Metabolom- und Transkriptom-Analysen zur Charakterisierung von pflanzlichen Substanzen und daraus hergestellten Zubereitungen

Metabolomic and Transcriptomic Analyses in the Characterization of Herbal Substances and their Preparations

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Orland A., Knapp K., Krämer E., Pfeiffer M., Stolte F., Kehraus S., Frötschl R., König G. M., Knöß W.: **New approaches in the characterization of medicinal plants (poster),** 59th International Congress and Annual Meeting of the Society for Medicinal Plant and Natural Product Research, Turkey (Antalya), Sep 4-9, 2011

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Abbreviations:

δ	NMR chemical shift [ppm]
1D	one dimensional
18S rRNA	18S ribosomal RNA
AFLP	amplified fragment length polymorphism
BLAST	basic local alignment search tool
C	cytosine
°C	degree Celsius
C 18	C-18 modified silica gel
$CDCL_3$	deuterated chloroform
DMSO-*d6*	deuterated dimethyl sulfoxide
DAD	diode array detector
DCM	dichloromethane
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide
DMSO	dimethyl sulphoxide
EDQM	European Directorate for the Quality of Medicines & HealthCare
ELSD	evaporative light scattering detector
EtOH	ethanol
ETS	external transcribed spacer
FCS	fetal bovine serum
GC	gas chromatography
^1H	proton
h	hour
HLD-C	high-density lipoprotein C
HMPC	Committee on Herbal Medicinal Products
HPLC	high performance liquid chromatography
IC_{50}	test concentration giving 50% survival

IPA	ingenuity pathway analysis
ITS	internal transcribed spacer
lincRNA	long intergenic non-coding RNA
MeOH	methanol
mRNA	messenger ribonucleic acid
NMR	nuclear magnetic resonance
PBS	phosphate buffered saline
PC	principal component
PCA	principal component analysis
PCR	polymerase chain reaction
RIN	RNA integrity number
qRT-PCR	quantitative reverse transcriptase polymerase chain reaction
RFLP	restriction fragment length polymorphism
s	second
TCM	traditional Chinese medicine
TLC	thin-layer chromatography
tRNA	transfer ribonucleic acid
WHO	World Health Organization

1 Introduction

1.1 Traditional medicine and medicinal plants

Plant-based systems play an essential role in health care. Medicinal plants have a long tradition in various cultures all around the world. Although modern medicine is well developed and accepted in most parts of the world, there is a high demand for herbal medicine and remedies.

Folk medicine or traditional medicine used to prevent or cure diseases has a long history and is based on acquired knowledge developed over generations. The World Health Organization (WHO) defined traditional medicines as "*the sum total of the knowledge, skills, and practices based on the theories, beliefs, and experiences indigenous to different cultures, whether explicable or not, used in the maintenance of health as well as in the prevention, diagnosis, improvement or treatment of physical and mental illness.*" (WHO, 2010). In traditional medicine, herbal substances and preparations thereof are frequently applied. The European Medicines Agency established the Committee on Herbal Medicinal Products (HMPC). Its task is to assist in the harmonization of procedures and provisions concerning herbal medicinal products within the European Union and further integrating herbal medicinal products in the European regulatory framework.

According to the European directive 2001/83/EC as amended (including directive 2004/24/EC), herbal substances include mainly whole, fragmented or cut plants, plant parts, fungi and lichen in an unprocessed, usually dried, form. Subsequently, herbal preparations are obtained by subjecting herbal substances to treatments such as extraction, distillation, expression, fractionation, purification, concentration or fermentation.

The European Directorate for the Quality of Medicines & HealthCare (EDQM), responsible for the elaboration of the European Pharmacopeia, defined the term herbal drug and herbal drug preparation synonymous to herbal substance and herbal preparation.

In Germany, about 2,000 herbal medicinal products are registered or approved at the Federal Institute for Drugs and Medical Devices (BfArM). Plant parts or plant material in the crude or processed state are regarded as active substance. Herbal substances or their preparations are administered as tea, extracts, tablets, capsules, solutions, juice, suppository, salves or cream.

1.2 Quality control and characterization of herbal substances and preparations thereof

The composition of phytochemical constituents within an herbal substance depends on many factors like the plant parts used, genetic variation, collection period, makeup of soil, climatic conditions, drying and storing methods and geographical location (Bandaranayake W., 2006; Bauer R., 1998).

For safe use the quality of herbal substances is a significant characteristic as it determines the biological activities. The quality of herbal medicinal products is assured by a defined manufacturing process and additionally subjected to quality control. Medicinal plants comprise a mixture of various phytochemical constituents; thus, the appropriate quality control is challenging as a plant may comprise up to 200,000 metabolites (Fiehn O., 2001).

The quality criteria for herbal substances are based on a clear definition of the raw material documented in pharmacopeial monographs. When assessing the quality, sensory features, moisture, ash, physical constants, solvent residues and adulterations have to be checked to prove identity and purity. Additionally, herbal substances are tested for contaminations, such as heavy metals, pesticide residues, aflatoxins, and radioactivity. The identity is frequently analyzed by fingerprint methods like thin-layer chromatography (TLC) and the constant composition is ensured by the determination of selected marker compounds, e. g. using high performance liquid chromatography (HPLC) and gas chromatography (GC).

1.3 Omics-techniques for the comprehensive characterization of herbal substances and preparations thereof

1.3.1 Omics-techniques and systems biology

The suffix “omics” is used for high-throughput technologies frequently used in various disciplines. It describes the analysis of the totality of the field of science. In recent publications omics-technologies are used in a broader sense but it still implies the investigation of a high number of samples.

The application of these techniques was achieved due to the IT-based data evaluation using systems biology. This discipline has emerged since the third millennium. It is an interdisciplinary field of science and combines knowledge and tools from biology, computer sciences, medicine, physics, chemistry and engineering (Medina M., 2013).

Various classes of biological compounds, from genes through mRNA to proteins and metabolites, can be analyzed by the respective omic approaches, namely, genomics, transcriptomics, proteomics, or metabolomics (fig. 1).

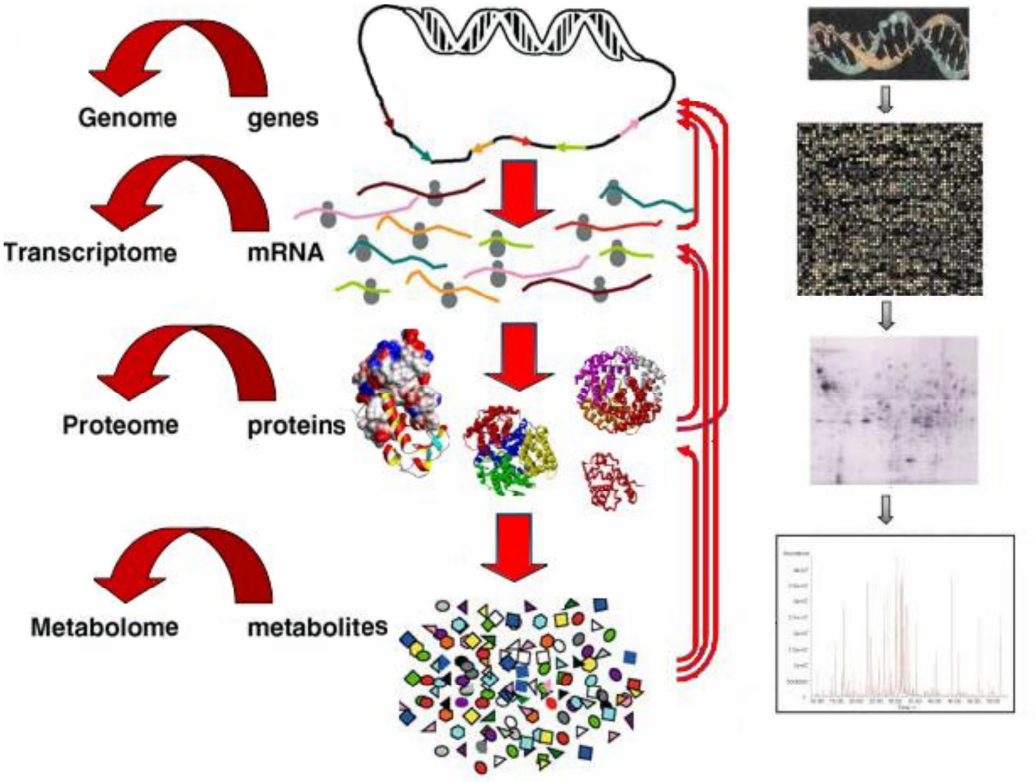


Fig. 1: Scheme of the 'omic' organization (adopted from Buszewski B., 2009). The flow of information is from genes to transcripts to proteins to function.

The term genomics was first coined by Thomas Roderick, meaning the study and comparison of genomes across species (Kuska B., 1998).

The transcriptome comprises the set of all RNAs (mRNA, tRNA, rRNA and other non-coding RNA) present in a single cell or organism. Techniques investigating the transcriptome usually investigate the gene expression level; hence, the information is passed from DNA via messenger RNA (mRNA).

First Marc Wilkins coined the term proteomics, to describe an entire organism's protein complement expressed by a genome (Yadav S., 2007). In contrast to the genome, the transcriptome and the proteome are highly dynamic. The study of the proteome is rather complex. A single gene can be processed to result in several different mRNAs or proteins.

Accordingly, metabolomics is a scientific field investigating small molecules within a biological sample. Variations in metabolite composition may be taken as the downstream result of changes in gene expression and protein translation; therefore, it is expected to be amplified relative to changes in the transcriptome and proteome (Silvestri E., 2011).

1.3.2 Analysis of herbal preparations by metabolomic fingerprinting

The term metabolome is defined as the complete set of small-molecule metabolites to be found within a biological sample, such as a single organism (Oliver S., 1998). The highly dynamic metabolome includes small molecule metabolites essential for the maintenance and growth of a cell or organism and comprises classes of compounds related to hormones, metabolic intermediates, signaling molecules and secondary metabolites (Sumner L., 2003). Metabolomic techniques are very useful for studying plant biochemistry, chemotaxonomy, ecology, pharmacology and quality control of herbal substances (van der Kooy F., 2009).

The most common methods in the investigation of the metabolome are nuclear magnetic resonance spectroscopy (NMR), chromatographic methods and mass spectrometry. Furthermore, hyphenated techniques like LC/MS or LC/NMR are likely to have an increased impact (Ulrich-Merzenich G., 2007; Verpoorte R., 2005).

For the investigation of the metabolome several applications are known (Fiehn O., 2000; Fiehn O., 2002). Metabolic profiling aims at the qualitative and quantitative analysis of a group of related compounds. Methods related to metabolomics aim at the qualitative and quantitative analysis of all metabolites within one organism. Metabolic or metabolomic fingerprinting aims at measuring a fingerprint of metabolites within an organism. This technique is applied without identification of all compounds, but rather used for sample classification by rapid global analysis (Ulrich-Merzenich G., 2007; Verpoorte R., 2005).

In a previous PhD project in our group, a metabolomic fingerprint method was successfully established. Crude herbal preparations were investigated by ^{1}H-NMR analysis in combination with principal component analysis (PCA) to evaluate ^{1}H-NMR data (Daniel C., 2009). PCA is a mathematical procedure. It is an unbiased technique and useful in the reduction of dimensionality of data obtained by ^{1}H-NMR analysis. In addition, PCA is useful in pattern recognition and applied to classify data. The score plot is a summary of the relationship among the samples. The loading plot summarizes the variability and is an additional tool to interpret the patterns displayed in the score plot.

1.3.3 Investigation of gene expression profiling

Genes are defined as the smallest unit of DNA or RNA needed for the final product of gene expression. The origin of gene expression in eukaryotes, prokaryotes and some viruses is DNA, which is transcribed into RNA. The final products of gene expression are proteins.

The regulation of genes is essential for all organisms and involved in processes related to cellular differentiation and morphogenesis. Furthermore, genes are regulated in response to

an environmental stimulus, which leads to an increase or decrease of the production of functional gene products (RNA or protein).

The transcriptome is dynamic and continuously responding to physiological and environmental conditions. According to the database NCBI, the human transcriptome comprises 20,724 protein-coding genes (www.ncbi.nlm.nih.gov/gene; 22. 3. 2014). The number of non-protein-coding genes bridges the total number of about 48,000 entries plus isoforms and transcript variants. Approximately 50% of the genes are non-coding for a protein. But this does not implicate that such RNAs do not contain information nor have any function. Non-coding RNAs seem to be particularly abundant in roles that require highly specific nucleic acid recognition without complex catalysis, such as in directing post-transcriptional regulation of gene expression or in guiding RNA modifications (Eddy S., 2001).

Microarray technology:

There are several techniques available to analyze gene expression, like serial analysis of gene expression (SAGE), northern blot, quantitative reverse transcription PCR (qRT-PCR) and expressed sequence tags (ESTs) (Malone J., 2011).

Moreover, high-throughput systems are available, such as next-generation sequencing or microarray analysis. The term microarray itself, also known as “biochip”, simply describes that a high number of molecules are arranged on an extremely small space, commonly on a glass slide (fig. 2) (Mülhardt C., 2013; Ulrich-Merzenich G., 2007).

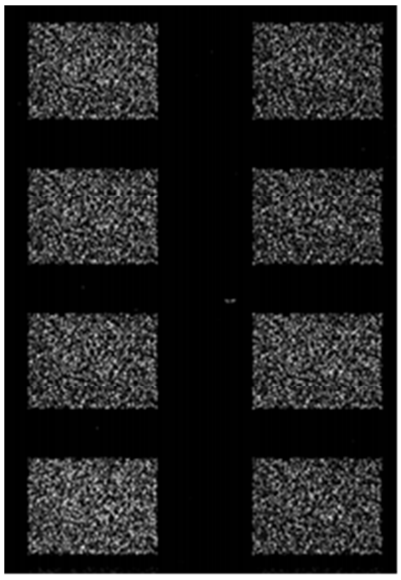


Fig. 2: A hybridized microarray slide using one color labeling. The Agilent 8x60K microarray (Whole Genome Gene Expression Microarrays v2, Agilent Technologies, 8x60K) has eight arrays per slide, each with 50,599 features.

The hybridisation-based DNA microarray technology has been implemented into various fields, due to low costs, ease in use and evaluation and optimized quality control (Brazma A., 2001). In 1987, one of the first manuscripts was published that described microarray experiments including the simultaneous investigation of 96 sequences (Kulesh D., 1987). Nowadays, gene expressions of thousands of genes up to the whole transcriptome are simultaneously analyzed.

In the early stage, there was much concern about the quality of microarray results. Recent investigations pointed out that an appropriate study design, considering cell or tissue type with adequate sample number, and sample storage are important factors for good quality. It was shown that experimental variation has a major impact on the reliability of gene expression results. Regarding quality control, sample preparation, RNA extraction, labeling, hybridization and chip assignment have to be performed according to a standardized procedure (McHale C., 2013). Furthermore, interlaboratory tests, comparisons and knowledge in statistical data evaluation are important to obtain biological relevant data (Bammler T., 2005; Irizarry R., 2005). Quality control practices were designed to overcome biases and experimental variability. The MicroArray Quality Control consortium (MAQC) developed guidelines and provided quality control standards, which are widely accepted by the scientific community (Shi L., 2006; Shi L., 2010).

The application of DNA microarray technology is diverse. Researchers and the pharmaceutical industry use microarray technique in diagnostics, pharmacogenomics, and epigenetic studies (Heller M.J., 2002). Gene expression profiling is used to discover the mechanism of action, toxicity or early effects of cells, risk assessment and the discovery of biomarkers predictive for certain diseases (Cui Y., 2010; Currie R., 2012; McHale C., 2013).

In herbal drug research the applications of DNA microarrays are related to pharmacodynamics, discovery of biomarkers, toxicogenomics as well as the elucidation of the molecular mechanism of action and the discovery of new drugs (Chavan P., 2006). Further, this technique is used for the prediction of safety, assessment of side effects (Lord P., 2006; Ulrich-Merzenich G., 2012) and the correct botanical identification and authentication of crude plant materials as part of standardization and quality control (Chavan P., 2006).

1.4 Identification of herbal substances by DNA markers

Macroscopic and microscopic tests and thin-layer chromatography are frequently employed to ensure identity and purity by phenotypic characteristics of herbal substances.

Furthermore, herbal substances can be identified by genotypic characteristics by the use of molecular markers.

A molecular marker is a particular sequence of DNA showing variability between different species in the nucleic acid sequence. The genotype is phenotypically neutral and mostly unaffected by environmental conditions or prolonged storage of the herbal substance. The use of molecular markers is independent of the state of grinding of the herbal substance (Kersten T., 2013).

However, subsequent treatment and processing of herbal substances have shown limitations for the use of molecular markers. DNA is often degraded during manufacturing of finished herbal medicinal products. Additionally, the quality of DNA is frequently affected during processing of herbal medicine derived from traditional Chinese medicine (Daniel C., 2009).

For reliable identification of herbal substances, the appropriate marker sequences have to be selected. They must be conserved within a species and are highly variable between closely related species. The length of the marker region is an important characteristic, since the amount of information has to be reasonable for proper identification. However, the amplification of shorter fragments is more feasible, particularly from processed herbal substances. Furthermore, for the reliable identification valid reference data are highly important (Erickson D., 2008).

1.4.1 Identification of herbal substances by locus-specific DNA markers

For DNA barcoding of medicinal plants several marker regions are known. In plants, chloroplasts, mitochondria and the nucleus have their own genetic information.

Several marker sequences located in the DNA of the chloroplast (matK, rbcL, psbA, trnH) have successfully been applied for the identification of plants (Gao T., 2011; Liu Y., 2012; Liu Y., 2013; Saarela J., 2013).

Cytochrome oxidase 1 (Cox1) is a marker sequence located in the mitochondria and frequently used for barcoding investigations in animals. For the identification of plants it was shown to be insufficient as the variability of the mitochondrial DNA is too low (Hebert P., 2003; Kerr K., 2007).

The internal transcribed spacer region (ITS) and the closely related external transcribed spacer region (ETS) are located in the region of the 18S-5.8S-26S nuclear ribosomal cistron (fig. 3).

Fig. 3: Graphic scheme of a part of the nuclear ribosomal cistron. The arrows indicate the binding site of the universal plant specific primers (modified from Kersten T., 2013). ETS: external transcribed spacer, ITS: internal transcribed spacer, NTS: non-transcribed spacer.

ITS and ETS refer to segments of non-functional RNA involved in the maturation of RNA. In a previous research project in our group, universal plant specific deoxyoligonucleotides were established to investigate the potential to identify herbal substances (Kersten T., 2013).

1.5 Herbal substances characterized in the present study

Within the study presented, three different herbal substances were characterized by their metabolomic profile and functional activity.

1.5.1 *Chelidonium majus* L.

The dried aerial parts of *Chelidonium majus* L. (Papaveraceae) (fig. 4) are used as herbal substance. From the aerial parts and the roots more than twenty isoquinoline alkaloids like chelidonine, protopine, coptisine, berberine, stylopine and sanguinarine have been isolated (Colombo M., 1996).

Fig. 4: Chelidonii herba.

Chelidonii herba is monographed in the European Pharmacopeia. According to the monograph (8.0, 07/2012:1861), the herbal substance is specified to contain not less than 0.6% of alkaloids, expressed as chelidonine, in relation to the dry weight of the herbal substance.

