3D models. An example of 2D can be a triculture of PHH with 3T3-J2 fibroblasts and liver sinusoidal endothelial cells overlaid with Matrigel, which displayed a stable phenotype with increased albumin and urea secretion for 3 weeks [102]. While a 3D model of PHH co-culture with liver sinusoidal endothelial

cells, Kupffer cells and hepatic stellate cells remained integrated throughout the subsequent 14-day culture period and this configuration seemed to have a better functionality than a single PHH spheroid [103].

2.2.2.4 Liver-on-chip

To mimic the 3D architecture and organ-specific microenvironment, an "organ-on-chip" microfluidic approach can be taken. Matrix-coated porous membranes with a layer of endothelial cells and populated by the desired co-culture, connected by wells containing the preferred perfusion medium, can be used to predict clearance, toxicity and mechanism of action of drugs [104, 105]. The microfluidic system can be directly connected to analytical instruments and detect metabolic changes in real time. An example is a study using NMR spectroscopy and Liver-on-chip model to study metabolic response of hepatocytes to hepatotoxic drug flutamide and its metabolite hydroxyflutamide [106]. Furthermore, biochips containing primary hepatocytes can be used to monitor pharmacokinetic parameters of drugs and these results were described to resemble the data from clinical trials [107]. Despite that, many issues remain to be solved, especially in terms of lobule zonation or inducing and maintaining heterogenous expression of drug metabolising enzymes [104, 108]. Due to limitations in production methods, it is complicated to arrange cells and extracellular matrix components into organized 2D/3D structures. The creation of biliary system in a liver-on-chip device is especially important to recapitulate the liver microenvironment and enhance hepatocyte functionality [109].

2.2.2.5 Isolated perfused liver

Isolated perfused liver (IPL) is a long established and widely used model in various scientific fields, ranging from pharmacological and toxicological studies to physiology and pathophysiology analysis [110]. This technique is considered as an *ex vivo* model and differs significantly from both *in vitro* and *in vivo* models. Its application is deeply connected with liver graft preservation, especially in cases involving marginal organs such as fatty livers or organs from non-heart beating donors, that would otherwise be discarded [111, 112]. The advantages of perfused liver are preservation of the hepatic architecture and polarity, the natural cell–cell relationships and zonal heterogeneities. Further, the vascular integrity and the bile flow can be also assessed, allowing the evaluation of the non-parenchymal cell involvement [113, 114]. A precise control of the

experimental condition can be induced in IPL by avoiding extra-hepatic factors, such as blood constituents and neuro-hormonal substances. Furthermore, exposure of the liver to different substances and real-time evaluation of the liver damage are possible as well [113].

To obtain accurate and reproducible results, great skill in microsurgical techniques is necessary. The correct setting of perfusion system is another factor that can influence the gained results. Just like the other liver models, standardized protocols and equivalent practice among laboratories are essential to gain reproducible data. In IPL, this demand is multiplied because of the necessary high technical expertise involved [114]. Representative set up of ILP is presented in Figure 7 [115].