The German Commission E indicated Chelidonii herba for the use in spastic discomfort of bile ducts and gastrointestinal tract. Several reports describe cases related to liver toxicity (De Smet P., 2002; Stickel F., 2003; Strahl S., 1998; Teschke R., 2012). Thus, since 2008, Chelidonii herba has been affected by a graduated scheme in Germany stating a negative benefit-risk profile (Bundesinstitut für Arzneimittel und Medizinprodukte, 2009). Preparations containing Chelidonii herba with a daily dosage of more than 2.5 mg/day are excluded from the market. The assessment of the HMPC for Chelidonii herba considered a negative

benefit-risk profile for the oral use not excluding product-specific applications (Committee on Herbal Medicinal Products, 2011).

1.5.2 *Actaea racemosa* L.

The dried rhizome of *Actaea racemosa* L. (syn. *Cimicifuga racemosa* (L.) Nutt., Ranunculacea) is used as herbal substance (fig. 5).

Fig. 5: Cimicifugae racemosae rhizoma.

Herbal preparations of Cimicifugae racemosae rhizoma contain a complex mixture of triterpene glycosides, the major constituents are actein, 23-*epi*-26-deoxyactein (syn. 27-deoxyactein), acetylshengmanol-3-*O*-xyloside, cimigenol 3-*O*-arabinoside and cimigenol 3-*O*-xyloside (Bedir E., 2000; Chen S., 2002c; He K., 2006; Shao Y., 2000; Watanabe K., 2002).

Cimicifugae racemosae rhizoma is included in the European Pharmacopeia (8.0, 07/2012:/2069). According to the monograph, the content of triterpenes glycosides is specified to a content of at least 1.0%.

Furthermore, the herbal substance is monographed in the American Herbal Pharmacopeia (USP35-NF30S1) and in the Chinese Pharmacopeia 2010. The monographs of the different pharmacopeias differ in the plant source used. In the Chinese Pharmacopeia, *C. heracleifolia* Kom., *C. dahurica* (Turcz.) Maxim. and *C. foetida* L. are used species, while in the European Pharmacopeia and American Herbal Pharmacopeia *A. racemosa* (L.) is described as the plant source. In the European Pharmacopeia monograph of Cimicifugae racemosae rhizoma, a TLC method was developed to detect contaminations with closely related species, such as *C. americana* Michx., *C. foetida*, *C. dahurica* and *C. heracleifolia*.

In the monograph of the Commission E, published in 1989, Cimicifugae racemosae rhizoma was indicated for premenstrual and menopausal disorders. The HMPC reassessed this herbal substance in 2010 (Committee on Herbal Medicinal Products, 2010b). According to the HMPC monograph on this herbal substance, Cimicifugae racemosae rhizoma is considered to be safe under appropriate labelling (Committee on Herbal Medicinal Products, 2010a). Its use is limited to postmenopausal women with a benefit of the herbal substance used for the relief of menopausal complaints such as hot flushes and profuse sweating (Committee on Herbal Medicinal Products, 2010a).

Questions have been raised about the safety of Cimicifugae racemosae rhizoma. Cases of liver toxicity in patients taking this herbal substance were reported, subsequent evaluation found some products to be adulterated with other related herbal species (Patel N. and Derkits R., 2007; Gardiner P., 2008; Joy D., 2008; Pierrard S. 2009; Vannacci A., 2009; Painter D., 2010). In Germany, a graduated scheme has been effective since 2009, directing

warnings and precautions regarding the risk of hepatotoxicity and the consumption of Cimicifuga containing medicines (Bundesinstitut für Arzneimittel und Medizinprodukte, 2009).

1.5.3 *Silybum marianum* (L.) Gaertn.

The mature fruits of *Silybum marianum* (L.) Gaertn. (Asteraceae) (fig. 6) and preparations thereof have been marketed for several decades.

Fig. 6: Silybi mariani fructus.

The group of constituents described to be responsible for the pharmacological activity is a mixture called silymarin. Silymarin comprises polyphenolic molecules including the flavonolignans silibinin, isosilibinin, silidianin, silichristin and isosilochristin. In Silybi mariani fructus, silymarin is identified to a content of 1.5-3% (Halbach G., 1971).

The European Pharmacopeia contains a monograph of Silybi mariani fructus (8.0, 01/2014:1860). According to the monograph, a content of at least 1.5% of silymarin, expressed as silybin, is specified for the herbal substance.

Silybi mariani fructus is traditionally used. The Commission E described the use in cases of toxic liver damage, liver diseases and supportive treatment of chronic inflammatory liver diseases. In Germany, silymarin is approved for the treatment of *Amanita phalloides* poisoning administered as an antidote in large doses.

2 Scope of the present study

The present thesis was performed in cooperation of the Institute of Pharmaceutical Biology at the University of Bonn and the BfArM. The project focused on the characterization of herbal substances and preparations thereof.

For quality control and characterization of herbal substances and their preparations, currently applied standard methods have shown limitations. Hence, the potential of recently emerging new methodologies was investigated to comprehensively characterize herbal substances and their preparations. In the last decades, progress was achieved in molecular and analytical methods. Therefore, a metabolomics-based approach together with gene expression profiling was used to characterize herbal substances and preparations thereof.

The focus of the project was to bridge data about functional characteristics obtained by gene expression profiling and the metabolomic profile of herbal preparations. Human liver cells exposed to herbal preparations were investigated by microarrays with a focus on a specific gene expression fingerprint and systems biological data evaluation. In a previous research project, it was shown that a metabolomics-based approach is an appropriate method to characterize the chemical composition of herbal preparations. Therefore, it was used for phytochemical profiling of samples in addition to standard methods described in the European Pharmacopeia. The models investigated were chosen from medicinal plants for which either safety concerns associated with liver toxicity were reported (*C. majus* and *A. racemosa*) or for which therapeutic use associated with liver diseases were reported (*S. marianum*).

The proof of herbal identity is mandatory for quality control of herbal substances and their preparations. Hence, in a second approach, a combination of a PCR-based method and the metabolomics-based approach was used to identify and classify herbal substances and herbal preparations. In this subproject, the potential of the combination of both methods was evaluated more in detail using herbal substances from Asian therapeutic systems.

3 Combining metabolomic analysis and microarray gene expression analysis in the characterization of the medicinal plant *Chelidonium majus* L.

3.1 Abstract

Background and objective: Even though herbal medicines have played an important role in disease management and health for many centuries, their present frequent use is challenged by the necessity to determine their complex composition and their multitarget mode of action. In the present study, modern methods were investigated towards their potential in the characterization of herbal substances. As a model the herbal substance Chelidonii herba was used, for which several reports on liver toxicities exist. Extracts of Chelidonii herba with different solvents were chemically analyzed and functionally characterized by experiments with HepG2 liver cells.

Methods: Chelidonii herba was extracted with four solvents of different polarity (dichloromethane, water, ethanol, and ethanol 50% (V/V); four replicates each). The different extracts were metabolomically characterized by ^{1}H-NMR fingerprinting analysis and principal component analysis (PCA). The content of alkaloids was additionally determined by RP-HPLC-DAD. Functional characterization was achieved by the determination of cell proliferation and by transcriptomics-based techniques (Whole Genome Gene Expression Microarrays v2, Agilent Technologies) in HepG2 cells after exposure to the different extracts.

Results: Based on data from ^{1}H-NMR fingerprints and RP-HPLC analyses the different extracts showed a divergent composition of constituents depending on the solvent used. HepG2 liver cells responded differentially to the four extracts. Microarray analysis revealed a significant regulation of genes and signal cascades related to biotransformation. Also liver-toxic signal cascades were activated. Neither the activated genes nor the proliferation response could be clearly related to the differing alkaloid content of the extracts.

Conclusion: Different manufacturing processes lead to different herbal preparations. A systems biology approach combining a metabolomic plant analysis with a functional characterization by gene expression profiling in HepG2 cells is an appropriate strategy to characterize variations in plant extracts. Safety assessments of herbal substances may benefit from such complementary analyses.

Gene abbreviations:

AhRR: aryl-hydrocarbon receptor repressor; AKR1B10: Aldo-keto reductase family 1 member B10; AKR1C4: Aldo-keto reductase family 1 member C4; BAAT: Bile Acid-CoA: Amino Acid N-Acyltransferase; CHST10: Carbohydrate sulfotransferase 10; CHST14: Carbohydrate sulfotransferase 14; Cyp1A1: Cytochrome P450 family 1 member A1; CYP19A1: Cytochrome P450 family 19 member A1; Cyp1B1: Cytochrome P450 family 1 member B1; CYP24A1: Cytochrome P450 family 24 member A1 (1,25-dihydroxyvitamin D_3 24-hydroxylase); CYP26A1: Cytochrome P450 family 26 member A1; CYP27C1: Cytochrome P450 family 27 member C1; CYP2A7: Cytochrome P450 family 2 member A7; CYP2B6: Cytochrome P450 family 2 member B6; CYP2A7: Cytochrome P450 family 2 member A7; CYP2C18: Cytochrome P450 family 2 member C18; CYP3A4: Cytochrome P450 family 3 member A4; CYP3A7: Cytochrome P450 family 3 member A7; CYP4V2: Cytochrome P450 family 4 member V2; CYP8B1: Cytochrome P450 family 8 member B1; EGR2: Early growth response protein 2; GLYATL1: Glycine-N-Acyltransferase-Like 1; GSTO2: glutathione S-transferase omega 2; GSTP1: Glutathione S-transferase P; HMOX1: heme oxygenase (decycling) 1; HS3ST3A1: Heparan sulfate glucosamine 3-O-sulfotransferase 3A1; HTR3A: 5-hydroxytryptamine receptor 3A; IGFBP1: Insulin-like growth factor-binding protein 1; KCNAB3: Voltage-gated potassium channel subunit beta-3; SULT1C3: Sulfotransferase 1C3; SULT1E1: Sulfotransferase 1C3; SULT4A1: Sulfotransferase 1C3; UGT2A3: Uridine 5'-diphospho-glucuronosyltransferase 2A3; UGT2B11: Uridine 5'-diphospho-glucuronosyltransferase 2B11

3.2 Introduction

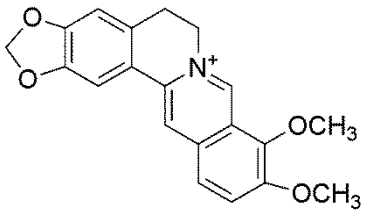
Medicinal plants and products derived thereof have a long tradition of therapeutic use and are widely accepted in the population. The quality of medicinal plants is a basic requirement for the safety of herbal preparations. In terms of quality requirements, the authentication of herbal substances is indispensable. In this context, molecular biological methods were tested successfully for their applicability in the identification of herbal substances (Kersten T., 2008). For safety assessment, several established *in vitro* and *in vivo* "toxicological" methods are available: for example the comet assay determines DNA strand breakage or the T-cell-dependent antibody response assesses the antibody response to immunization. However, in the field of regulatory science there exists the necessity to investigate novel technologies, including functional genomics, proteomics, metabolomics, high-throughput screening and systems biology in order to replace current toxicology assays used for drug approval (Hamburg M., 2011). In future investigations it is important to characterize the toxicant/s with additional complementary methods. Thus, the chemical profile of a plant-derived extract, together with data on its cellular responses, toxicological findings and extrapolated effects of dose-response investigations have to be taken into account for the compilation of an overall picture (Hartung T., 2011; Krewski D., 2010; Wilkening S., 2003).

In contrast to single substances, plant extracts present a challenge, as they are complex mixtures. Often, plant extracts are characterized by marker substances used for the standardization of herbal substances. But multicomponent mixtures can have synergistic effects, which may arise due to the combination of many components of an herbal preparation (Wagner H., 2006) not covered by the determination of marker substances alone.

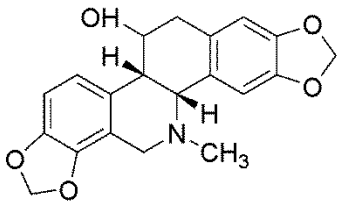
In this study Chelidonii herba was used as a model for our investigations. In literature, there are several reports on liver toxicity associated with application of herbal preparations derived from *Chelidonium majus* L. (Papaveraceae) (De Smet P.A., 2002; Stickel F., 2003; Strahl S., 1998; Teschke R., 2012).

The secondary metabolites most abundant in *C. majus* L. are alkaloids, more than 20 of which are chemically identified. The most important alkaloids (fig. 7) are the benzophenanthridine alkaloids (sanguinarine, chelerythrine, chelidonine) and protoberberines (berberine, coptisine). But also organic acids like chelidonic acid (Shen Z., 2001), citric acid, malic acid, or succinic acid were isolated from *C. majus* L., as well as saponins, choline and histamine, ferulic acid, caffeic acid and *p*-coumaric acid (Hahn R., 1993) are present.

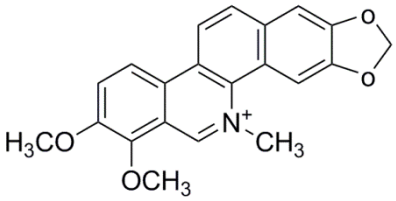
Berberine



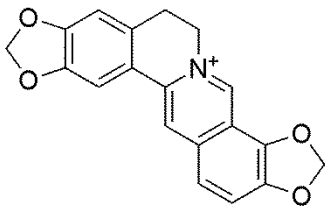
Chelidonine



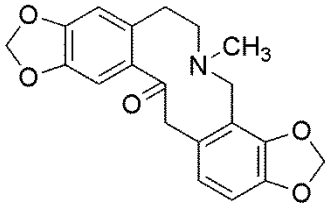
Chelerythrine



Coptisine



Protopine



Sanguinarine

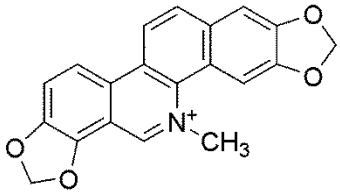


Fig. 7: Chemical structure of typical alkaloids in *C. majus* L..

In the European Pharmacopoeia there is a minimum content of 0.6% alkaloids specified, expressed as chelidonine for Chelidonii herba. For the investigations performed in the current study, four extracts with solvents of different polarities were used. The complex mixture of chemical constituents was characterized by ^{1}H-NMR fingerprint analysis, which is a comprehensive approach in the characterization of the plant metabolome (Daniel C., 2008). The content of the alkaloids chelidonine, protopine, coptisine, berberine, sanguinarine and chelerythrine was quantified by RP-HPLC. The chemical profile was correlated to effects on liver cells. Therefore, the liver proliferation was investigated in response to the different extracts and effects were further characterized by a transcriptomic approach. Alterations in the gene expression were monitored by microarrays, permitting the assessment of complete gene expression profiles induced by different compounds or extracts. By systems biological data evaluation it was possible to place data into a biological context.

3.3 Materials and methods

Extraction of Chelidonii herba:

The herbal substance was complying with the monograph in Ph. Eur. 8.0, 07/2012:1861. It was purchased from a local pharmacy and powdered by a mill. Chelidonii herba was extracted with four different extraction solvents (ethanol, ethanol 50% (V/V), dichloromethane and water). Respectively, 1.0 g of plant material was extracted with 10 ml of the appropriate solvent, frequently stirring at room temperature for 10 min. After filtering, the extraction procedure was repeated. The solvents were eliminated under reduced pressure and the dried extract was resolved in 500 µl deuterated dimethyl sulphoxide. Four replicates were performed for each extract. The sample material Chelidonii herba in analogy to voucher specimens was deposited at the Institute of Pharmaceutical Biology, University of Bonn.

Quantification of alkaloids in Chelidonii herba extracts with high performance liquid chromatography (HPLC):

Liquid/liquid extraction cartridges (Chem Elut CE 1010) were used for sample preparation. For separation of alkaloids a Synergy 4u Polar-RP (80Å, 250 x 4.6 mm column, Phenomenex) was used with a cartridge (SequrityGuard Cartidges, Polar RP 4 x 3.0 mm ID, Phenomenex). Alkaloids were detected by DAD-UV at 285 nm. Two different mobile phases were used: solvent A, 90% H_2O adjusted to a pH of 2.5 with phosphoric acid/10% methanol (V/V) and solvent B, 30% H_2O adjusted to a pH of 2.5 with phosphoric acid/70% methanol (V/V). Alkaloids were eluted using a linear gradient from 30% B to 60% B for the first 6 min, followed by a linear gradient from 60% B to 100% B in 23 min. Subsequently the column was washed for 6 min with 100% B and re-equilibrated to the starting conditions first by a linear gradient from 100% B to 30% B in 3 min and then an isocratic gradient (30% B) for 10 min. The solvent flow rate was adjusted to 1.0 ml/min. Spectral data for all peaks were recorded in the range of 200-600 nm. The alkaloids were identified by the comparison of retention times and UV spectra to the respective reference substances. Protopine was shown to elute first (17.3 min), followed by chelidonine (19.6 min) and coptisine (23.2 min). The peak obtained at 26.1 min was identified as berberine, subsequently sanguinarine (29.9 min) and chelerythrine (32.3 min) eluted.

A validated method of the German Homeopathic Pharmacopeia was applied. Regarding validation based on ICH guideline, linearity, precision of the instrument, precision of the method and limit of detection was determined (International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use, 2005). The

determination of linearity indicated R^2=0.99 for all used reference substances. Precision of the instrument showed a relative standard deviation of 3.43% and 5.65% for coptisine and protopine, respectively. Standard deviation was calculated based on six replicates of the Chelidonii herba extracts, each injected twice (10µl).

^{1}H-NMR spectroscopy and PCA:

^{1}H-NMR spectra of extracts were recorded with a Bruker advance 300 DPX instrument (temperature 25 °C, 64 scans). Calibration of spectra was performed according to signals of incomplete deuterated solvents. Data were processed using TOPSPIN software. Data evaluation by PCA was done with AMIX software (Bruker). Data from δ 0 to δ 10 ppm were included. The region from δ 2.2 to δ 5.5 ppm was excluded to eliminate the effects of signals from water and the solvent peak of DMSO (2.54 ppm) in the extract. The bucket width was 0.05 ppm and data were integrated according to the sum of intensities. Four replicates of each extract were recorded by ^{1}H-NMR.

Preparation of total RNA:

Total RNA was isolated from HepG2 cells using RNeasy Mini Kit (Qiagen, Germany) following the manufacturer's instructions.

Whole genome microarray:

The quality of RNA was examined by the RIN-values (Agilent 2100 Bioanalyzer). The concentration and purity of extracted RNA was measured using an UV-visible spectrometer (NanoDrop 1000; Thermo Fisher Scientific, Waltham, MA) by absorption at wavelengths of 260 and 280 nm. RNA samples with a 260/280 nm absorption ratio > 1.8 and RIN > 9.5 were used in subsequent microarray analysis. For microarray profiles, fluorescence labeled cRNA samples were prepared from 100 ng RNA using reverse transcriptase. The amplification reaction with simultaneous introduction of Cy3-dCTP to the amplified complementary RNA (cRNA) was performed using a Quick Amp Labeling Kit for One-Color (Agilent Technologies). The concentration of the purified samples and the Cy3 dye incorporation efficiency was evaluated using a NanoDrop 1000 spectrophotometer. After fragmentation (60 °C, 30 min), the single colour cRNA samples were hybridized to a DNA chip (Whole Genome Gene Expression Microarrays v2, Agilent Technologies, 8x60K, 27958 Gene RNAs, 7,419 lincRNAs) at 65 °C for 17 hours in a hybridization oven (Agilent Technologies). Four independent experiments were performed under each experimental condition.

qRT-PCR:

After reverse transcription by random priming the resulting cDNA was used for qRT-PCR. Following initial denaturation (95 °C, 10 min), amplification was performed over 45 cycles (Light Cycler 480, Roche) with denaturation at 95 °C for 10 s and annealing with primers at temperatures shown in (tab. 1).

Tab. 1: Primer used for qRT-PCR for validation of microarray results.

Primer/Gene name	NCBI accession code	Primer sequence (5´→3´)	Annealing temperature [°C]
HMOXI_1	NM_002133.2	CCCACGCCTACACCCGCTAC	65
HMOXI_2	NM_002133.2	GGTGGCACTGGCAATGTTGG	65
EGR2_1	NM_000399.3	CCTTTGACCAGATGAACGGAGT	64
EGR2_2	NM_000399.3	GCTGGCACCAGGGTACTGA	64
IGFBP1_1	NM_000596.2	CACAGCAGACAGTGTGAGACAT	52
IGFBP1_2	NM_000596.2	ATTTCATCTGGTTTCAGTTTTGTAC	52
AhRR_1	NM_020731	CAGTTACCTCCGGGTGAAGA	59
AhRR_2	NM_020731	CCAGAGCAAAGCCATTAAGA	59
Cyp1A1_1	NM_000499.3	AACCTTTGAGAAGGGCCACA	55
Cyp1A1_2	NM_000499.3	GACCTGCCAATCACTGTGTC	55
Cyp1B1_1	NM_000104	CACTGCCAACACCTCTGTCTT	53
Cyp1B1_2	NM_000104	CAAGGAGCTCCATGGACTCT	53
HTR3A_1	NM_213621.3	CCCAAGCCACCAAGACTGATA	57
HTR3A_2	NM_213621.3	GCCACATGGACCAGAGCATAAC	57
GAPDH_1	NM_002046.4	CCACCCATGGCAAATTCCATGGCA	57
GAPDH_2	NM_002046.4	CTAGACGGCAGGTCAGGTCCACC	57

Elongation was performed at 72 °C for 20 s. The size of PCR fragments was analyzed by agarose gel electrophoresis. Evaluation of gene expression was done with Light Cycler 480 Software 1.5 and Cp-values were normalized to GAPDH.

Data processing and statistics:

Hybridized and washed microarrays were scanned using an Agilent Scanner (Agilent Technologies) with Feature Extraction Software (Agilent Technologies). Fluorescence intensity data were imported to GeneSpring GX version 12.5 (Agilent Technologies) with the quantile scaling normalization. Before analysis, four data-filtration steps were conducted to exclude low-quality data to guarantee the accuracy of the statistical analysis. In the first step, spots with lower intensities than the threshold, which was determined based on the intensities of the Agilent RNA Spike-Mix, were filtered out to exclude spots weaker than background noise. In the second step, spots with saturated intensities and near-background intensities were filtered out using the 'flag' function of the Feature Extraction Software. In the third step, spots with large variance among the four repeated experiments (coefficient value > 50%) were filtered out. The final filtration step was conducted based on the fold increase. Statistical analysis of the genes remaining after the four filtration steps was performed with a *t*-test corrected using the Benjamini and Hochberg false discovery rate (Benjamini Y., 1995). Genes were considered as up- or down-regulated with a fold ≥ 2 and ≤ -2 and a $p < 0.05$.

These processed data were used for further analysis by Ingenuity Systems Inc. Reedwood City, USA. Ingenuity data base was used as reference set. In the networks interaction 70 molecules were set and all data sources were integrated in data analysis. Data were compared to the human reference data (Tissues and Primary Cells not otherwise specified, Cells not specified and other cells; Organ systems liver and other cells not otherwise specified).

Treatment of HepG2 cells with plant extracts:

HepG2 cells were purchased from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures GmbH and were used between passages 3 and 12. The cells were maintained in T75 flasks in RPMI 1640 medium supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin solution (100 U/ml) and 1% L-glutamine. The cells were constantly incubated in humidified atmosphere at 5% CO_2 and 37 °C.

When the cells were about 80% confluent, they were treated with trypsin and harvested by centrifugation. Cells were counted and an equal number (1.0×10^6 cells/5 ml media) was transferred to each experimental plate (5 cm Ø) for treatment with the different extracts on the following day. Cells were treated with the extracts in a concentration of 1:501. The

vehicle treated cells (1% DMSO) served as a control group. Four replicates were performed for each experimental condition.

Cell growth and proliferation assay using xCELLigence system:

About 7500 HepG2 cells were seeded to each well. After 24 h, medium containing the extracts in a concentration of 1:501 adjusted to 1% DMSO or medium containing 1% DMSO as solvent control was added, respectively. The cell index was monitored every 15 min.

3.4 Results

NMR fingerprint analysis:

The spectra of the ethanolic, ethanolic 50% (V/V), dichloromethane and aqueous Chelidonii herba extracts were recorded by ^{1}H-NMR in order to characterize the groups of constituents of Chelidonii herba. This method is a comprehensive approach, where the complexity of extracts can be shown, in contrast to methods, where just marker substances are analyzed. The data of the ^{1}H-NMR spectra were statistically evaluated by PCA (fig. 8).

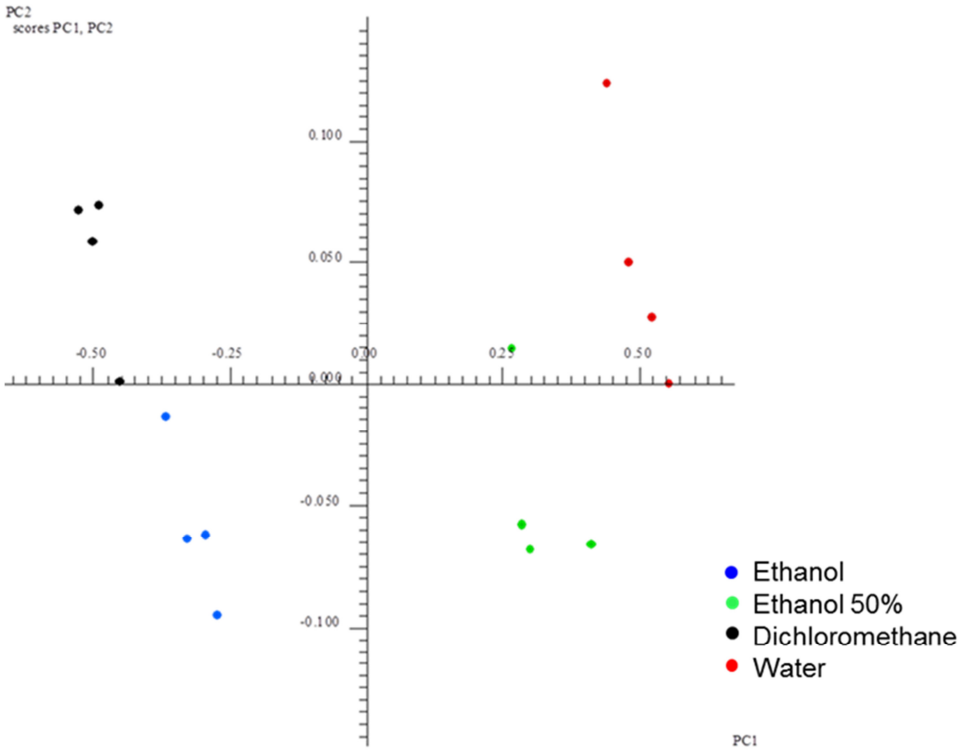


Fig. 8: Score plot of PCA for PC1 and PC2 of the ^{1}H-NMR spectra of the extracts of Chelidonii herba performed with different solvents. Each extract was recorded in four replicates.

Discrimination between the four different extracts was achieved. Replicates of the different extracts are clustering with regards to their extraction solvent. The distribution of the clusters demonstrates that the ethanolic and dichloromethane extract as well as the aqueous and the ethanolic 50% (V/V) extract were similar to each other, respectively.

To identify classes of compounds responsible for the distribution, the loading plot (fig. 9) was evaluated.

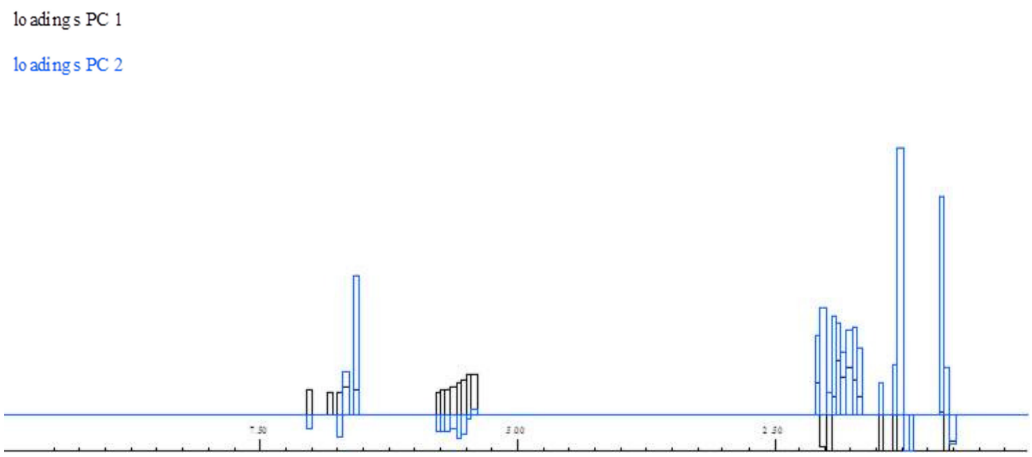


Fig. 9: Loadings for PC1 (blue) and PC2 (black). The scaling corresponds to the chemical shifts [ppm] of the ^{1}H-NMR spectra. Data in the range of δ 2.2 to δ 5.5 ppm were excluded.

The range between δ 2.2 and δ 5.5 ppm was excluded, because of water residues in the extracts. The main differences between the extracts are found in the region from 0-2.2 ppm, where most of the protons of terpenoids, steroids and organic acids resonate. In the region between δ 5.25 and δ 7.25 ppm, protons attached to phenylpropanoids, flavonoids, phenolics and tannins cause resonance signals. In this region differences between the extracts can be observed. ^{1}H-NMR resonances of alkaloids like chelidonine, protopine, coptisine, sanguinarine, chelerythrine and berberine are expected in the range of δ 7.5 to δ 10 ppm. As the loading plot shows, discrimination of PCA does not result from the different alkaloid content of the extracts.

Quantification of Chelidonii herba alkaloids by RP-HPLC:

A more detailed characterization of the different Chelidonii herba extracts was done by quantifying chelidonine, protopine, sanguinarine, coptisine, chelerythrine and berberine of the Chelidonii herba extracts by RP-HPLC (tab. 2). The dichloromethane extract showed the highest total alkaloid content (8.11 mg/ml extract; 4.06 mg/g dry weight). The content of the alkaloid chelidonine (7.15 mg/ml extract) was higher compared to the other Chelidonii herba extracts. The total alkaloid content of the ethanolic extract (5.93 mg/ml extract; 2.96 mg/g dry weight) and the ethanolic 50% (V/V) extract (4.74 mg/ml extract; 2.37 mg/g dry weight) was higher compared to the aqueous extract (0.94 mg/ml extract; 0.47 mg/g dry weight).

Tab. 2: Alkaloid composition of different extracts of Chelidonii herba. Quantification of alkaloids was obtained by RP-HPLC-DAD (285 nm).

	Ethanolic extract		50 % (V/V) ethanolic extract		Dichloro-methane extract		Aqueous extract	
		± SD		± SD		± SD		± SD
Protopine (mg/ml)	0.44	0.06	0.72	0.09	0.10	0.02	0.14	0.08
Chelidonine (mg/ml)	3.67	1.40	2.16	0.16	7.15	2.36	0.31	0.17
Coptisine (mg/ml)	1.32	0.40	1.43	0.24	0.40	0.07	0.17	0.11
Berberine (mg/ml)	0.14	0.01	0.15	0.00	0.13	0.00	0.13	0.01
Sanguinarine (mg/ml)	0.33	0.04	0.25	0.05	0.31	0.07	0.18	0.02
Chelerythrine (mg/ml)	0.03	0.00	0.02	0.01	0.02	0.01	0.00	0.01
total alkaloids (mg/ml)	5.93		4.74		8.11		0.94	
total alkaloids (mg/g dry weight)	2.96		2.37		4.06		0.47	

HepG2 cell proliferation:

Effects of different Chelidonii herba extracts on liver cells were investigated. The ethanolic and dichloromethane extract containing Chelidonii herba extract were applied with 11.84 µg/ml and 16.19 µg/ml content of total alkaloids, respectively. HepG2 cells were exposed to the 50% (V/V) ethanolic and the aqueous extract in a concentration of 9.46 µg/ml and 1.88 µg/ml, respectively. Each extract was applied to the cells in a dilution of 1:501 adjusting 1% of DMSO, respectively. The aqueous extract did not exhibit cytotoxic effects on HepG2 cells compared to the solvent control. After six hours of exposure to the aqueous extract 105% of the HepG2 cells were viable in relation to DMSO control (fig. 10).

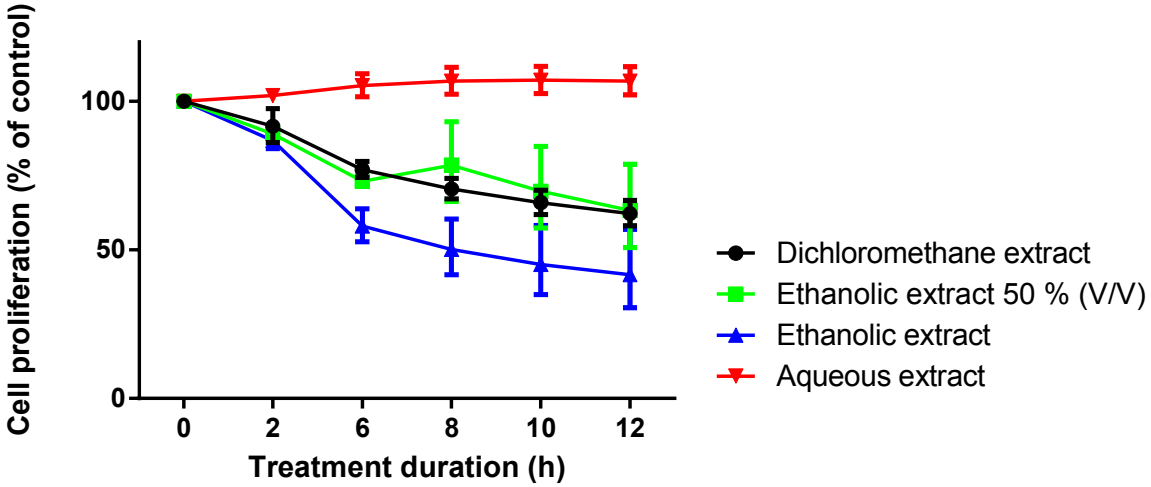


Fig. 10: Effects of different Chelidonii herba extracts on HepG2 liver cells. Cell proliferation is set into relation to control and treatment start. Extracts were applied to the cells in a concentration of 1:501. Values are expressed as mean ±SEM of at least 3 to 12 replicates.

The strongest effects on proliferation of the liver cells was observed for the ethanolic extracts (ethanol and ethanol 50% (V/V)) of Chelidonii herba. After six hours of exposure to the ethanolic extract 58% and 73% of the HepG2 cells were viable, respectively. Less cytotoxic effects were observed for the dichloromethane extract. 77% of the HepG2 cells were viable after six hours of exposure to the dichloromethane extract.

Whole genome microarrays:

In order to explore cellular effects of the different Chelidonii herba extracts at the genetic level, expression profiling was carried out in HepG2 cells. The initial data analysis revealed that each treatment with respect to the type of extract showed a distinct expression profile. In total, 2,861 genes were regulated due to the treatment with the dichloromethane Chelidonii herba extract, the regulation of 1,871 genes was different from that of the control for the aqueous Chelidonii herba extract, 1,850 genes were differently modulated when the cells were exposed to the ethanolic extract, and the expression of 695 genes was altered as a result of the treatment with the ethanolic 50% (V/V) extract.

Data evaluation with IPA showed that signal cascades associated with toxicity and biotransformation were activated (fig. 11). Signal cascades involved in oxidative stress, fatty acid metabolism or liver damage were induced. An increase in the permeability transition or the transmembrane potential of mitochondria and mitochondrial membranes was activated significantly for at least one condition in HepG2 cells. Following exposure to the ethanolic extract seven signal cascades were activated. Respectively, six and two signal cascades

were activated for the ethanolic 50% (V/V) and the dichloromethane extract. No signal cascades were induced in HepG2 cells after exposure to the aqueous extract.

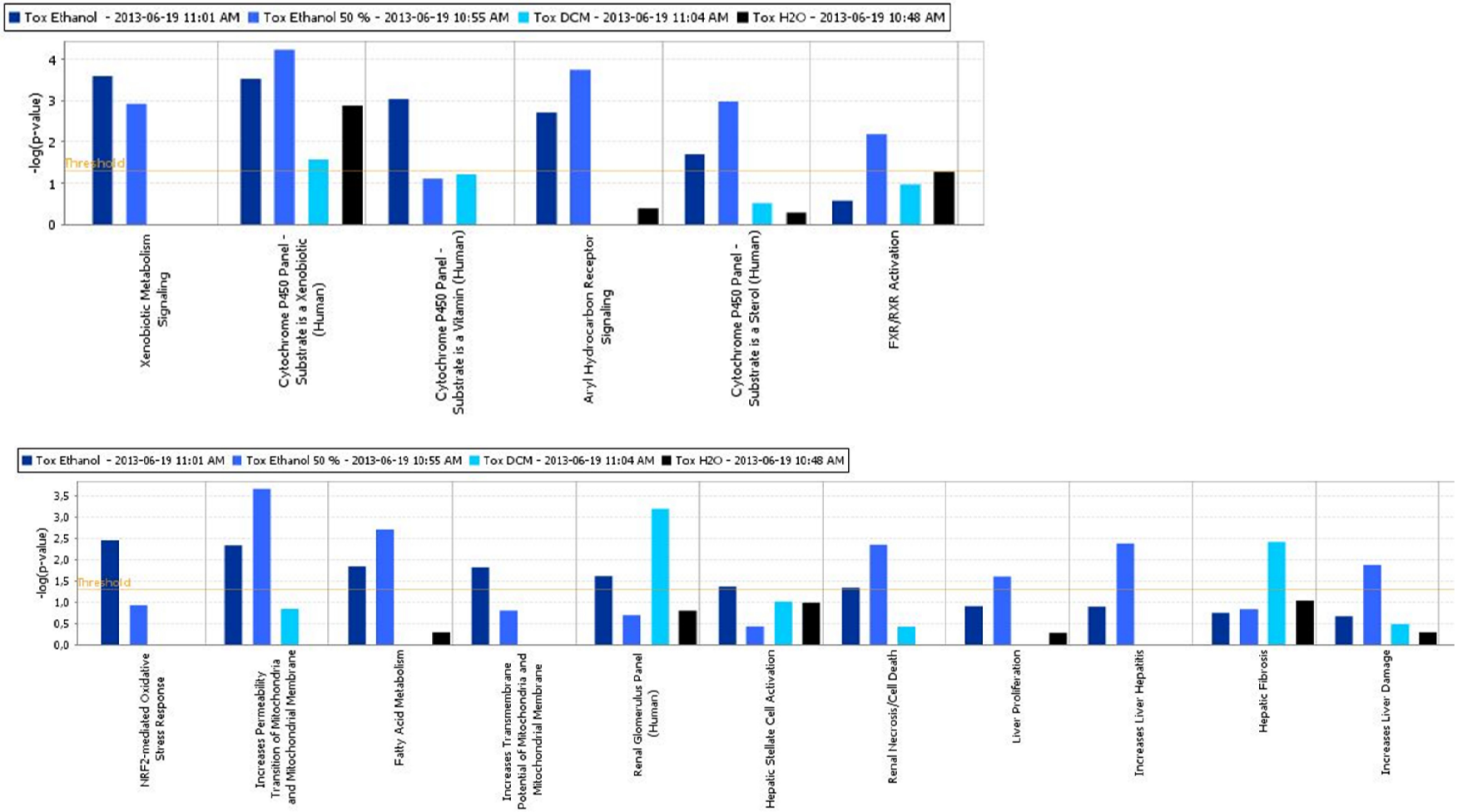


Fig. 11: Signal cascades involved in biotransformation (upper part) and liver toxicity (lower part) of HepG2 cells after exposure to the different Chelidonii herba extracts. All extracts were applied in a dilution of 1:501 and data were obtained by IPA data evaluation. Dark blue colour: treatment with ethanolic extract, light blue colour: treatment with ethanolic 50% (V/V) extract, turquoise colour: treatment with dichloromethane extract, black colour: treatment with aqueous extract of Chelidonii herba. Effects were considered as statistically significant, if a threshold of p > -log 1.31.