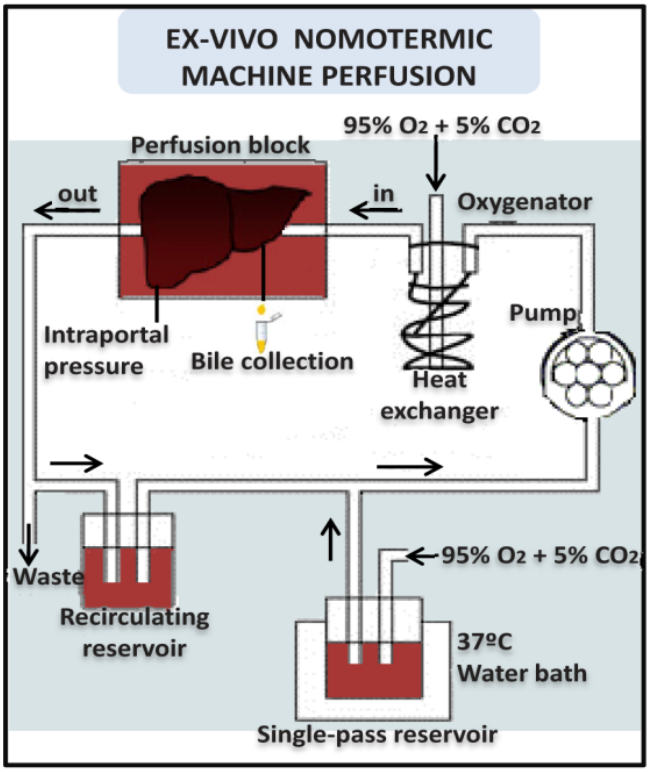


Figure 5. *Ex vivo* set up of isolated perfused liver

Nowadays, these techniques are applied not only to model liver toxicity or to study kinetics of xenobiotics, but they are used to preserve liver for organ transplantation from donors, where simple cold storage is not enough, for example, the liver from non-heart-beating donors or those containing fat due to alcohol or obesity. Using machine perfusion preservation may enhance the donor pool by reclaiming liver that would be otherwise unsuitable. The reason for this is a simple organ shortage [116].

2.3 Hepatotoxicity prediction using mammalian *in vivo* models

For classification of drug-induced liver injury (DILI), several systems exist. The general division can be into a direct injury by the compound and/or its metabolites, or over-sensitization of liver cells to damage, induced by cytokines. While the former is mostly dose-dependent and predictable, the latter one is unpredictable and idiosyncratic [117]. An example of drug with predictable and dose-dependent DILI is acetaminophen. Overdose of acetaminophen is related with hepatic injury that occurs due to the activity of the toxic *N*-acetyl-*p*-benzoquinone imine metabolite, produced by the CYP2E1 pathway of acetaminophen metabolism. Exposure of reactive oxygen species to organelles can lead to apoptosis and necrosis of hepatocytes [118]. In contrast, idiosyncratic DILI is dependent on many host, environmental, and compound-specific risk factors. Other variables, such as age, sex, obesity, alcohol consumption, diabetes mellitus, and chronic liver disease can increase the risk of idiosyncratic DILI [119].

Animal studies are essential for studying liver toxicity, because modulation of immune system and its influence on liver injury development is something that can be hardly reproduced *in vitro*. Despite the generation of new predictive cell culture model systems, experiments in animal models are an unavoidable part of the pre-clinical drug development, based on the assumption that basic processes are comparable among the tested species and humans [120, 121]. Nevertheless, evidence suggests that preclinical animal studies can predict only up to 70% of human toxicity [122]. The traditional animal studies fail to identify all possible adverse effects and many compounds pass safely through animal testing. Hepatotoxicity discovered during clinical trials or during post-marketing phase can lead to liver injuries for certain populations of patients and/or drug withdrawal from market [123]. This discordance between man and animal test species has been attributed to differences in the metabolism and metabolic clearance pathways [124].

Animal models for studying DILI are not limited to rodents only (rats, mice, rabbits and guinea pigs), but include also larger non-rodent animals, such as pigs, sheep or monkeys [125]. No evidence was provided, whether there is a stronger correlation of DILI between humans and rodent or non-rodent animal models [125, 126]. Still, the decision about the most valid animal model for toxicity testing may be highly dependent

on the group of compounds studied [126]. All of these findings suggest that it is necessary to examine DILI in both rodent and non-rodent animals [118].

2.4 Humanized mouse model

To make animal models for liver injury testing more similar to humans, immunodeficient mice have been engrafted with primary human hepatocytes or iHPSC, to produce a humanized mouse model [127]. These specific mice can also be used to study drug metabolism and liver regeneration, because they express human-specific enzymes and similarly generate human-specific metabolites of tested substrates [128]. What is even more remarkable, human specific hepatitis viruses can infect these mice [129, 130]. In some models, human liver cells are transplanted into immunodeficient mice that express a urokinase-type plasminogen activator (uPA) transgene in their liver. Although this facilitates the growth of transplanted human liver cells, it also causes continuing and progressive damage to liver parenchymal cells. These uPA-dependent models have very significant disadvantages that limit their utility for many applications, including a very poor breeding efficiency, renal disease, and a very narrow time window for transplantation. Other types of knockouts exist, yet each of them has their own pros and cons [131].

2.5 Zebrafish as model organisms for studying DILI

The zebrafish (*Danio rerio*) is a promising animal model for assessing drug-induced toxicity in a variety of organ systems [132]. Although zebrafish liver can also be used to study drug-induced toxicity, it is a less frequently used model in comparison to other zebrafish organs [133]. Despite not being mammals, lower-order vertebrates like zebrafish have similar molecular and cellular processes that can accurately model human physiology [134]. Optical transparency of zebrafish embryo, that grows outside of uterus, has allowed us to study embryonic lethal phenotypes. This is something that is hardly possible with mammalian models [135].