The signal pathway, which was significantly regulated by all four treatments, is "*Cytochrome P450 Panel-Substrate is a Xenobiotic*". All other signal pathways related to biotransformation were activated at least by one extract or by two extracts. Genes involved in biotransformation that were regulated significantly in at least one condition are shown in (tab. 3).

Tab. 3: Fold changes of genes related to biotransformation after exposure to the different Chelidonii herba extracts on HepG2 cells. Fold changes of genes, which are related to biotransformation were obtained from microarray experiments by data evaluation (Genespring 12.5). Values in bold indicate statistical significance by at least twofold and p ≤ 0.05.

Gene	Dichloromethane extract	Ethanolic extract	Ethanolic extract 50% (V/V)	Aqueous extract
AKR1B10	1.5822	**2.0252**	1.9929	1.2447
AKR1C4	-1.161	**2.7486**	**2.1811**	-1.2813
BAAT	**-5.5898**	**-2.4609**	**-2.0001**	**-2.994**
CHST10	**-2.7535**	-1.5089	-1.2222	-1.8048
CHST14	**-2.2993**	-1.4761	-1.072	-1.6347
CYP19A1	1.1568	**2.2508**	**2.4009**	1.1475
CYP1A1	1.5768	**2.9473**	**3.2499**	1.4064
CYP1B1	**2.9442**	**3.2531**	**4.1636**	**2.2513**
CYP24A1	**2.31**	**3.9019**	**2.7159**	-1.244
CYP26A1	**3.5888**	**3.5292**	1.856	1.04
CYP27C1	1.1677	-1.0281	**2.8781**	1.223
CYP2A7	1.4945	1.4648	1.5285	**2.5271**
CYP2B6	1.5528	**2.2208**	1.4477	1.6054
CYP2C18	1.1694	1.5978	**3.0097**	1.4221
CYP3A4	**5.4536**	**6.4891**	**5.4454**	**3.2177**
CYP3A7	**-2.6684**	-1.1983	-1.1357	**-2.8465**
CYP4V2	**-2.8117**	1.1533	1.1997	-1.654
CYP8B1	-1.7735	**-3.4139**	**-2.1441**	1.1904
GLYATL1	**-3.0002**	**-4.1781**	**-2.4801**	**-2.4961**
GSTO2	-1.7483	-1.5308	-1.3509	**-2.1757**
GSTP1	**3.8747**	**2.8508**	**2.8556**	**2.5285**
HS3ST3A1	-1.0795	-1.0437	1.2999	**2.3288**
KCNAB3	**-2.3195**	-1.7664	-1.5293	-1.7743
SULT1C3	-1.7304	**-2.0568**	-1.9581	1.1393
SULT1E1	**-5.7103**	**-2.9995**	**-2.6536**	**-3.2872**
SULT4A1	**2.00**	1.9249	1.6726	1.5822
UGT2A3	**-2.2794**	-1.4233	-1.1904	-1.9758
UGT2B11	**-2.6812**	**-2.4336**	-1.8786	**-2.1003**

Exposure of cells to the dichloromethane and the ethanolic extracts led to significant regulation of 15 and 16 genes relevant to biotransformation, respectively. Regarding biotransformation, 12 differently regulated genes were identified for the ethanolic 50% (V/V) extract, and the regulation of 11 genes was altered following exposure to the aqueous extract.

qRT-PCR:

Data obtained by microarray were verified by qRT-PCR for selected genes (fig. 12). Results of qRT-PCR display minor differences in fold changes, but overall confirmed the results obtained by microarray analysis.

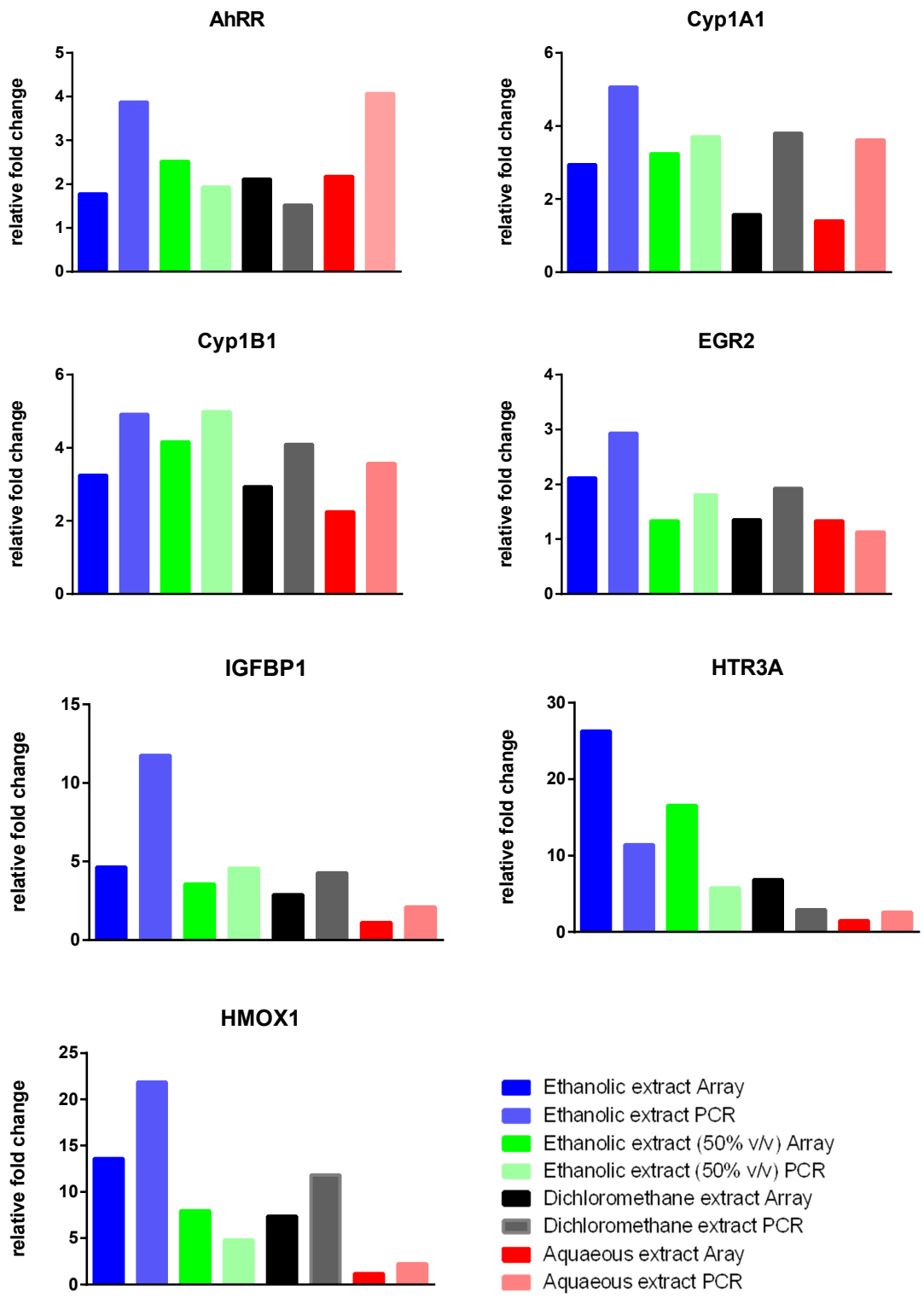


Fig. 12: Fold changes of the gene expression measured by microarray and qRT-PCR. Data obtained by qRT-PCR were normalized to GAPDH and set into relation to solvent control (AhRR: aryl-hydrocarbon receptor repressor, Cyp1A1: Cytochrome P450 family 1 member A1, Cyp1B1: Cytochrome P450 family 1 member B1, IGFBP: Insulin-like growth factor-binding protein 1, EGR2: Early growth response protein 1, HTR3A: 5-hydroxytryptamine receptor 3A, HMOX: heme oxygenase (decycling) 1).

3.5 Discussion

The quality and safety of herbal substances are important aspects in drug approval. Clinical double-blinded studies are rare in the field of herbal medicinal products, compared to studies on approved chemically-defined substances. Technical development and the avoidance of animal experiments are driving forces to investigate the applicability of new methods in the characterization of plant extracts; hence, to get a first insight for their potential prospects in regulatory affairs of herbal substances/herbal preparations.

In the present study, the ^{1}H-NMR fingerprint of Chelidonii herba extracted with solvents of different polarities was analyzed and the alkaloid content was determined. Cell proliferation of HepG2 liver cells was investigated, as it is considered that Chelidonii herba may have toxic effects on the liver, as several reports in literature described (Benninger J., 1999; De Smet P., 2002; Stickel F., 2003; Strahl S., 1998).

Plant extracts are always multicomponent mixtures. ^{1}H-NMR fingerprints of Chelidonii herba extracts showed that the latter differ in their composition regarding the groups of constituents. According to the ^{1}H-NMR spectra and the loading plot), discrimination of the different extracts resulted from plant constituents associated with a big range of metabolites suggested to include terpenoids, steroids, organic acids, phenylpropanoids, flavonoids, phenolics and tannins. Resonance signals corresponding to the above-named plant constituents were obtained in the ^{1}H-NMR spectra of the dichloromethane and the ethanolic Chelidonii herba extracts.

For both ethanolic extracts the alkaloid content quantified by RP-HPLC differed only by about 1 mg/ml extract (ethanolic extract 5.93 mg/ml extract; ethanolic 50% (V/V) 4.74 mg/ml extract). The dichloromethane extract showed the highest content of the total alkaloids (8.11 mg/ml). Only a minor amount of alkaloids was extracted with water (0.94 mg/ml). As investigations of cell proliferation demonstrated, each extract of Chelidonii herba displayed different effects. Although the highest amount of total alkaloids was found in the dichloromethane extract, the strongest inhibition of proliferation of HepG2 cells was observed for the treatment with the ethanolic extract, suggesting that not exclusively alkaloids cause toxicity. This is also supported by the fact that relative proliferation rates of cells treated with the dichloromethane and the ethanolic 50% (V/V) extract were comparable (77 and 73% relative cell proliferation, respectively), though the total alkaloid content in the dichloromethane extract was nearly twice as high as in the ethanolic 50% (V/V) extract. Earlier studies, reported in literature, analyzed cytotoxic effects of the different alkaloids in HepG2 cells. IC_{50} values of chelidonine were 34.50 µM (12.19 µg/ml) after 24 h (El-Readi M., 2013), the corresponding IC_{50} values of berberine and coptisine were 129.56 µM (48.17

µg/ml) and 202.33 µM (64.81 µg/ml), respectively (Yi J., 2013). Sanguinarine and chelerythrine showed IC_{50} values between 5 and 10 µM (24 h) (Zdarilova A., 2006), while protopine did not show a significant change in relation to the control at a concentration of 75 µM after 24 h (Vrba J., 2011). In this study, the dichloromethane extract was very rich in chelidonine in relation to the other investigated Chelidonii herba extracts. Even with regard to the IC_{50} values of the quantified alkaloids, the phytochemical characteristics indicate that the alkaloid content is not exclusively responsible for the antiproliferative effect of the extracts.

Microarray data evaluation of the present study showed that each extract caused a distinct expression pattern. For *Equisetum arvense* derived from different origins, discriminatory results in hierarchical cluster analysis of gene expression profiling in *Saccharomyces cerevisiae* were observed (Cook R., 2013). Discriminatory expression profiles in blood samples and subsequent different mode of actions were also observed in rats after their treatment with different fractions of a *Salix spec*. extract (Ulrich-Merzenich G., 2012). This indicates the specificity of transcriptome investigations.

By further data evaluation obtained with the different Chelidonii herba extracts the biological processes correlated to liver toxicities were analyzed. Most significant activated signal cascades related to toxicity were found in biotransformation processes. HepG2 cells exposed to the dichloromethane (15 genes) or the ethanolic extract (16 genes) showed the highest number of regulated genes related to biotransformation processes. For the ethanolic 50% (V/V) extract 13 genes and for the aqueous extract 11 genes were induced, respectively. It is noticeable that the higher the quantity of alkaloids was, but also the more plant constituents were extracted as indicated by ^{1}H-NMR fingerprint, the more genes were regulated in microarray experiments that play a role in biotransformation.

As microarray results showed, mRNA levels of Cyp1A1 and Cyp1B1 were induced in HepG2 cells. Protopine was shown to induce the formation of mRNA of Cyp1A1 in HepG2 cells without changing the Cyp1A1 protein or activity (Vrba J., 2011). But Cyp1A1 is inducible by various herbal substances, e.g. by the flavonoids quercetin and kaempferol (Ciolino H., 1999), catechins like epigallocatechin-3-gallate (Palermo C., 2005) or alkaloids like berberine (Chatuphonprasert W., 2011; Lo S., 2013; Salminen K., 2011; Vrzal R., 2005; Zhou C., 2011). Cyp3A4 has a share of 30% of the relative abundance of cytochromes in the liver and plays a dominant role in drug metabolism (Rendic S., 1997). Exposure of HepG2 cells to all four Chelidonii herba extracts induced Cyp3A4 gene expression, which was shown earlier to be potentially mediated by alkaloids (Salminen K., 2011; Zhou C., 2011).

Further data evaluation with IPA linked to liver-toxic effects confirmed the liver proliferation results. Most significantly regulated signal cascades and most antiproliferative effects, as cell

proliferation showed, were observed for the ethanolic extract. According to the microarray results, oxidative stress plays a role in hepatotoxicity. Oxidative stress can damage proteins, DNA and lipids in cells. Nrf2 is a transcription factor which is able to transactivate detoxifying enzymes and antioxidant enzymes (Itoh K., 1997).

The most significantly activated cascade ("*Increase in the Permeability Transition of Mitochondria and Mitochondrial Membranes*") indicates that mitochondria were depolarized. This is a key event in necrotic and apoptotic cell death (Lemasters J., 1998). The signal cascade "*Increase in the Transmembrane Potential of Mitochondria and Mitochondrial Membranes*" was observed for exposure to the ethanolic extract. The transmembrane potential of mitochondria is essential for the ATP production of the cell. Mitochondrial dysfunction may lead to alterations in the transmembrane potential; subsequently this may lead to apoptosis.

Both ethanolic extracts of Chelidonii herba altered the fatty acid metabolism in HepG2 cells. The liver is involved in important aspects of lipid metabolism (fatty acid β-oxidation, lipogenesis and lipoprotein uptake and secretion). Hepatic stellate cell (HSC) activation was significantly activated by the ethanolic extract. HSCs may be activated by hepatotoxins, initiating a cascade of pro-inflammatory events. Continued liver injury may lead to tissue fibrosis and liver cirrhosis. Significant results for "*Hepatic Fibrosis*" were observed for the dichloromethane extract. "*Liver Proliferation*" and "*Increased Liver Damage*" were activated after exposure to the ethanolic 50% (V/V) extract of Chelidonii herba.

The data evaluated with IPA indicate that extracts of Chelidonii herba induce also nephrotoxic cascades. The signal cascade "*Renal Glomerulus Panel*" was activated for the ethanolic and dichloromethane extract of Chelidonii herba. The signaling cascade "*Renal necrosis/Cell Death*" was significantly regulated for the ethanolic and ethanolic 50% (V/V) extract of Chelidonii herba in HepG2 cells.

Overall, several signal cascades attributable to hepatotoxicity are regulated. But the relevance of *in vitro* results obtained with cell culture models for human is difficult to estimate and still under debate. In previous studies, biotransformation properties of HepG2 cells and primary liver cells were compared and showed similar results (Wilkening S., 2003). The relevance of data obtained by microarray experiments should be further proven on the protein or cellular level.

Herbal preparations are defined according to the manufacturing process. Extracts obtained with the different solvents showed a different phytochemical composition and subsequently different biological activities. This emphasizes the importance of standardized manufacturing processes in herbal drug preparation.

In conclusion, the results demonstrate that a combination of the metabolomic characterization and the analysis of the transcriptome by microarrays is an appropriate strategy to characterize plant extracts. The approach provides complementary information, which often may be more representative for the characterization of a complex mixture than analysis of single compound or groups of constituents. The analysis of the microarray experiments with bioinformatics tools can be differentially attributed to specific extracts. For safety assessment of extracts of Chelidonii herba, it is particularly interesting that signal cascades correlated with biotransformation or toxicity has been identified. However, the current set of available data is limited and not permitting to draw any regulatory conclusion. Further studies are necessary to evaluate the suitability of different cell culture models and their relation to results obtained by liver proliferation studies.

4 A combined approach to characterize Cimicifugae racemosae rhizoma by metabolomic fingerprinting and gene expression profiling

4.1 Abstract

Background: Herbal substances and preparations thereof are used traditionally around the globe. In the last years, technical progress on metabolomics and transcriptomics has been achieved. Cimicifugae racemosae rhizoma is an herbal substance for which several reports associated to liver-toxic effects exist. In the present study, metabolomic and transcriptomic methods were used to characterize the chemical profile and functional effects of Cimicifugae racemosae rhizoma complementary to standard methods. The triterpene glycosides of Cimicifugae racemosae rhizoma extracts were quantified. Extracts of different composition were prepared, as three solvents of different polarity were used (ethanol, dichloromethane and ethanol 50% (V/V) (four replicates each). In addition to the standard method described in the European Pharmacopeia, the extracts were characterized by ^{1}H-NMR analysis and PCA. HepG2 cell proliferation was investigated after exposure to different extracts. Gene expression profiles of HepG2 cells were investigated by microarrays.

Results: By HPLC-ELSD it was demonstrated that the content of triterpene glycosides varied in the different extracts (total triterpene glycoside content: ethanolic extract 6.1 mg/ml; dichloromethane extract 4.4 mg/ml, ethanolic 50% (V/V) extract 2.9 mg/ml). ^{1}H-NMR spectrometric analysis of the different extracts showed that they contained varying herbal constituents. The different extracts were applied to HepG2 cells in a concentration of 1:501 (total triterpene content applied to HepG2 cells: ethanolic extract: 12.2 µg/ml, dichloromethane extract: 8.8 µg/ml, ethanolic 50% (V/V) extract: 5.8 µg/ml) and provoked different effects on HepG2 cell proliferation. Gene expression data showed that all different extracts influenced signal cascades related to cholesterol biosynthesis and induced statin-related responses. Additionally, signal cascades assignable to liver toxicity (e.g. liver necrosis, cell death) and the induction of several cytochromes were identified, mainly for the ethanolic and the dichloromethane extract.

Conclusion: ^{1}H-NMR analysis and gene expression profiling techniques are useful for the comprehensive characterization of herbal preparations. The results of the present study support the necessity of appropriate quality assurance and characterization of herbal

substances and preparations thereof. For the assessment of quality and safety of herbal medicinal products a comprehensive approach by using ^{1}H-NMR spectroscopy analysis and the investigation of the transcriptome approach proved to be valuable and complementary to already established methods.

Gene abbreviations:

CYP1A2: cytochrome P450, family 1, subfamily A, polypeptide 2; CYP2C9: cytochrome P450, family 2, subfamily C, polypeptide 9; CYP2D6: cytochrome P450, Family 2, subfamily D, polypeptide 6; CYP51A1: cytochrome P450, family 51, subfamily A, polypeptide 1; DHCR7: 7-dehydrocholesterol reductase; EDN1: endothelin 1; FDFT1: farnesyl-diphosphate farnesyltransferase 1; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; HMGCR: 3-hydroxy-3-methylglutaryl-CoA reductase; HMGCS: 13-hydroxy-3-methylglutaryl-CoA synthase 1; HMOX1: heme oxygenase (decycling) 1, IDI1: isopentenyl-diphosphate delta isomerase 1; IGFBP1: insulin-like growth factor-binding protein 1; LDLR: low density lipoprotein receptor; LSS: lanosterol synthase (2,3-oxidosqualene-lanosterol cyclase); MSMO1: methylsterol monooxygenase 1; MVD: mevalonate (diphospho) decarboxylase; MVK: mevalonate kinase; SQLE: squalene epoxidase; SREBP: sterol regulatory element binding transcription factor

4.2 Background

In modern medicine substantial progress could be observed in recent decades, i.e. the use of recombinant proteins. Nevertheless, traditional herbal substances considerably contribute to the human health care. However, it is mandatory to apply safe herbal medicines with adequate quality. Herbal substances and preparations thereof are multicomponent mixtures; hence, they are very different from chemically-defined medicinal products. The active principle from herbal substances is often unknown and the complex mixtures are regarded as active substance. The multicomponent nature of herbal medicines complicates their analytical evaluation.

For regulatory affairs of herbal medicines research on characterization of herbal preparations is of major interest, since the currently applied standard methods have shown limitations. It has already been described that the phytochemical characterization using marker substances is not always sufficient for an adequate characterization of herbal substances and preparations thereof. Hence, methods related to metabolomics, i. e. the complete phytochemistry, and transcriptomics, i. e, the overall biological response need to be further examined for their usefulness in evaluating the quality and safety of an herbal medicine.

NMR spectroscopy is a frequently used metabolomic technique (Kim H., 2010; Schripsema J., 2010; Wang X., 2011). The basis of this approach is a broad-spectrum analysis that takes into account the entire NMR-sensitive metabolite content, extracted from the sample in question. In the present research, a multivariate statistical analysis method, i. e. principal component analysis (PCA), was applied to analyze ^{1}H-NMR spectra of herbal extracts (Daniel C., 2008). PCA is an unsupervised and therefore unbiased technique for multivariate analysis.

The functional effects of the herbal preparations were characterized by determination of the cell proliferation rate of HepG2 liver cells after exposure to differently prepared extracts containing Cimicifugae racemosae rhizoma. Additionally, transcriptomics methods, such as microarrays were used to identify differently regulated genes and place them into a biological context by systems biological data evaluation.

Cimicifugae racemosae rhizoma (*Actaea racemosa* L., syn. *Cimicifuga racemosa* L.; Ranunculaceae) is a widely used herbal substance and has been linked to a variety of benefits for specific health problems in women. According to the HMPC monograph on Cimicifugae racemosae rhizoma, the drug is indicated for the relief of menopausal complaints such as hot flushes and profuse sweating (Committee on Herbal Medicinal

Products, 2010a). Questions have been raised about the safety of Cimicifugae racemosae rhizoma. Cases of liver toxicity were described for patients who were taking products derived from this herbal substance; subsequent evaluation found some products to be adulterated with other related herbal species (Patel N. and Derkits R., 2007; Gardiner P., 2008; Joy D., 2008; Pierrard S. 2009; Vannacci A., 2009; Painter D., 2010). Since 2009, a graduated scheme has been effective concerning the risks of hepatotoxicity and the consumption of herbal preparations containing Cimicifugae racemosae rhizoma in Germany. This graduated scheme implies precautions and warnings with regard to the application of Cimicifuga containing preparations. Since the association of hepatotoxicity with application of Cimicifugae racemosae rhizoma is possible, this is an interesting model object to correlate the chemical profile and effects on liver cells with differently prepared extracts.

Chemical constituents of a Cimicifugae racemosae rhizoma extract may comprise at least three major natural product groups: Cycloartenal-type triterpenes, phenolics and flavonoids (Chen S., 2002c; Li J., 2006). According to the HMPC monograph on Cimicifugae racemosae rhizoma, there is an ongoing discussion on the constituent(s) responsible for the effectiveness of Cimicifugae racemosa rhizoma. Therefore, the whole extract is considered as the active pharmaceutical ingredient.

According to the HMPC monograph on Cimicifugae racemosae rhizoma, their preparations contain a complex mixture of 40-70 mg/g herbal substance of triterpene glycosides. The major constituents are actein, 23-*epi*-26-deoxyactein (syn. 27-deoxyactein), cimiracemoside A, acetylshengmanol-3-*O*-xyloside, cimigenol 3-*O*-arabinoside and cimigenol 3-*O*-xyloside (fig. 13) (Bedir E., 2000; Chen S., 2002c; He K., 2006; Shao Y., 2000; Watanabe K., 2002).

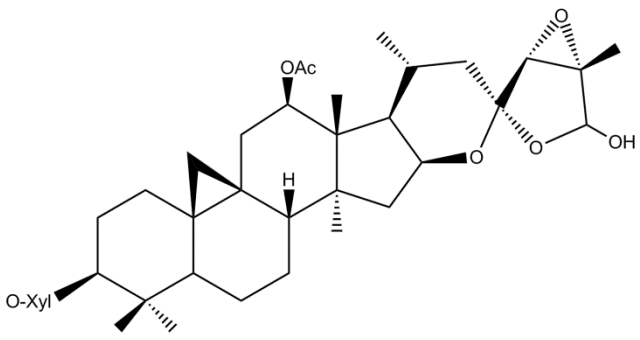


Fig. 13: Chemical structure of actein.

Caffeic, ferulic, isoferulic, fukinolic, fukiic and cimicifugic acid (Kruse S., 1999), as well as cimiracemates belong to the group of phenolics and were found in minor amounts (Chen S.,

2002a; Chen S., 2002b). Guanidine alkaloids like cimipronidine were detected in Cimicifugae racemosae rhizoma only in smaller quantities (Fabricant D., 2005).

The occurrence of the flavonoids formononetin and kaempferol has not been verified (Jiang B., 2006; Jiang B., 2011; Kennelly E., 2002; Struck D., 1997), except for two references (Al-Amier H., 2012; Panossian A., 2004), which reported formononetin to occur in a ppm range. In current reports on Cimicifugae racemosa rhizoma, kaempferol was not detected (Struck D., 1997). For these natural products the involvement in estrogenic effects was discussed (Jarry H., 1985).

4.3 Materials and Methods

Plant material and preparation of extracts:

Samples of Cimicifugae racemosae rhizoma were provided by Phytolab GmbH & Co. KG. In analogy to voucher specimen, herbal substance has been deposited at the University of Bonn, Institute of Pharmaceutical Biology.

Cimicifugae racemosae rhizoma was extracted with three different extraction solvents (ethanol, ethanol 50% (V/V), dichloromethane). Four replicates were performed for each extract. Respectively 2.0 g of plant material was extracted with 20 ml of the solvent, frequently stirring at room temperature for 20 min. After filtering, the extraction procedure was repeated using 20 ml of the respective solvent. The extract was divided into two parts and the solvents were eliminated under reduced pressure. One half of the dried extract was solved in 500 µl deuterated dimethyl sulphoxide for ^{1}H-NMR measurement. The remaining half of the dried extract was solubilized in 2.0 mL 50% (V/V) methanol and used for HPLC analysis.

^{1}H-Nuclear magnetic resonance (NMR) spectroscopy:

^{1}H-NMR spectra of extracts were recorded with a Bruker advance 300 DPX instrument (temperature 25 °C, 64 scans). Four replicates of each extract were recorded by ^{1}H-NMR. Calibration of spectra was performed according to signals of incompletely deuterated solvents. Data were processed using TOPSPIN software.

Principal component analysis:

PCA was performed using TopSpin™ software (Bruker). Data from δ 0 to δ 10 ppm were included. The region from δ 2.3 to δ 5.0 ppm was excluded to eliminate the effects of water

signals and the solvent peak of DMSO (2.54 ppm) in the extract. The bucket width was 0.05 ppm and data were integrated according to the sum of intensities.

Quantification of triterpene glycosides in Cimicifugae racemosae rhizoma extracts:

The triterpene glycosides were quantified using HPLC and evaporative light scattering detector (ELSD). HPLC analysis was carried out on an Agilent 1100 series instrument equipped with a quaternary pump, a four-channel-online degasser and an autosampler. ELSD chromatograms were recorded on a Sedere Sedex-75 (Alfortville, France) and chromatographic data were collected by Chemstation software (Agilent). The ELSD detector was set to 40 °C and the measurements were carried out in gain 9. The carrier gas was compressed air.

With respect to the quantification of triterpene glycosides a validated method described in the European Pharmacopeia was used. Regarding validation based on ICH guideline, linearity, precision of the instrument and precision of the method was determined (International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use, 2005). The determination of linearity was performed using glycyrrhizic acid (R^2=0.99), as described in the monograph, and additionally by using 27-deoxyactein (R^2=0.99). Linearity was analyzed using concentrations in the range of 76.5 to 765.2 µg/ml and 50.7 to 505.6 µg/ml for glycyrrhizic acid and 27-deoxyactein, respectively. Precision of the instrument showed a relative standard deviation of 1.85% for 27-deoxyactein after six injections of this compound. Precision of the method showed a relative standard deviation of 5.9% after injection of six replicates of the dichloromethane Cimicifugae racemosae rhizoma extract. The injection volume was 10 µl and the investigated extracts were injected twice.

The different Cimicifugae racemosae rhizoma extracts were filtered via 45 µm membranes. Chromatography was performed on a Zorbax Eclipse XDB C18 column (250 x 4.6 mm i.d., 5 µm) with a flow rate of 1 mL/min. The column was equipped with an analytical guard column.

The mobile phase consisted of 0.1% (V/V) formic acid (A) and 0.1% formic acid/acetonitrile 50% (V/V) (B) The program of chromatography showed minor changes according to the pharmacopeial monograph: 0 min 50% B, 50 min 80% B. Finally, the column was washed for 3 min with 95% B and re-equilibrated to 50% B for 5 min.

Treatment of HepG2 cells with plant extracts:

HepG2 cells were purchased from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures GmbH and were used between passage 3 and 12. The

cells were maintained in T75 flasks in RPMI 1640 medium supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin solution (100 U/ml) and 1% L-glutamine. The cells were constantly incubated in humidified atmosphere at 5% CO_2 and 37 °C.

When the cells were to about 80% confluent, they were treated with trypsin and harvested by centrifugation. Cells were counted and an equal number (1.0 x 10^6 cells/5 ml media) were transferred to each experimental plate (5 cm Ø) for treatment with the different extracts on the following day. Cells were treated with the extracts in a concentration of 1:501. The vehicle treated cells (1% DMSO) served as a control group. Four replicates were performed for each experimental condition.

Cell growth and proliferation assay using xCELLigence system:

About 7,500 HepG2 cells were seeded to each well. After 24 h, medium containing the extracts in a concentration of 1:501 adjusted to 1% DMSO or medium containing 1% DMSO as solvent control was added, respectively. The cell index was monitored every 15 min.

Whole genome microarray:

The quality of RNA was examined by the RIN-values (Agilent 2100 bioanalyzer). The concentration and purity of extracted RNA was measured using an UV-visible spectrometer (NanoDrop 1000; Thermo Fisher Scientific, Waltham, MA) by absorption at wavelengths of 260 and 280 nm. RNA samples with a 260/280 nm absorption ratio > 1.8 and RIN > 9.5 were used in subsequent microarray analysis. For microarray profiles, fluorescently labeled cRNA samples were prepared from 100 ng RNA using reverse transcriptase. The amplification reaction with simultaneous introduction of Cy3-dCTP to the amplified complementary RNA (cRNA) was performed using a Quick Amp Labeling Kit for One-Color (Agilent Technologies). The concentration of the purified samples and the Cy3 dye incorporation efficiency was evaluated using a NanoDrop 1000 spectrophotometer. After fragmentation (65 °C, 30 min), the single colour cRNA samples were hybridized to a DNA chip (Whole Genome Gene Expression Microarrays v2, Agilent Technologies, 8x60K, 27,958 Gene RNAs, 7,419 lincRNAs) at 65 °C for 17 hours in a hybridization oven (Agilent Technologies). Four independent experiments were performed under each experimental condition.

qRT-PCR:

After reverse transcription by random priming the resulting cDNA was used for qRT-PCR. Following initial denaturation (95 °C, 10 min), amplification was performed over 45 cycles (Light Cycler 480, Roche) with denaturation at 95 °C for 10 s and annealing with primers at

temperatures shown in tab. 4. Elongation was performed at 72 °C for 20 s. The size of PCR fragments was analyzed by agarose gel electrophoresis. Gene expression was evaluated with Light Cycler 480 Software 1.5 and C_P-values were normalized to GAPDH.

Tab. 4: Primer used for qRT-PCR for validation of microarray results.

Primer/Gene Assignment	NCBI accession code	Primer sequence (5´→3´)	Annealing temperature (°C)
HMGCR_1	NM_000859	TGATTGACCTTTCCAGAGCAAG	52
HMGCR_2	NM_000859	CTAAAATTGCCATTCCACGAGC	52
EDN1_1	NM_001955.4	CCTGGACATCATTTTGGGTCAA	62
EDN1_2	NM_001955.4	TCATGGTCTCCGACCTGGT	62
HMOXI_1	NM_002133.2	CCCACGCCTACACCCGCTAC	65
HMOXI_2	NM_002133.2	GGTGGCACTGGCAATGTTGG	65
IGFBP1_1	NM_000596.2	CACAGCAGACAGTGTGAGACAT	52
IGFBP1_2	NM_000596.2	ATTTCATCTGGTTTCAGTTTTGTAC	52
GAPDH_1	NM_002046.4	CCACCCATGGCAAATTCCATGGCA	57
GAPDH_2	NM_002046.4	CTAGACGGCAGGTCAGGTCCACC	57

Data processing and statistics:

Hybridized DNA chip slides were scanned using an Agilent Scanner (Agilent Technologies) with Feature Extraction Software (Agilent Technologies). Fluorescence intensity data were imported to GeneSpring GX version 12.5 (Agilent Technologies) with the quantile scaling normalization. Subsequently data were filtered to exclude low-quality data to guarantee the accuracy of the statistical analysis. In the first step, spots with lower intensities than the threshold, which was determined based on the intensities of the Agilent RNA Spike-Mix, were filtered to exclude weaker spots than background noise. Entities with saturated intensities and near-background intensities were filtered using the 'flag' function of the Feature Extraction Software. The final filtration was conducted based on the fold increase.

Statistical analysis of the remaining entities was performed with a t-test corrected using the Benjamini and Hochberg false discovery rate (Benjamini Y., 1995). Genes were considered as up- or down-regulated with a fold ≥ 2 and ≤ -2 and $p \leq 0.05$.

These processed data were used for further analysis by Ingenuity systems Inc. Reedwood city, USA. Filters were set for SurePrint G3 Human CGH Microarray 8*60K as reference set. In the networks interaction 70 molecules were set and all data sources were integrated in data analysis. Data were compared to all species (human, rat and mouse) and all available tissues and types of cells.

4.4 Results

^{1}H-NMR spectroscopy and PCA:

In total ^{1}H-NMR spectra from twelve samples of Cimicifugae racemosae rhizoma, which were extracted with three different solvents, were analyzed. Differences in the spectra of the ethanolic, ethanolic 50% (V/V) and dichloromethane Cimicifugae racemosae rhizoma extracts were visually noticeable with regard to the extraction solvents, respectively. According to the spectra obtained for the ethanolic and dichloromethane extracts resonance signals assignable to terpenoids, phenylpropanoids and flavonoids were obtained.

The differences of the ^{1}H-NMR spectra were further ascertained by a pattern recognition approach. The different Cimicifugae racemosae rhizoma extracts were discriminated by PCA (fig. 14).

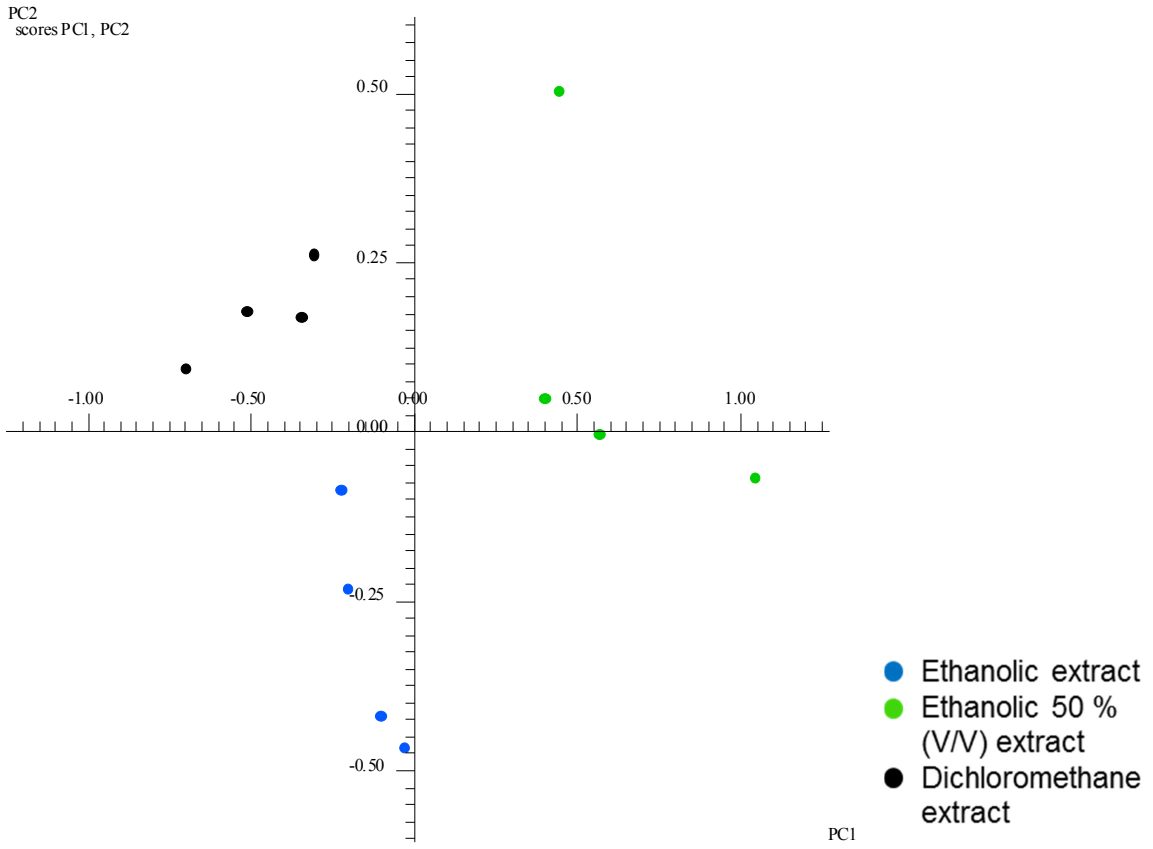


Fig. 14: Score plot of PCA for PC1 and PC2 of the ^{1}H-NMR spectra of the extracts of Cimicifugae racemosae rhizoma. The extracts were prepared with different solvents; four replicates each. The extracts were recorded in DMSO-*d6*.