The tri-lobed liver of zebrafish is similar to the liver of mammals with regard to biological functions, which include the processing of lipids, vitamins, proteins and

carbohydrates and the synthesis of serum proteins [136]. However, bile ducts, portal veins and hepatic arteries are not organized in portal tracts, but randomly organized throughout the liver parenchyma of zebrafish. Similarly, zebrafish hepatocytes are not organized in plates, but in tubules. The lack of lobular arrangement impairs morphological differentiation, because the vessels are histologically identical [137].

When choosing for a proper toxicity model, characterization of metabolic properties of selected species is very important, because it can determine whether the tested compound will be toxic or not [138]. The zebrafish embryonic liver is completely developed and functional by 72 hours post fertilization, as judged by organ appearance and functional markers, such as phase I and phase II biotransformation enzymes [139]. The CYP enzyme families 1–4, which predominantly metabolise exogenous compounds, are more diverse in zebrafish than in humans [140]. Formation of transgenic zebrafish larvae expressing human CYP3A4 has increased the applicability of zebrafish in pharmacokinetic and toxicity testing [141].

Zebrafish hepatotoxicity assays have been mostly observational, because the necrotic cells can be visually identified due to change in appearance. The major shortcoming is the low sensitivity to detect early toxicity [142, 143]. For practical reasons, the toxicity research is moving from observational to mechanism-based toxicity assays. Several potential injury biomarkers for DILI in zebrafish model were suggested after exposure to model hepatotoxicants, either being apoptosis- or metabolism-related [144-146].

2.6 Predictive modelling

Computational modelling approaches have been created to help in evaluation of the efficacy, toxicity, and metabolism of pharmaceutical ingredients [147]. The aim of computational methods is to complement *in vitro* and *in vivo* toxicity tests to potentially minimize the need for animal testing, reduce the cost and time of toxicity tests, and improve toxicity prediction and safety assessment. In recent years, machine-learning algorithms for prediction models have been developed to obtain much better predictions [148, 149]. Predictive models can be divided into two basic categories: models from homogenous data and models from heterogenous data. Homogenous data models can be

further subdivided (based on the type of data) into chemical structure-based *in silico* models, *in vitro* assay-based models and toxicogenomic-based models [118, 150]. There exist various methods to predict the toxicity, both qualitatively and quantitatively. each method has its strengths, limitations, scope of application, and specificity of interpretation. Nevertheless, selection of the most suitable approach is dependent on the studied problem [149].

2.7 microRNAs as biomarkers of DILI

An ideal biomarker of DILI should cover the specific response to DILI and no other type of liver injury. It should be sensitive and detectable at the early stages of disease, without the use of specialised equipment. To facilitate the early diagnosis, it should be easily obtained without the use of invasive procedures, optimally from the body fluids. It should be conserved and translational between humans and preclinical models [151].

MicroRNAs (miRNAs) have been shown as a capable source of biomarkers for a wide range of pathologies, across many organs. These small, 18-25 nucleotides long, non-coding RNA molecules are important epigenetic regulators of post-transcriptional gene expression [152]. They present an attractive non-invasive biomarker, as they can be easily either actively secreted or passively released to body fluids, following organ damage. In blood, miRNAs are incorporated into extracellular vesicles (exosomes or apoptotic bodies) or they are protein-bound (argonaute-2, lipoproteins) [153]. It was described that a single miRNA can potentially regulate single or multiple mRNA and vice versa [154].

Among the liver-enriched miRNAs, the most highlighted miRNAs are probably miR-122 and miR-192 [155]. Especially for miR-122 circulating in blood, its potential as a biomarker of DILI was subsequently highlighted in a cohort study with acetaminophen overdosed patients. It was shown that miR-122 can be used independently or alongside the aminotransferases in the prognosis of DILI [156]. Despite being liver specific, miR-122 expression changes are not unique only to DILI, but its serum expression can be elevated even after other liver injuries. This specific miRNA also plays a role in hepatocellular carcinoma, hepatocyte phenotype or hepatitis C infection [157, 158].