With regard to the extraction solvent, replicates of the different extracts showed cluster formation. The first principal component (PC1) indicated 58.0% of the explained variance and the second principal component displayed 17.2%. No natural compound was precisely identified responsible for the differentiation of the extracts by the visual investigation of the spectra or by the loading plot (fig. 15).

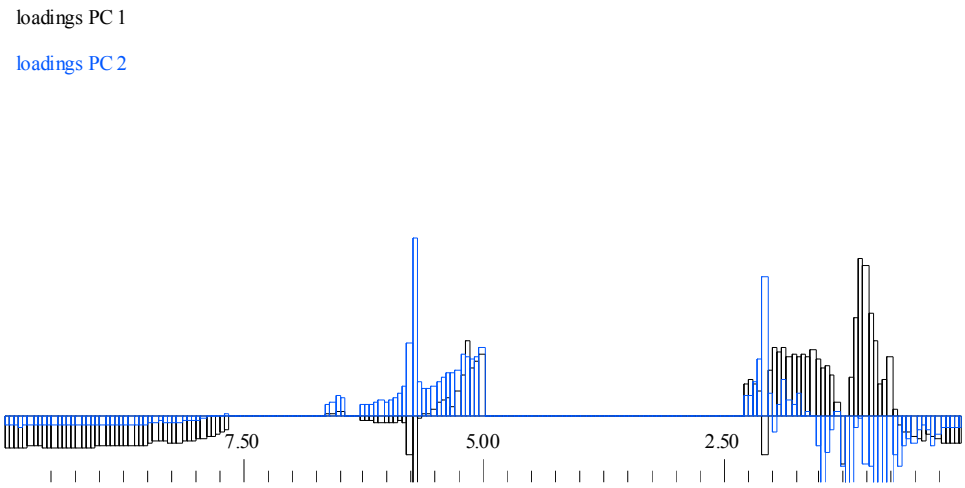


Fig. 15: Loadings for PC1 (blue) and PC2 (black). The scaling corresponds to the chemical shifts [ppm] of the 1H-NMR spectra. Signals in the range of δ 2.2 to δ 5.5 ppm were excluded.

To identify classes of compounds responsible for the distribution, the loading plot was evaluated. Signals in the range between δ 2.3 and δ 5.0 ppm were excluded, because of overlapping signals from water residues in the extracts. According to the loading plot, the main differences between the extracts were found for resonance signals (δ 0- δ 2.3 ppm), which could be associated with protons from terpenoids, steroids, and organic acids. In the region between δ 5.0 and δ 6.7 ppm, protons attached to phenylpropanoids, flavonoids, and phenolics cause resonance signals and in this region differences between the extracts were observed. In the range of δ 7.5 to δ 10 ppm protons associated with alkaloids resonate. The loading plot indicated that for the classification of the extracts compounds like alkaloids contributed only to a limited amount.

Quantification of triterpene glycosides in Cimicifugae racemosae rhizoma extracts:

The triterpene glycosides in Cimicifugae racemosae rhizoma were quantified according to the method described in the monograph 7.5/2069 in the European Pharmacopeia (with minor modifications). As demonstrated in tab. 5 the ethanolic extract showed the highest content of triterpene glycosides (6.1 mg/ml and 12.2 mg/g of dry weight of Cimicifugae racemosae rhizoma). 24.6% of the content of triterpene glycosides corresponded to 27-deoxyactein. The dichloromethane extract contained 4.4 mg/ml triterpene glycosides, whereof 24.7% was 27-deoxyactein. The triterpene glycoside content in the ethanolic extract 50% (V/V) obviously was lower (2.9 mg/ml) and 26.5% was 27-deoxyactein.

Tab. 5: Quantification of triterpenes glycosides of different Cimicifugae racemosae rhizoma extracts. Triterpenes glycosides were separated and detected by HPLC-ELSD.

	Total triterpene glycosides (mg/ml extract)	Total triterpene glycosides (mg/g dry weight)	27-Deoxyactein (mg/ml extract)	Total 27-Deoxyactein (mg/g dry weight)
Dichloromethane extract	4.4	8.8	1.1	2.2
Ethanolic extract	6.1	12.3	1.5	3.0
50% (V/V) ethanolic extract	2.9	5.8	0.8	1.5

Cell proliferation of HepG2 cells after exposition to different Cimicifugae racemosae rhizoma extracts:

The different Cimicifugae racemosae rhizoma extracts were investigated for effects on HepG2 liver cell proliferation (fig. 16). After six hours of exposure to the 50% (V/V) ethanolic extract 81% of cells were proliferating in relation to DMSO control. A stronger effect on hepatocellular cell proliferation was observed for the dichloromethane extract. Here, after six hours of exposure, 72% of cells were proliferating in relation to the control. The ethanolic extract showed an even more marked effect on cell proliferation (61% after six hours of exposure) than the dichloromethane Cimicifugae racemosae rhizoma extract.

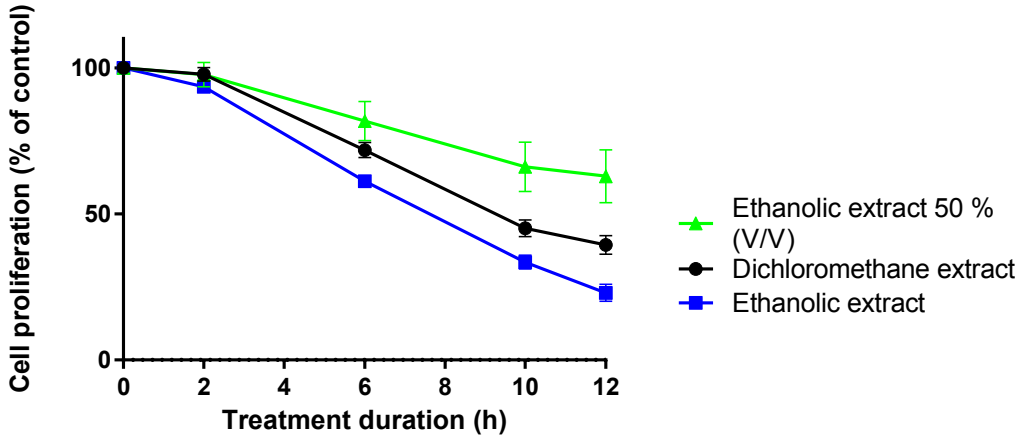


Fig. 16: Effects of HepG2 liver cells after exposure to different Cimicifugae racemosae rhizoma extracts. Cell proliferation was set into relation to control and treatment start. Extracts were applied to the cells in a concentration of 1:501. Values are expressed as mean ±SEM of at least 3 to 12 replicates.

Microarray data analysis:

Gene expression of HepG2 cells, exposed to the different Cimicifugae racemosae rhizoma extracts, was investigated at a time point of six hours of exposure. Preliminary investigations indicated that it is an eligible time point to cover the primary gene response.

Microarray analysis revealed that exposure of the ethanolic extract induced most effects on gene expression, 522 genes were differently regulated. The dichloromethane extract significantly altered the regulation of 348 genes, while the ethanolic 50% (V/V) changed the expression of 51 genes in relation to control.

Using IPA significantly regulated genes were investigated for affected signal cascades (fig. 17).

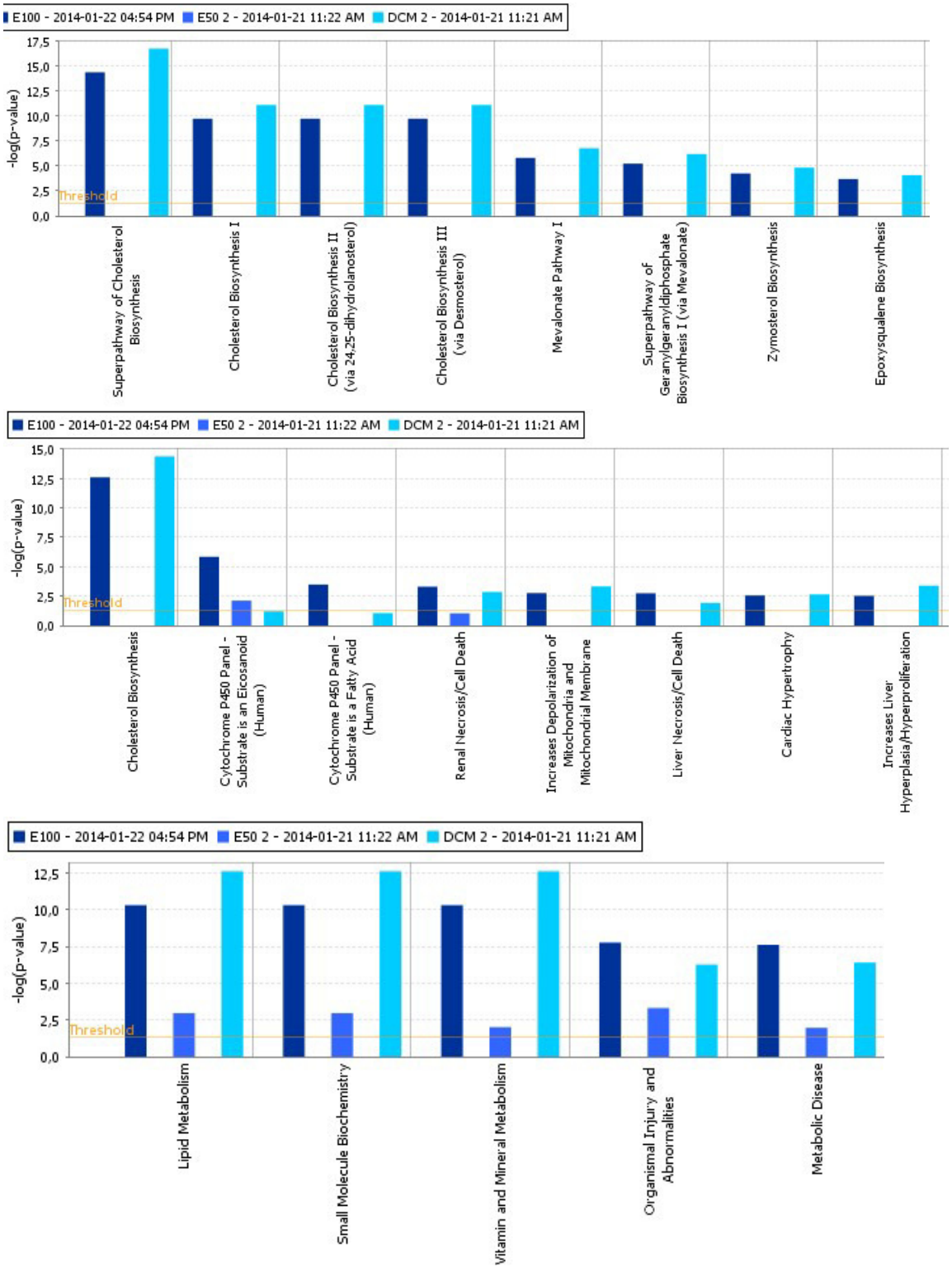


Fig. 17: Signal cascades involved in canonical pathways (upper part), related to diseases and biofunctions (middle part), biotransformation and liver toxicity (lower part) of HepG2 cells after exposure to different Cimicifugae racemosae rhizoma extracts. All extracts were applied in a dilution of 1:501; data were obtained by IPA data evaluation. Dark blue color: treatment with ethanolic extract, light blue color: treatment with ethanolic 50% (V/V) extract, turquoise colour: treatment with dichloromethane extract. Effects were considered as statistically significant, if values were exceeding a threshold of p > -log 1.31.

The ethanolic and dichloromethane extract significantly altered signal cascades and networks involved in the sterol biosynthesis, terpenoid biosynthesis and fatty acid and lipid biosynthesis.

For HepG2 cells exposed to Cimicifugae racemosae rhizoma extracts the following signal cascades related to diseases and biofunctions emerged: "*Lipid Metabolism*", "*Small molecule Biochemistry*" and "*Vitamin and Mineral Metabolism*". Associated with these processes are the synthesis of cholesterol, sterols, lipids, terpenoids and steroids, the metabolism of cholesterol, membrane lipid derivatives and steroids, steroidogenesis and the concentration of lipids and long chain fatty acids. Related to metabolic diseases, dislipidemia, hypertriglyceridemia and hypercholesterolemia can be allocated to this context. Furthermore, the signal cascade "*Organismal Injury and Abnormalities*" showed significant results for all three different extracts.

As there is a special interest for signal cascades related to toxicity of Cimicifugae racemosae rhizoma, the data were further analyzed with this regard (fig. 17). The relevance on cholesterol biosynthesis is reflected. Furthermore, involvement of cytochromes related to processes in metabolism or biotransformation is demonstrated, at least for the ethanolic extract. The impairment of mitochondria in toxicity-related signal cascades was shown for exposure of the ethanolic and the dichloromethane extracts, while no statistical significant effects were observed for the ethanolic 50% (V/V) extract. "*Liver Necrosis/Cell Death*", "*Renal Necrosis/Cell Death*" and "*Cardiac Hypertrophy*" and the signal cascade "*Increase in Liver Hyperplasia/Hyperproliferation*" were altered significantly for the ethanolic and dichloromethane extracts but not for the ethanolic 50% (V/V) extract.

Cellular responses and affected signal cascades to cholesterol biosynthesis were characterized more in detail (tab. 6). Several significantly regulated genes demonstrated to have an impact in cholesterol biosynthesis, in the sterol regulatory element-binding protein (SREBP), but also in the statin-related mediated cellular answer.

Tab. 6: Fold changes of genes related to cholesterol biosynthesis in HepG2 cells after exposure to the different Cimicifugae racemosae rhizoma extracts. Fold changes of genes, which are related to cholesterol biosynthesis, SREBP and statin-related cellular responses were obtained from microarray data evaluation (Genespring 12.5). Values in bold indicate statistical significance by at least twofold change in gene regulation and p ≤ 0.05.

Gene Name	NCBI accession code	Dichloromethane extract	Ethanolic extract	Ethanol 50% (V/V) extract
Cholesterol biosynthesis				
IDI1	NM_004508	**2.62**	**2.81**	1.94
HMGCS1	NM_002130	**2.84**	**2.91**	**2.16**
HMGCR	NM_000859	**2.59**	**2.62**	**2.11**
MVK	NM_000431	**2.15**	**2.27**	1.67
MVD	NM_002461	**3.36**	**3.28**	**2.73**
FDFT1	NM_004462	**2.21**	**2.29**	1.83
DHCR7	NM_001360	**2.19**	**2.43**	1.79
LSS	NM_001001438	**2.12**	**2.50**	1.62
SQLE	NM_003129	**2.56**	**2.58**	**2.29**
CYP51A1	NM_000786	**2.16**	**2.30**	1.72
MSMO1	NM_006745	**2.71**	**3.09**	1.98
Statin-related				
HMGCR	NM_000859	**2.59**	**2.62**	**2.11**
LDLR	NM_000527	**4.06**	**4.92**	**2.18**
FDFT1	NM_004462	**2.21**	**2.29**	1.83
SQLE	NM_003129	**2.56**	**2.58**	**2.29**
SREBP				
HMGCR	NM_000859	**2.59**	**2.62**	**2.11**
LSS	NM_001001438	**2.12**	**2.50**	1.62
HMGCS1	NM_002130	**2.84**	**2.91**	**2.16**
MVD	NM_002461	**3.36**	**3.28**	**2.73**
IDI1	NM_004508	**2.62**	**2.81**	1.94
FDFT1	NM_004462	**2.21**	**2.29**	1.83
SQLE	NM_003129	**2.56**	**2.58**	**2.29**
CYP51A1	NM_000786	**2.16**	**2.30**	1.72
LDLR	NM_000527	**4.06**	**4.92**	**2.18**

qRT-PCR:

Data obtained by microarray analysis were verified by qRT-PCR for IGFBP1 (insulin-like growth factor-binding protein 1), HMGCR (3-hydroxy-3-methylglutaryl-CoA reductase), HMOX1 (heme oxygenase (decycling) 1 and EDN1 (endothelin 1) (fig. 18). Results of qRT-PCR displayed minor differences in fold changes, but confirmed the results obtained by microarray analysis.

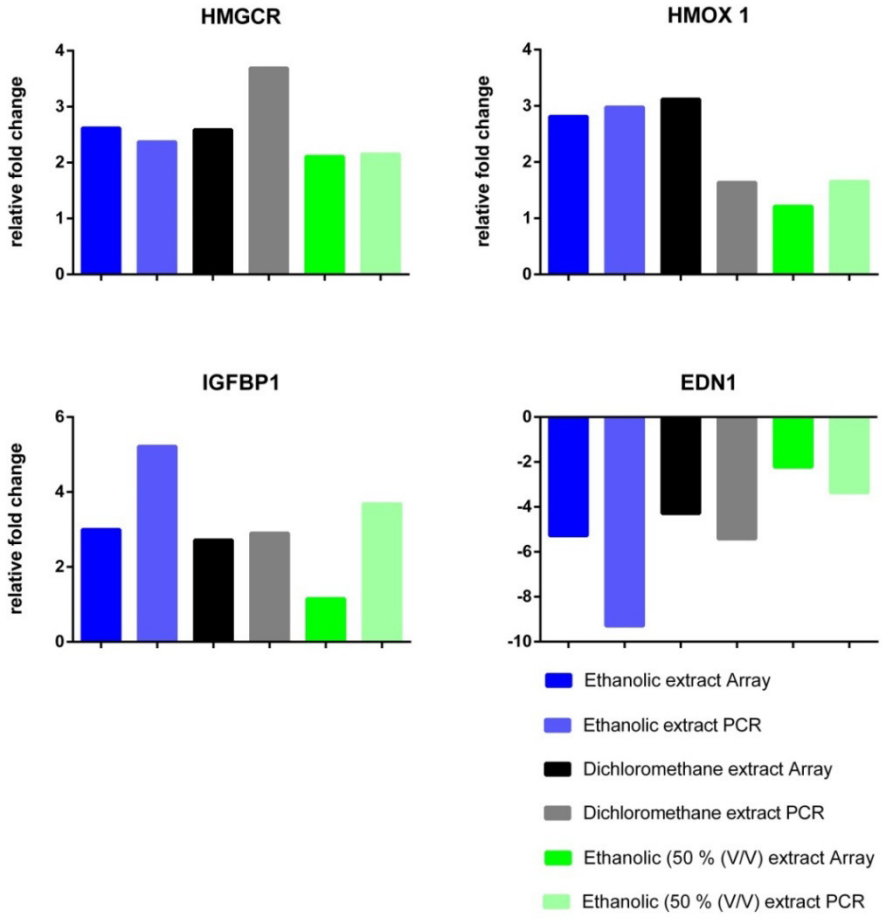


Fig. 18: Fold changes of gene expression measured with microarray and qRT-PCR. Data obtained with qRT-PCR were normalized to GAPDH and set into relation to the solvent control (IGFBP1: insulin-like growth factor-binding protein 1, HMGCR: 3-hydroxy-3-methylglutaryl-CoA reductase, HMOX1: heme oxygenase (decycling) 1, EDN1: endothelin 1).