Despite many candidates described in the literature, miRNAs are not perfect biomarkers. In clinical practice, the use of whole blood for miRNA analysis would be optimal. However, blood haemolysis leads to the release of blood-cell rich miRNAs, contaminating the sample [159]. Frequently used technique for miRNA quantification is reverse transcription-quantitative polymerase chain reaction (RT-qPCR). This technique requires normalisation to avoid bias, for example because of the variation in RNA isolation, conditions during sample collection or inter-individual differences. Inter-individual variation is another factor that needs a lot of consideration and many studies to assess baseline expression levels across different population groups for individual miRNAs [160].

3 Objectives

This dissertation thesis is composed as a commented collection of scientific articles introducing to the reader the observed effects of selected mono- and sesquiterpenes on model liver *in vitro* systems, including viability tests, gene and protein expression changes and bioinformatic prediction of novel targets for studied compounds.

Specific aims of experimental part of the dissertation thesis were:

- Literary review of known information about hepatotoxicity of mono- and sesquiterpenes
- Optimisation of precision-cut liver slices preparation, culture and viability testing
- Studying the toxicity of monoterpenes (*R*)-pulegone and (*R*)-menthofuran
- Selection and validation of reference genes for gene expression studies in human liver slices using RT-qPCR
- Studying the agonism of six selected sesquiterpenes (*cis*-nerolidol, *trans*-nerolidol, farnesol, α-humulene, β-caryophyllene, caryophyllene oxide) towards pregnane X receptor and their effect on gene and protein expression of various cytochrome P450 isoforms
- Studying the toxicity of sesquiterpenes alantolactone and germacrone towards differentiated HepaRG cells and bioinformatic prediction of novel targets of their biological activity

4 Works included in Dissertation thesis

I. **Zárybnický** T, Boušová I, Ambrož M, Skálová L. Hepatotoxicity of monoterpenes and sesquiterpenes. Arch Toxicol. 2018, 92(1):1-13. Review. (IF 2018: **5.741**)

II. **Zárybnický** T, Matoušková P, Lancošová B, Šubrt Z, Skálová L, Boušová I. Inter-Individual Variability in Acute Toxicity of R-Pulegone and R-Menthofuran in Human Liver Slices and Their Influence on miRNA Expression Changes in Comparison to Acetaminophen. Int J Mol Sci. 2018, 19(6):1805. (IF 2018: **4.183**)

III. **Zárybnický** T, Matoušková P, Ambrož M, Šubrt Z, Skálová L, Boušová I. The Selection and Validation of Reference Genes for mRNA and microRNA Expression Studies in Human Liver Slices Using RT-qPCR. Genes. 2019, 10(10):763. (IF 2018/2019: **3.331**)

IV. Šadibolová M, **Zárybnický** T, Smutný T, Pávek P, Šubrt Z, Matoušková P, Skálová L, Boušová I. Sesquiterpenes Are Agonists of the Pregnane X Receptor but Do Not Induce the Expression of Phase I Drug-Metabolizing Enzymes in the Human Liver. Int J Mol Sci. 2019, 20(18):4562. (IF 2018/2019: **4.183**)

V. **Zárybnický T**, Matoušková P, Skálová L, Boušová I. The hepatotoxicity of alantolactone and germacrone: their influence on cholesterol and lipid metabolism in differentiated HepaRG cells. (Submitted manuscript)

5 Authors contribution

This doctoral dissertation is based on the papers referred as number 1-5. The candidate is a first author of four publications, no. 1, 2, 3 and 5.

- In publication I, the candidate:
  - summarized publications covering the topic of the review
  - wrote a draft and participated in revising the manuscript

- In publication II, the candidate:
  - participated in the design of experiments
  - prepared precision-cut human liver slices
  - performed toxicity testing and measured the gene expression changes of selected miRNAs using RT-qPCR
  - wrote a draft and participated in revising the manuscript

- In publication III, the candidate:
  - participated in the design of experiments
  - prepared precision-cut human liver slices
  - evaluated the viability of precision-cut human liver slices
  - measured the gene expression of tested reference genes using RT-qPCR and performed stability testing
  - wrote a draft and participated in revising the manuscript

- In publication IV, the candidate:
  - participated in the design of experiments
  - prepared precision-cut human liver slices
  - measured gene expression changes of studied biotransformation enzymes using RT-qPCR
  - wrote parts of a manuscript concerning his experiments

- In publication V, the candidate:
  - participated in the design of experiments
  - performed culture of dHepaRG cells and toxicity testing