4.5 Discussion

There is a high interest in the investigation of Cimicifugae racemosae rhizoma with different compositions of constituents and the correlation to their functional activity since the

components responsible for the pharmacological activity of Cimicifugae racemosae rhizoma so far have not been finally identified.

Indeed, the extraction solvents used in the present study showed an impact on the composition of the different extracts, which was generally shown by ^{1}H-NMR analysis and the content of triterpene glycosides investigated by HPLC. The spectra obtained by ^{1}H-NMR indicated that various sets of constituents were extracted suggesting to include triterpene glycosides, phenylpropanoids and flavonoids. Therefore, the correlation of the functional activity to the chemical profile has to be considered for the whole extract and not exclusively for the amount of triterpene glycosides.

In the present study, different extracts of Cimicifugae racemosae rhizoma exhibited different effects on cell proliferation. For actein an IC_{50} value of 27 µg/ml was reported for HepG2 cells (Einbond L., 2009) and 5.7 µg/ml in MDA-MB-453 after 96 hours of exposure (Einbond L., 2008). In the human breast cancer cell MDA-MB-453 it was shown that the inhibitory effects on cell proliferation was primarily due to the triterpenes glycosides (Einbond L., 2008). The results obtained in the present study demonstrated the strongest antiproliferative effects for the ethanolic extract (61% of relative cell proliferation after six h). For this extract the highest amount of triterpenes was obtained (6.1 mg/ml). The less triterpene glycosides were extracted in the different extracts the less antiproliferative effects were observed. But the ^{1}H-NMR spectra of the dichloromethane and the ethanolic Cimicifugae racemosae rhizoma extracts displayed additional signals attributable to phenylpropanoids and flavonoids. Regarding toxicity mediated by Cimicifugae racemosae rhizoma, triterpene glycosides and also cinnamic acid esters were previously investigated for their apoptosis-inducing properties indicating that both natural products contribute to apoptotic effects (Hostanska K., 2004a; Hostanska K., 2004b). Hence, it can be suggested that the antiproliferative effects observed in the present study were not exclusively attributable to triterpene glycosides.

In order to identify putative mechanisms in HepG2 cells treated with preparations from Cimicifugae racemosae rhizoma, genome-wide transcriptional alterations were investigated. One of the major findings of the microarray analysis was the influence on expression of genes related to cholesterol biosynthesis upon the treatment with the different extracts.

There are several reports on the ability of Cimicifugae racemosae rhizoma or isolated compounds thereof to affect lipid synthesis. On the level of transcriptomics, a Cimicifugae racemosae rhizoma extract with a concentration of 40 µg/ml (total triterpene content 10.53%, about 3.8 µg/ml triterpenes) mediated alterations on cholesterol biosynthesis in MCF7 breast cancer cells (Einbond L., 2007). In rats actein was applied as a pure compound (35.7 mg/kg). There, it was shown that genes related to cholesterol and fatty acid biosynthesis as well as a

statin-related response was observed (Einbond L., 2009). In rats which were fed with 35.7 mg kg^{-1} Cimicifugae racemosae extract (enriched with triterpenes, 27% triterpene glycosides, about 9.6 mg kg^{-1} total triterpene glycosides) it was observed that the cholesterol biosynthesis was altered on the transcriptomics level. The content of free fatty acids and triglycerides increased in the liver and decreased in the serum (Einbond L., 2012). In a clinical trial investigating a combination of *Cimicifugae racemosae rhizoma* (0.0364 ml, equivalent to 1 mg triterpene glycosides) and *Hypericum perforatum* (84 mg dried extract, equivalent to 0.25 mg hypericine) HLD-C serum levels increased (Chung D., 2007). Furthermore, it was reported that the fraction containing saponins (2.05 mg/day/animal) prepared from a Cimicifugae racemosae rhizoma extract was responsible for the reduction of cholesterol levels (Seidlova-Wuttke D., 2012). However, in a clinical study on perimenopausal and postmenopausal women no effects on the level of serum cholesterol were found after twelve months of administration of an ethanolic extract of Cimicifugae racemosae rhizoma (128 mg/d standardized to 7.27 mg triterpene glycosides) (Geller S., 2009).

Additionally, with respect to liver-toxic effects, liver and kidney necrosis related to cell death were induced by exposure to the ethanolic and dichloromethane extract. It was reported that Cimicifugae racemosae rhizoma extracts with a dose of 1,000 mg kg^{-1} (total triterpene content ≥ 6%, about 60 mg kg^{-1} per day of triterpenes) caused liver-toxic effects in rats evidenced by microvesicular steatosis (Lüde S., 2007). A *Cimicifuga* extract applied to rats at a dose of 300 mg kg^{-1} (total triterpene content 2.5%, about 7.5 mg kg^{-1} per day of triterpenes) did not affect liver morphology or liver function indices (Mazzanti G., 2008). The outcome of a study investigating liver-toxic effects indicated the involvement of mitochondrial toxicity. The mitochondrial membrane potential was decreased in HepG2 cells after exposure to a Cimicifugae racemosae rhizoma extract with a total triterpene content about 6 µg/ml (≥ 100 µg/ml, total triterpene content ≥ 6%) (Lüde S., 2007). In the present study, microarray results obtained by IPA indicated that mitochondria and mitochondrial membranes depolarized in HepG2 cells after exposure of the ethanolic (total triterpene content about 12.2 µg/ml) and dichloromethane extract (total triterpene content about 8.8 µg/ml).

The microarray results in this study showed a lot of similar cellular effects for the dichloromethane and ethanolic extract, e. g. responses affecting cholesterol biosynthesis or mitochondrial stress responses. These effects were not observed after exposure to the ethanolic 50% (V/V) extract. A major difference between the ethanolic and dichloromethane extract was the expression of genes related to cytochromes. The gene expression profile of cells exposed to the ethanolic and ethanolic 50% (V/V) extract showed the induction of genes related to the "*Cytochrome P450 Panel-Substrate is an Eicosanoid*". Exclusively for the ethanolic extract the signal cascade "*Cytochrome P450 Panel-Substrate is a Fatty Acid*"

was significantly activated. Thereby, it can be assumed that phytochemical compounds influencing induction of cytochromes were extracted using ethanol. In previous studies it was shown that preparations of Cimicifugae racemosae rhizoma changed the activities of CYP1A2, CYP2B CYP2C9, CYP3A and CYP2D6 (Huang Y., 2010; Yokotani K., 2013). It was demonstrated that the relevant extract inhibited the activity of cytochromes, but it was also shown that pure compounds like cimicifugic acid a and b and especially fukinolic acid inhibited cytochrome activity even stronger (Huang Y., 2010). In the present study the respective cytochromes were not altered at the transcriptional level; however, extracts were prepared with different solvents. The results emphasize the impact of various secondary metabolites on the activity of cytochromes.

Conclusively, the results of the present study demonstrated that the metabolomics approach investigated in this study is an efficient technique to comprehensively investigate the phytochemical fingerprint of herbal extracts and to complement established methods for the characterization of herbal preparations. With regard to the extraction solvents, extracts with differing extract compositions were obtained. At the transcriptional level, several effects were observed in common for extracts with a similar composition of components. But even for these extracts relevant differences were obtained, like a differential induction of genes related to cytochromes. The present study supports the importance of appropriate quality assurance and characterization of herbal preparations. The comprehensive investigation of herbal substances and preparations thereof by metabolomic and transcriptomic analyses are meaningful; the application of these techniques for regulatory affairs has to be examined with further research.

5 Differential transcriptional profiles mediated by exposure to silybin and a preparation derived of Silybi mariani fructus

5.1 Abstract

Objective: Milk thistle is an medicinal plant that originates from the Mediterranean region. Based on traditional use, milk thistle has been used as an herbal remedy, especially for diseases related to the liver and the gallbladder. Several studies suggest that silymarin protects the liver from toxins and that these flavonolignans exhibit anti-oxidative and anti-inflammatory activity.

Materials and Methods: In the study presented, Silybi mariani fructus was extracted (70% (V/V) ethanol) and chemically characterized by means of ^{1}H-NMR analysis and quantification of silymarin with RP-HPLC-DAD. The cell proliferation rate was investigated after exposure to the 70% (V/V) ethanolic Silybi mariani fructus extract and silybin as a pure substance. Gene expression profiling (Whole Genome Gene Expression Microarrays v2, Agilent Technologies) was analyzed for both conditions (5 replicates each).

Results: ^{1}H-NMR analysis suggested that various groups of constituents, such as organic acids, amino acids, glycosides, and aromatic compounds, were present in the Silybi mariani fructus extract. The quantification of silymarin showed a content of 4.8 mg/ml. HepG2 cells were exposed to the extract corresponding to a concentration of 9.6 µg/ml silymarin. Silybin as a chemically-defined substance was applied in a concentration of 9.6 µg/ml. The cell proliferation did not exhibit significant differences in relation to vehicle control. Gene expression profiling revealed different cellular responses. Silybin altered signal cascades related to cell death, proliferation, and development, while the molecular and cellular functions affected by Silybi mariani fructus were related to drug metabolism, cell morphology, and cellular assembly and organization. Common to both conditions, signal cascades associated with lipid metabolism and small molecule biochemistry were activated. Furthermore, microarray data evaluation demonstrated the involvement of liver-toxic pathways common to both conditions, such as liver damage and liver regeneration. However, liver necrosis and liver cholestasis were altered exclusively after exposure to silybin. The exposure of HepG2 cells to Silybi mariani fructus activated the signal cascades liver fibrosis and glutathione depletion, which was not observed for cells exposed to silybin.

The expression of the solute carrier transporters SLC16A6, SLC24A4, and SLC7A11 was changed suggesting a putative role in drug resistance.

Conclusion: Although cell proliferation was basically not affected by silybin or Silybi mariani fructus extract, microarray data evaluation revealed differently activated cellular mechanisms. This emphasizes that cellular responses after application of multicomponent mixtures may differ widely from isolated substances thereof, and the appropriate characterization of herbal extracts is highly important.

Gene abbreviations

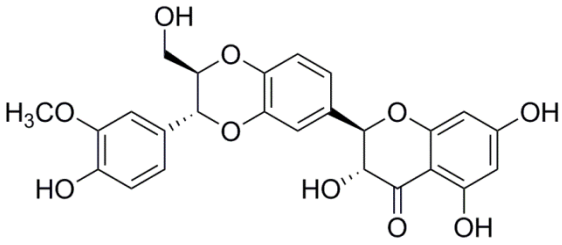
AhRR: Aryl-hydrocarbon receptor repressor; CYP1A1: Cytochrome P450 family 1 member A1; Cyp1B1: Cytochrome P450 family 1 member B1; SLC7A11: Solute Carrier Family 7, Member 11; SLC16A6: Solute Carrier Family 16, Member 6; SLC24A4: Solute Carrier Family 24, Member 4; SOS1: Son of sevenless homolog 1; TGF β: Transforming growth factor β

5.2 Introduction

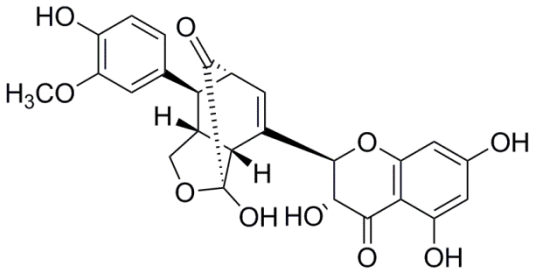
Herbal medicines are frequently used worldwide and their use in Europe is increasing (Jordan S., 2010). People from various nations rely on medicinal plants for their primary health care. In phytotherapy, therapeutic efficacy is based on the combined action of a mixture of constituents. The secondary metabolites produced by the plant act in various ways; the pharmaceutical effects may be exhibited by the synergistic or antagonistic interaction of many phytochemicals. Omics-technologies and systems biology may facilitate elucidating synergistic effects of herbal mixtures.

The mature fruits of *Silybum marianum* (L.) Gaertn. (Asteraceae) and preparations thereof have been marketed for several decades. The constituent described to be responsible for their pharmacological activity is silymarin (Flora K., 1998; van Erp N., 2005; Polyak S., 2010). Silybin, silychristin, silydianin and isosilybin are the main flavonolignan components of silymarin (fig. 19).

Silybin



Silidianin



Silichristin

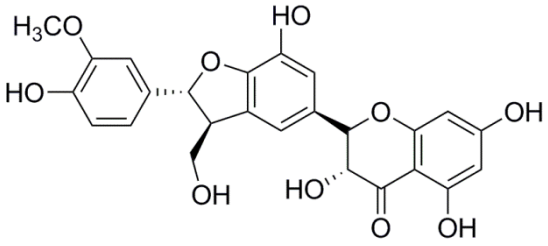


Fig. 19: Structure of typical flavonolignans in Silybi mariani fructus.

Within the fruit of the plant flavonoids such as dihydrokaempferol and taxifolin have been identified as well as sterols (cholesterol, campesterol and stigmasterol) (Abenavoli L., 2010). The content of lipids is high, in a range of 20-30% linoleic acid, oleic acid, and palmic acid have been identified. As further constituents, saponins, amines, sugars, and mucilages have been isolated from Silybi mariani fructus (Abenavoli L., 2010; Wagner H., 1974).

In the European Pharmacopeia 8.0, “Milk Thistle fruit” (01/2014:1860) and the herbal preparation “Milk Thistle dry extract, refined and standardized” (01/2014:2071) are monographed. For the fruit, a content of at least 1.5% silymarin is specified.

Silybi mariani fructus has been traditionally used, and Commission E monograph described its use against toxic liver damage, liver diseases, and chronic inflammatory liver diseases. Furthermore, Silybi mariani fructus has been traditionally used for treatment of the spleen and, gallbladder for ailments such as jaundice and gallbladder colic. In Germany, silybin has been approved for the treatment of *Amanita phalloides* poisoning (Wellington K., 2001).

The potential benefit of Silybi mariani fructus in health care for liver diseases still remains a controversial issue. Regarding safety of Silybi mariani fructus, there are no reports on adverse side effects.

Several reports suggest antioxidant, anticancer, hepato- and nephroprotective activity for Silybi mariani fructus. Silymarin is described as protecting against various chemicals and toxicants like lasalocid, irradiation, carbon tetrachloride, ethanol, and paracetamol (van Pelt J., 2003; Cho B., 2013; Garrido A., 1991; Radko L., 2013). The proposed mechanism of action is associated with the antioxidant function (Valenzuela A, 1986), inhibition of lipid peroxidation and scavenging of free radicals (Gyorgy I., 1992; Mira L., 1994), which may stabilize the membrane. Associated with cellular regeneration, silymarin has been shown to stimulate RNA polymerase I, leading to an increase in the protein synthetic apparatus and a higher turnover of structural and functional proteins (Machicao F., 1977). Furthermore, silymarin has shown protective effects via an increase in total glutathione content and glutathione-related enzymes (Valenzuela A., 1989).

The objective of this study was to evaluate cellular responses after exposure to a complex herbal extract of Silybi mariani fructus and the chemically-defined substance silybin. Contrary to the investigated herbal substances Chelidonii herba and Cimicifugae racemosae rhizoma described in chapter 3 and 4, Silybi mariani fructus is not considered to be liver-toxic. Hence, Silybi mariani fructus represented an interesting model object for evaluation of gene expression analysis. Silybi mariani fructus was extracted with 70% (V/V) ethanol. This solvent is frequently used in the preparation of commercially available preparations containing Silybi mariani fructus. The extract was investigated with ^{1}H-NMR analysis, as it has been shown to be a useful method for comprehensive characterization of complex herbal mixtures (Daniel C., 2008). Additionally, the silymarin content was quantified with RP-HPLC-DAD. For the investigation of cellular effects, HepG2 liver cells were used. Functional tests, including the analyses of the cell proliferation rate and gene expression profiling, were

performed to characterize cellular responses after exposure to the complex Silybi mariani extract and silybin, respectively.

5.3 Materials and Methods

Chemicals and reagents:

Silybin (≥98% [HPLC]), containing both A and B diastereomers, was purchased from Sigma-Aldrich (Hamburg, Germany).

Plant material:

The herbal substance was complying with the monograph European Pharmacopeia 8.0 “Milk Thistle fruit” (01/2014:1860). It was purchased from a local pharmacy and powdered by a mill. The sample material Silybi mariani fructus in analogy to voucher specimens was deposited at the Institute of Pharmaceutical Biology, University of Bonn.

Extraction of Silybi mariani fructus:

The plant material was powdered and 2.0 g were extracted with 20.0 ml of 70% (V/V) ethanol for 20 min. The extract was divided into two equal parts. The solvent was evaporated using a rotary evaporator, respectively. For ^{1}H-NMR measurement and HepG2 cells experiments, the residue was solubilized in 1.0 ml deuterated DMSO-*d6*. For the quantification of silymarin by HPLC, the dried extract was solubilized in 5 ml 50% (V/V) methanol.

Quantification of silymarin in Silybi mariani fructus extract:

Silymarin in the 70% (V/V) ethanolic Silybi mariani fructus extract was analyzed with RP-HPLC-DAD at wavelength 288 nm using DAD. The HPLC system was equipped with an 1100 quaternary pump, 1100 autosampler, 1100 column thermostat, and 1100 diode array detector (all components from Agilent Technologies).

With respect to the quantification of silymarin a validated method described in the European Pharmacopeia was used. Regarding validation based on ICH guideline, linearity, precision of the instrument, precision of the method and limit of detection was determined (International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use, 2005). The determination of linearity indicated R^2=1 after injection of silybin A and B in a range or 219.5 to 10.97 µg/ml. Precision of the instrument showed a relative standard deviation of 0.12% and 0.28% for silybin A and B, respectively.

Precision of the method showed a relative standard deviation of 8.22% after injection of six replicates of 70% (V/V) ethanolic Silybi mariani fructus extract (two injections of the extract). The lower limit of detection was 0.1 µg/ml for silybin A and B. 10µl of the extract used for quantification was injected twice.

Peaks corresponding to silymarin within the chromatogram were identified according to the monograph: “Milk thistle dry extract, refined and standardized” using the reference “milk thistle dry extract HRS” from the EDQM (Strasbourg, France). The chromatogram obtained for the 70% (V/V) ethanolic Silybi mariani fructus did not show major variation in retention times compared to the reference chromatogram from EDQM. Quantification of silymarin was performed with silybin (A+B) standard (Sigma-Aldrich, Hamburg, Germany). The peak identity of silidianin was confirmed with spike experiments with silidianin (PhytoLab GmbH & Co. KG, Vestenbergsgreuth, Germany).