- performed bioinformatic prediction of novel targets
- measured expression changes of studied genes using RT-qPCR
- measured changes in expression of studied proteins using western blot
- wrote a draft and participated in revising the manuscript

6 The publications not related to this doctoral dissertation

1. Hanousková B, Skála M, Brynychová V, **Zárybnický** T, Skarková V, Kazimírová P, Vernerová A, Souček P, Skálová L, Pudil R, Matoušková P. Imatinib-induced changes in the expression profile of microRNA in the plasma and heart of mice-A comparison with doxorubicin. Biomed Pharmacother. 2019, 115:108883. (IF 2018/2019: **3.743**)

2. Kagawa T, **Zárybnický** T, Omi T, Shirai Y, Toyokuni S, Oda S, Yokoi T. A scrutiny of circulating microRNA biomarkers for drug-induced tubular and glomerular injury in rats. Toxicology. 2019, 415:26-36. (IF 2018/2019: **3.547**)

3. Raisová Stuchlíková L, Králová V, Lněničková K, **Zárybnický** T, Matoušková P, Hanušová V, Ambrož M, Šubrt Z, Skálová L. The metabolism of flubendazole in human liver and cancer cell lines. Drug Test Anal. 2018, 10(7):1139-1146. (IF 2018: **2.799**)

7 Articles published in journals with impact factor associated with a topic of doctoral dissertation accompanied by candidate´s commentary

7.1 Publication I

Zárybnický T, Boušová I, Ambrož M, Skálová L. Hepatotoxicity of monoterpenes and sesquiterpenes. Arch Toxicol. 2018, 92(1):1-13. Review. (IF 2018: **5.741**)

Herbs and herbal products are extensively consumed world-wide and they are generally considered as safe. However, this may not always be true. In the presented review, we extensively summarized the known literature about hepatotoxic properties of several monoterpenes and sesquiterpenes that showed liver toxicity. This toxicity was mainly based on reactive metabolites formation and increased oxidative stress. There is a high probability that many other terpenes, without sufficiently known metabolism and effects in liver, could also exert hepatoxicity. Intensive research in terpenes metabolism and toxicity represent the only way to reduce the risk of liver injury induced by essential oils and other terpenes-containing products. Based on this review, several promising candidates were selected, that were studied further.

7.2 Publication II

Zárybnický T, Matoušková P, Lancošová B, Šubrt Z, Skálová L, Boušová I. Inter-Individual Variability in Acute Toxicity of R-Pulegone and R-Menthofuran in Human Liver Slices and Their Influence on miRNA Expression Changes in Comparison to Acetaminophen. Int J Mol Sci. 2018, 19(6):1805. (IF 2018: **4.183**)

The toxicity of monoterpenes (*R*)-pulegone (PUL) and (*R*)-menthofuran (MF), abundant in the *Lamiaceae* family, was verified in precision-cut human liver slices (PCLS) in comparison to acetaminophen (APAP) as a model hepatotoxicant. PUL has shown reproducible toxicity in all our experiments, with half-maximal effective concentration (EC_{50}) of PUL approximately 294 μM, using liver slices from five human donors. On the other hand, MF that was presumed to be a metabolite of PUL and one of the main reasons for PUL toxicity, has shown toxicity only in two out of five experiments and also in higher concentrations than PUL ($EC_{50} \geq 418$ μM).

Another aim of the study was to evaluate the expression changes of selected miRNAs in human PCLS after PUL, MF and APAP treatment. Because APAP and PUL share a similar phenotype of liver injury (centrilobular necrosis) and similarities in their structure, we expected similar profiles of miRNA expression changes. However, except marked decrease of miR-155-5p across tested samples, the microRNA expression changes in liver slices were considerably inter-individual.

Our experiments have shown that PUL toxicity and the role of MF may differ between species and deserve more attention. Also, marked inter-individual variabilities in all our results demonstrate the high probability of significant differences in the hepatotoxicity of tested compounds among people.

7.3 Publication III

Zárybnický T, Matoušková P, Ambrož M, Šubrt Z, Skálová L, Boušová I. The Selection and Validation of Reference Genes for mRNA and microRNA Expression Studies in Human Liver Slices Using RT-qPCR. Genes. 2019, 10(10):763. (IF 2018/2019: **3.331**)

The selection of a suitable combination of reference genes (RGs) for data normalisation is a crucial step for obtaining reliable and reproducible results from transcriptional response analysis using a reverse transcription-quantitative polymerase chain reaction (RT-qPCR). While it is known that changes happen inside PCLS during the culture, such as decrease of activity and expression of CYPs or activation of pro-fibrotic pathways, no-one has ever performed validation of RGs used for normalisation of RT-qPCR data. Afterall, RT-qPCR results are remarkably dependent on data normalisation, most frequently by RGs, which can influence the significance of observed change or even the trend of expression change.

The stability of RGs was verified in thirty-five human liver tissue samples and twelve PCLS. In PCLS, the RGs stability was compared between non-treated slices (controls) only and slices treated by β-naphthoflavone (10 μM) or rifampicin (10 μM) as CYP inducers. Regarding mRNA, the best combination of RGs for the PCLS controls was *YWHAZ* and *B2M*, while *YWHAZ* and *ACTB* were selected for the liver samples and treated PCLS.

Our study has shown that using a single RG for normalisation of RT-qPCR data in liver slices is not suitable. Similarly, we have shown that the combination of suitable RGs can change based on the studied sample and experimental design. Especially when working with human liver samples originating from biologically diverse human individuals. Before every data evaluation, the stability of RGs should be always checked.

7.4 Publication IV

Šadibolová M, **Zárybnický** T, Smutný T, Pávek P, Šubrt Z, Matoušková P, Skálová L, Boušová I. Sesquiterpenes Are Agonists of the Pregnane X Receptor but Do Not Induce the Expression of Phase I Drug-Metabolizing Enzymes in the Human Liver. Int J Mol Sci. 2019, 20(18):4562. (IF 2018/2019: **4.183**)

Sesquiterpenes are the main components of plant essential oils and bioactive compounds with numerous health-beneficial activities. On the other hand, previous studies have shown their interactions with CYP by inhibiting the activities of several CYP isoforms, especially CYP3A4.

The effect of six sesquiterpenes (farnesol, *cis*-nerolidol, *trans*-nerolidol, α-humulene, β-caryophyllene, and caryophyllene oxide) on pregnane X receptor and their modulatory effects on the expression of four phase I drug metabolism enzymes (CYP 3A4 and 2C, carbonyl reductase 1, and aldo-keto reductase 1C), at both the mRNA and protein levels, were evaluated.

In PCLS, the effects of studied sesquiterpenes on the expression of all the tested drug-metabolising enzymes at the mRNA and protein levels were mild or none, despite the fact that all of the tested sesquiterpenes significantly induced CYP3A4 expression via pregnane X receptor interaction in transfected HepG2 cells, used for gene reporter assay. Therefore, there is a low probability that these compounds could significantly interact with drug biotransformation.

7.5 Publication V

Zárybnický T, Matoušková P, Skálová L, Boušová I. The hepatotoxicity of alantolactone and germacrone: their influence on cholesterol and lipid metabolism in differentiated HepaRG cells. (Submitted manuscript)

Sesquiterpenes alantolactone (ALA) and germacrone (GER) are naturally occurring molecules that are being studied as potential anticancer agents. Using the differentiated HepaRG (dHepaRG) cells, a human hepatocyte-like model, the toxicity of ALA and GER was compared with results published on highly proliferative cancer cell lines after ALA and GER treatment. The results showed lesser toxicity for ALA to dHepaRG cells, but no difference for GER. Taking the reactive oxygen species formation into consideration, even low effective concentrations of ALA for antiproliferative effect would have a chance to induce liver injury because of oxidative stress.

A bioinformatic tool BATMAN-TCM was searched for new molecular targets of tested sesquiterpenes. Analysis of their common targets lead us to studying their effects on cholesterol metabolism and 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR), a major regulatory enzyme in mevalonate pathway. Although HMGCR protein, *Hmgcr* mRNA and sterol regulatory element-binding protein 2 were affected by ALA and GER treatment, multidose experiments did not show any change in total cholesterol level. The gene expression of aromatase (*Cyp19a1*), another target predicted by BATMAN-TCM, was measured by RT-qPCR. Although both compounds influenced *Cyp19a1* expression, the profile differed between ALA and GER treatment.

Dysregulation in lipid metabolism was detected by measuring the expression of major genes responsible for lipid sequestration (*Plin2*, *Plin4*), *de novo* lipogenesis (*Fasn*, *Scd*) and triglyceride synthesis (*Acacb*, *Gpam*) by RT-qPCR, with the phenotype profile suggesting peroxisome proliferator-activated receptor α agonism.

8 Conclusions

The presented doctoral thesis was interested in natural compounds from the group of monoterpenes and sesquiterpenes, their liver toxicity and other interactions with liver cells *in vitro*. Based on the selected objectives, presented results are summarized to the following points:

- The known literature about hepatotoxic properties of several monoterpenes and sesquiterpenes, that showed liver toxicity, was extensively summarized. This toxicity was mainly based on the reactive metabolites formation and increased oxidative stress. There is a high probability that many other terpenes, without sufficiently known metabolism and effects in liver, could also exert hepatoxicity.
- The model of precision-cut human liver slices was successfully optimized. The well-known hepatotoxicant PUL has shown reproducible toxicity in all our experiments, using liver slices from five human donors. On the other hand, MF that was presumed to be a metabolite of PUL and one of the main reasons for PUL toxicity, has shown toxic effects only in two out of five experiments and in higher concentrations than PUL. Testing the miRNA expression changes, except marked decrease of miR-155-5p across tested samples, the microRNA expression changes in liver slices were considerably inter-individual.
- Stability of RGs was compared between human liver samples, non-treated human PCLS (controls) only or PCLS treated by β-naphthoflavone (10 μM) or rifampicin (10 μM) as CYP inducers. The best combination of RGs for the PCLS controls was *YWHAZ* and *B2M*, while *YWHAZ* and *ACTB* were selected for the liver samples and treated PCLS. Using a single RG for normalisation of RT-qPCR data in PCLS is not suitable. Similarly, we have shown that the combination of suitable RGs can vary, based on the studied samples and experimental set up.
- The interaction of six sesquiterpenes (FAR, cNER, tNER, HUM, CAR, and CAO) with pregnane X receptor was tested in transfected HepG2 cells, used for gene reporter assay. All of the tested sesquiterpenes significantly induced CYP3A4 expression via pregnane X receptor interaction. The expression of four phase I drug metabolising enzymes (CYP 3A4 and 2C, carbonyl reductase 1, and aldo-keto reductase 1C), at both the mRNA and protein levels, were further evaluated. However, the effects of studied sesquiterpenes on

the expression of all the tested drug-metabolising enzymes in human PCLS, at the mRNA and protein levels, were mild or none.

- The toxicity of potential anticancer agents, sesquiterpenes ALA and GER, was evaluated in differentiated HepaRG cells. While ALA has shown lesser toxicity towards highly proliferative cancer cell lines, there was no difference for GER. Also, even low effective concentrations of ALA for antiproliferative effect would have a chance to induce liver injury because of oxidative stress. New molecular targets, HMGCR and CYP19A1, were predicted using a bioinformatic tool BATMAN-TCM. HMGCR expression was affected by ALA and GER treatment, but the effect was the most significant at toxic concentrations. Although both compounds influenced *Cyp19a1* mRNA expression, the profile differed between ALA and GER treatment, suggesting different mechanism of action.

9 Oral presentations

Zárybnický T, Lancošová B, Šubrt Z, Boušová I and Skálová L. Acute toxicity of (*R*)-pulegone and (*R*)-menthofuran in human liver slices. 8th Postgraduate and 6th Postdoc Conference, Faculty of Pharmacy, Charles University, 24.-25.1. 2018, Czech Republic. Book of abstracts, p. 66

Zárybnický T, Ambrož M, Šubrt Z, Matoušková P, Skálová L and Boušová I. Selection and validation of reference genes for mRNA and miRNA gene expression studies using RT-qPCR. 9th Postgraduate and 7th Postdoc Conference, Faculty of Pharmacy, Charles University, 23.-24.1. 2019, Czech Republic. Book of abstracts, p. 66

Zárybnický T, Matoušková and Boušová I. The toxicity of alantolactone and germacrone towards differentiated heparg cells and their influence on cholesterol metabolism. 10th Postgraduate and 8th Postdoc Conference, Faculty of Pharmacy, Charles University, 22.-23.1. 2019, Czech Republic. Book of abstracts, p. 24

10 Conference posters

Zárybnický T, Stuchlíková Raisová L, Králová V, Lněničková K, Matoušková P, Hanušová V, Ambrož M, Šubrt Z and Skálová L. Metabolism of flubendazole in human liver and cancer cell lines. XXIX. Xenobiochemické symposium, Czech Republic, Telč, 24.-26.5. 2017. Book of abstracts, p. 9

Zárybnický T, Lancošová B, Šubrt Z, Matoušková P and Skálová L. Acute toxicity of (*R*)-pulegone and (*R*)-menthofuran in human liver slices and their influence on miRNA expression *ex vivo*. XVIII. Interdisciplinary meeting of young life scientists, Czech Republic, Milovy, 14.-17.5. 2018. Book of abstracts, p. 42

Zárybnický T, Ambrož M, Šubrt Z, Matoušková P, Skálová L and Boušová I. Selection of suitable reference genes for gene expression studies in human liver slices. 23rd Interdisciplinary Toxicological Conference TOXCON 2018, Slovakia, Stará Lesná, 20.-22.6. 2018. Book of abstracts, p. 109

Zárybnický T, Skalická V, Kamasová T, Ambrož M, Matoušková P, Skálová L and Boušová I. The toxicity of germacrone and its influence on cholesterol metabolism in liver cells. XXX. Xenobiochemické symposium, Slovakia, Pezinok, 15.-17.5. 2019. Book of abstracts, p. 46

Zárybnický T, Skalická V, Kamasová T, Ambrož M, Matoušková P, Skálová L, Boušová I. The influence of alantolactone on cholesterol metabolism in liver cells. IUTOX 15th International Congress of Toxicology, USA, Hawaii, 15.-18.7. 2019. Book of abstracts, p. 256

11 Abbreviations

ALA	Alantolactone
BSEP	Bile salt export pump
CAR	β-Caryophyllene
CAO	Caryophyllene-oxide
cNER	*cis*-Nerolidol
CYP	Cytochrome P450
DILI	Drug-induced liver injury
DMSO	Dimethyl sulfoxide
EC_{50}	Half-maximal effective concentration
FAR	Farnesol
GER	Germacrone
HMGCR	3-Hydroxy-3-Methylglutaryl-CoA reductase
HUM	α-Humulene
IC_{50}	Half-maximal inhibitory concentration
IPL	Isolated perfused liver
MF	(*R*)-(+)-Menthofuran
miRNA	microRNA
NTCP	Na^+-taurocholate cotransporting polypeptide
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
OCT-1	Organic cation transporte-1
OATP-C	Organic anion transporting polypeptide-C
PHH	Primary human hepatocytes
PCLS	Precision-cut liver slices
PUL	(*R*)-(+)-pulegone
RT-qPCR	Reverse transcription-quantitative polymerase chain reaction
STAT3	Signal transducer and activator of transcription 3
tNER	*trans*-Nerolidol
uPA	urokinase-type plasminogen activator

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13 Supplements

13.1 Copies of published articles related to the topic of this doctoral thesis (I-V)

I. **Zárybnický** T, Boušová I, Ambrož M, Skálová L. Hepatotoxicity of monoterpenes and sesquiterpenes. Arch Toxicol. 2018, 92(1):1-13. Review. (IF 2018: **5.741**)

II. **Zárybnický T**, Matoušková P, Lancošová B, Šubrt Z, Skálová L, Boušová I. Inter-Individual Variability in Acute Toxicity of R-Pulegone and R-Menthofuran in Human Liver Slices and Their Influence on miRNA Expression Changes in Comparison to Acetaminophen. Int J Mol Sci. 2018, 19(6):1805. (IF 2018: **4.183**)

III. **Zárybnický T**, Matoušková P, Ambrož M, Šubrt Z, Skálová L, Boušová I. The Selection and Validation of Reference Genes for mRNA and microRNA Expression Studies in Human Liver Slices Using RT-qPCR. Genes. 2019, 10(10):763. (IF 2018/2019: **3.331**)

IV. Šadibolová M, **Zárybnický T**, Smutný T, Pávek P, Šubrt Z, Matoušková P, Skálová L, Boušová I. Sesquiterpenes Are Agonists of the Pregnane X Receptor but Do Not Induce the Expression of Phase I Drug-Metabolizing Enzymes in the Human Liver. Int J Mol Sci. 2019, 20(18):4562. (IF 2018/2019: **4.183**)

V. **Zárybnický T**, Matoušková P, Skálová L, Boušová I. The hepatotoxicity of alantolactone and germacrone: their influence on cholesterol and lipid metabolism in differentiated HepaRG cells. (Submitted manuscript)