The extracts were filtered via 45 µm membranes. For the separation of silymarin Luna C18 (150x4.6 mm,3 µm) (Phenomenex, Aschaffenburg, Germany), columns with a cartridge (SecurityGuard cartridges for C_{18} HPLC with 3.0 µM inner diameter) were used. The solvents (solvent A: phosphoric acid, methanol, water [0.5:30:70]; solvent B, phosphoric acid, methanol, water [0.5:80:20]) were used in a gradient with a flow of 1.0 ml/min. Separations were effected by a series of linear gradients of solvent B into solvent A as follows: elution starting with 12.0-20.0% B in A, 0-10 min; 20.0-25.0% B in A, 10-15 min; 25-40% B in A, 15-30 min; 40-75% B in A, 30-35 min; 75-100% B in A, 35-40 min;100-12% B in A, 40-25 min; 12% B in A 45-50 min.

^{1}H-Nuclear magnetic resonance (NMR) spectroscopy:

^{1}H-NMR spectra of extracts were recorded with a Bruker advance 300 DPX instrument (temperature 25 °C, 64 scans). Calibration of spectra was performed according to signals of incomplete deuterated solvents (DMSO [2.54 ppm]). Data from δ 0 to δ 10 ppm were considered for the analysis of ^{1}H-NMR fingerprint.

Preparation of total RNA:

Total RNA was isolated from HepG2 cells using RNeasy Mini Kit (Qiagen, Germany) following the manufacturer’s instructions.

Whole genome microarray:

The concentration and purity of extracted RNA was measured using an UV-visible spectrometer (NanoDrop 1000; Thermo Fisher Scientific, Waltham, MA) by absorption at wavelengths of 260 and 280 nm. RNA samples with a 260/280 nm absorption ratio > 1.8 and RIN > 9.5 were used in subsequent microarray analysis. For microarray profiles, fluorescently labeled cRNA samples were prepared from 100 ng RNA using reverse transcriptase. The amplification reaction with simultaneous introduction of Cy3-dCTP to the amplified complementary RNA (cRNA) was performed using a Quick Amp Labeling Kit for One-Color labeling (Agilent Technologies). The concentration of the purified samples and the Cy3 dye incorporation efficiency was evaluated using a NanoDrop 1000 spectrophotometer. After fragmentation (60 °C, 30 min), the single colour cRNA samples were hybridized to a DNA chip (Whole Genome Gene Expression Microarrays v2, Agilent Technologies, 8x60K, 27,958 Gene RNAs, 7,419 lincRNAs) at 65 °C for 17 hours in a hybridization oven (Agilent Technologies). Five independent experiments were performed under each experimental condition.

qRT-PCR:

After reverse transcription by random priming the resulting cDNA was used for qRT-PCR. Following initial denaturation (95 °C, 10 min), amplification was performed over 45 cycles (Light Cycler 480, Roche) with denaturation at 95 °C for 10 s. Primers with the respective annealing temperature are shown in tab. 7. Elongation was performed at 72 °C for 20 s. The size of PCR fragments was analyzed by agarose gel electrophoresis. Gene expression was evaluated with Light Cycler 480 Software 1.5 and Cp-values were normalized to GAPDH.

Tab. 7: Primer used for qRT-PCR for validation of microarray results.

Primer/ Gene name	NCBI accession code	Primer sequence (5´→´3´)	Annealing temperature (°C)
AhRR_1	NM_020731	CAGTTACCTCCGGGTGAAGA	59
AhRR_2	NM_020731	CCAGAGCAAAGCCATTAAGA	59
Cyp1A1_1	NM_000499.3	AACCTTTGAGAAGGGCCACA	55
Cyp1A1_2	NM_000499.3	GACCTGCCAATCACTGTGTC	55
Cyp1B1_1	NM_000104	CACTGCCAACACCTCTGTCTT	53
Cyp1B1_2	NM_000104	CAAGGAGCTCCATGGACTCT	53
GAPDH_1	NM_002046.4	CCACCCATGGCAAATTCCATGGCA	57
GAPDH_2	NM_002046.4	CTAGACGGCAGGTCAGGTCCACC	57
SOS1_1	NM_005633	GAGTGAATCTGCATGTCGGTT	57
SOS1_1	NM_005633	CTCTCATGTTTGGCTCCTACAC	57

Data processing and statistics:

Hybridized DNA chip slides were scanned using an Agilent Scanner (Agilent Technologies) with Feature Extraction Software (Agilent Technologies). Fluorescence intensity data were imported to GeneSpring GX version 12.5 (Agilent Technologies) with the quantile scaling normalization.

Light intensities were filtered to exclude low-quality data to guarantee the accuracy of the statistical analysis. Spots with lower intensities than the threshold, which was determined based on the intensities of the Agilent RNA Spike-Mix, were filtered to exclude spots weaker than background noise. Spots with saturated intensities and near-background intensities were excluded. The final filtration was conducted based on the fold increase. Statistical analysis of the remaining genes was performed with a t-test corrected using the Benjamini and Hochberg false discovery rate (Benjamini Y., 1995). Genes were considered as up- or down-regulated with a fold ≥ 2, ≤ -2, and $p < 0.05$.

These processed data were used for further analysis by Ingenuity Systems Inc. Reedwood City, USA (Qiagen). Ingenuity data base was used as reference set. In the networks interaction 70 molecules and all data sources were integrated in data analysis. Data were compared to the human reference data.

Treatment of HepG2 cells with plant extracts:

HepG2 cells were purchased from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures GmbH and were used between passage 3 and 12. The cells were maintained in T75 flasks in RPMI 1640 medium supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin solution (100 U/ml) and 1% L-glutamine. The cells were constantly incubated in humidified atmosphere at 5% CO_2 and 37 °C.

When the cells were about 80% confluent, they were exposed to trypsin and harvested by centrifugation. Subsequently, they were counted and an equal number of cells (1.0 x 10^6 cells/5 ml media) was transferred to each experimental plate (5 cm Ø). For treatment on the following day medium containing Silybi mariani fructus extract and silybin (9.6 µg/ml), each adjusted to 1% DMSO were applied to the liver cells, respectively. Cells were exposed to the extract in a concentration of 1:501 (9.6 µg/ml silymarin). The vehicle treated cells (1% DMSO) served as control group. Five replicates were performed for each experimental condition for microarray investigations.

Cell growth and proliferation assay using xCELLigence system:

About 7500 HepG2 cells were seeded to each well. After 24 h, medium containing the extract in a concentration of 1:501, or silybin (9.6 µg/ml), each adjusted to 1% DMSO was added, respectively. As solvent control medium containing 1% DMSO was applied. The cell index was monitored every 15 min.

5.4 Results

^{1}H NMR analysis:

The 70% (V/V) ethanolic Silybi mariani fructus extract was subjected to ^{1}H-NMR analysis. The spectrum (fig. 20) showed a signal from water (δ 3 to δ 6 ppm), but this signal did not interfere with the evaluation of important groups of metabolites. The signals near to the signal of water may be attributed to metabolites such as primarily saccharides and amino acids. In the spectrum of the Silybi mariani fructus extract, intense signals in the region of

δ 0.5 to δ 2.5 ppm were assigned. Protons attached to organic acids such as fatty acids resonate in this region. The remaining part (δ 5.5-δ 10.0 ppm) is considered to be the aromatic region, which contained various smaller signals. Protons attached to flavonoids, flavonolignans and phenolics resonate in the region from δ 5.5-δ 7.5 ppm. Several signals were observed in this region. ^{1}H-NMR resonances for alkaloids were expected in the downfield region (δ 7.5 to 10 ppm). Only smaller signals associated with this region were observed.

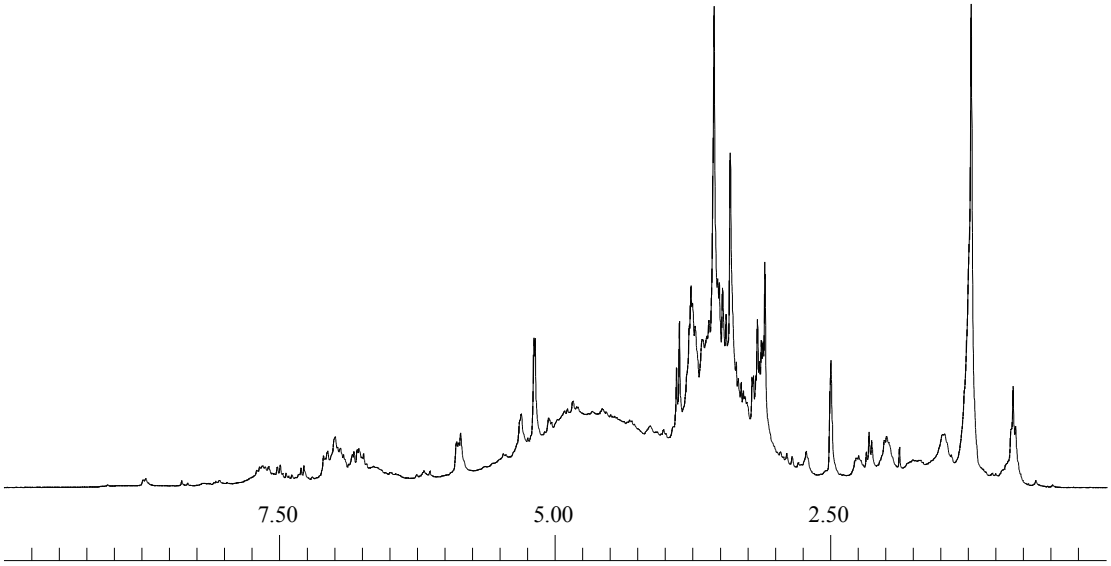


Fig. 20: ^{1}H-NMR spectrum of 70% (V/V) ethanolic Silybi mariani fructus extract. The spectrum was analyzed in DMSO-*d6.*

Quantification of silymarin in Silybi mariani fructus extract:

The content of silymarin was quantified in the extract with RP-HPLC-DAD (288 nm) (tab. 8).

Tab. 8: Quantified silymarin in the 70% (V/V) ethanolic Silybi mariani fructus extract.

	Extract (mg/ml)
Silicristin	0.7
Silidianin	1.5
Silybin A	0.7
Silybin B	1.0
Isosilybin A	0.6
Isosilybin B	0.3
Total silymarin	4.8

In the extract, a total silymarin content of 4.8 mg/ml (9.6 mg/g dry weight) was determined. According to the monograph of the European Pharmacopeia “Milk thistle dry extract, refined and standardized”, the percentage content of silicristin and silidianin was 46%, with reference to total silymarin. The percentage content of silybin A and silybin B was 35%, 19% for isosilybin A and isosilybin B with reference to total silymarin, respectively.

Cell proliferation of HepG2 cells:

Proliferation of HepG2 cells was investigated after exposure to 70% (V/V) ethanolic extract of Silybi mariani fructus (9.6 µg/ml silymarin) and silybin (9.6 µg/ml) as an isolated constituent thereof, each adjusted to 1% DMSO, respectively. The relative cell proliferation of HepG2 cells (fig. 21) was basically not affected due to exposure to the Silybi mariani fructus extract and silybin.

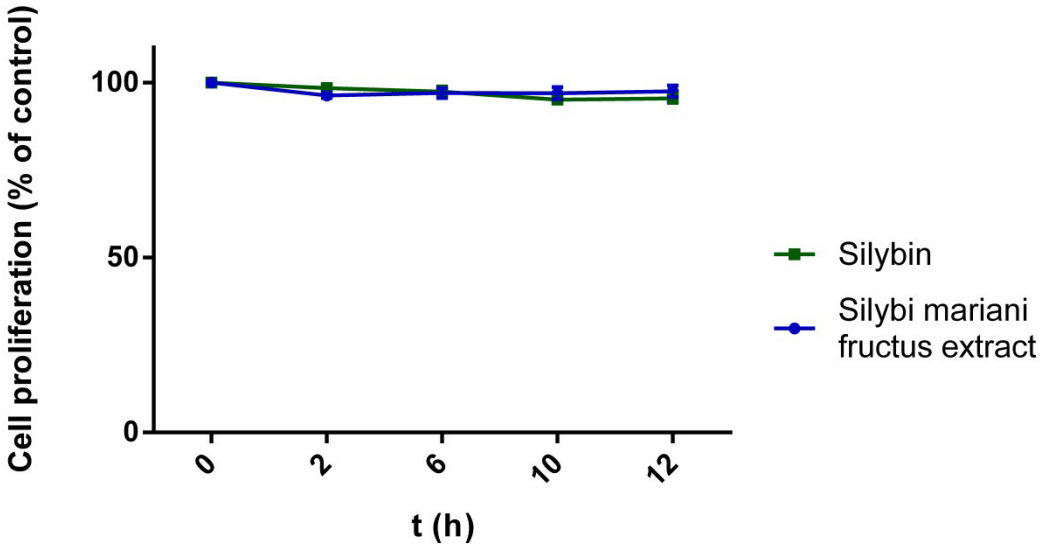


Fig. 21: Cell proliferation of HepG2 liver cells after exposure to 70% (V/V) Silybi mariani fructus extract and silybin, respectively. Cell proliferation was set into relation to the control and treatment start. The Silybi mariani fructus extract (silymarin 9.6 µg/ml) and silybin (9.6 µg/ml) were applied to the cells. Values are expressed as the mean ±SEM of at least 4 to 6 replicates.

Gene expression profiling of HepG2 cells after exposure to 70% (V/V) ethanolic Silybi mariani fructus extract and silybin:

The cellular transcription profile was investigated six hours after exposure to silybin and 70% (V/V) ethanolic Silybi mariani fructus extract. Data evaluation with Genespring 12.5 revealed 148 altered biological entities after exposure to silybin and 161 after exposure to the herbal preparation, respectively.

The significantly regulated genes were further analyzed with IPA to investigate significantly altered signal cascades. The molecular and cellular functions affected exclusively by Silybi mariani fructus extract were "*Drug Metabolism, Cell Morphology, Cellular Assembly, and Organization*". The signal cascades "*Lipid Metabolism"* and *"Small Molecule Biochemistry*" were altered by both Silybi mariani fructus and silybin. The signal cascades "*Cell Death and Survival, Cellular Development and Cellular Growth and Proliferation*" were altered by exposure to silybin.

Signal cascades related to toxicities indicated that different processes had been altered (fig. 22). The exposure to silybin changed pathways involved in the damage of mitochondria ("*Increases Permeability Transition of Mitochondria and Mitochondrial Membrane, Increases Damage of Mitochondria, Increases Depolarization of Mitochondria and Mitochondrial Membrane*") and "*TGF-β-Signaling*". Furthermore, signal cascades such as "*Hepatic Stellate*

Cell Activation" and "*Increases Liver Hepatitis*" were activated by the exposure to silybin. Processes related to biotransformation and drug metabolism were also altered differently. Exposure to silybin induced changes in "*PXR/RXR Activation*" and "*Cytochrome P450 Panel-Substrate is a Vitamin*", while exposure to the Silybi mariani fructus extract induced the signal cascades "*Aryl Hydrocarbon Receptor Signaling, Xenobiotic Metabolism Signaling, Fatty Acid Metabolism, Cytochrome P450 Panel-Substrate is a Sterol"* and *"Cytochrome P450 Panel-Substrate is a Xenobiotic*".

Hepatotoxicity-associated signal cascades were also affected differently by the Silybi mariani fructus extract and the pure compound silybin. The signal cascades "*Liver Necrosis*" and "*Liver Cholestasis*" were exclusively induced after exposure to silybin. The complex extract exclusively changed "*Liver Hyperplasia/Hyperproliferation, Liver Fibrosis*" and "*Glutathione Depletion in Liver*". In common to both conditions, the signal cascades "*Liver Damage, Liver Inflammation/Hepatitis, and Liver Regeneration*" were affected.

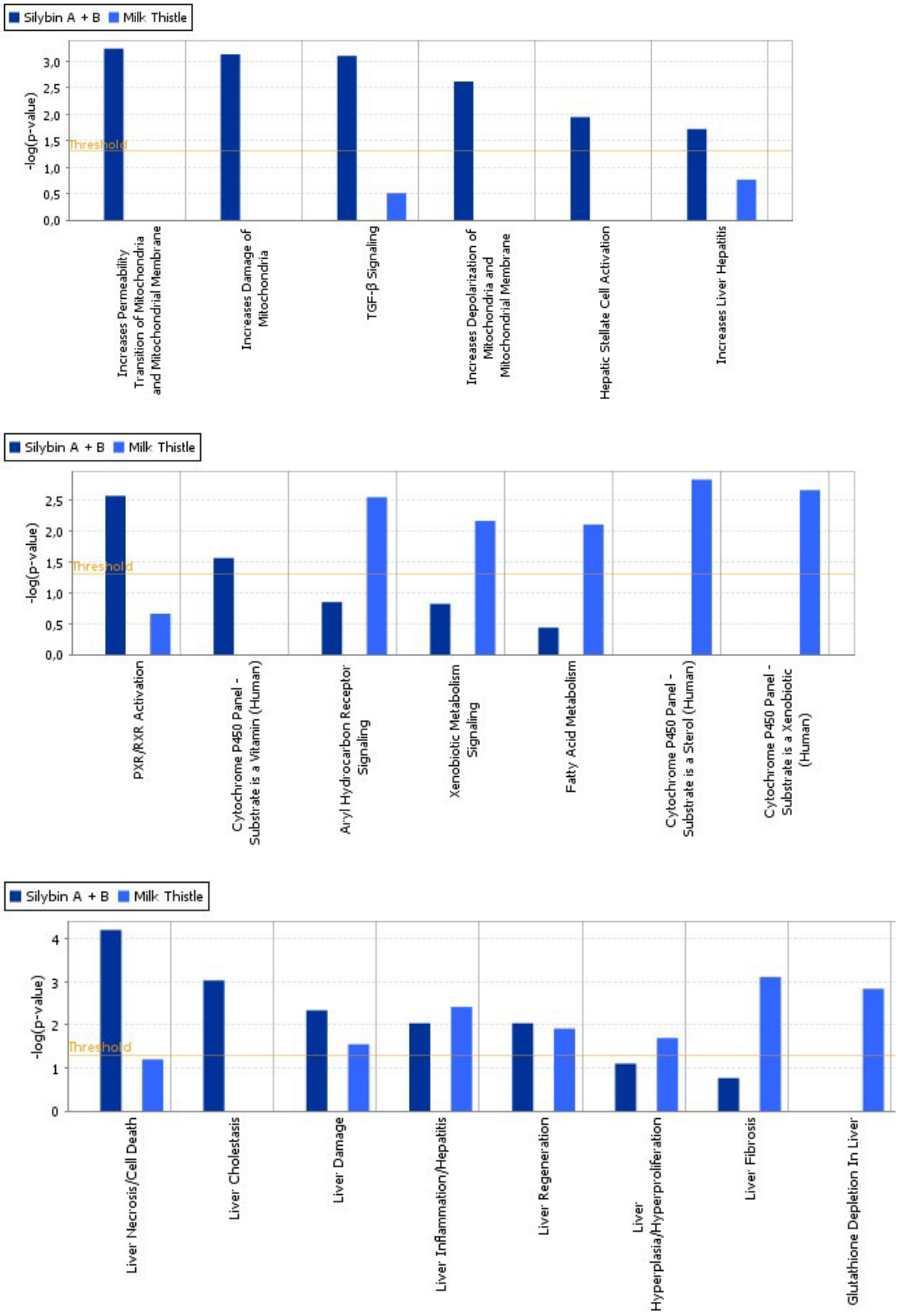


Fig. 22: Signal cascades involved in liver toxicity (upper part), related to metabolism (middle part) and liver-toxic diseases (lower part) in HepG2 cells after exposure to silybin (9.6 µg/ml) and 70% (V/V) ethanolic Silybi mariani fructus extract (9.6 µg/ml silymarin), respectively. The data were obtained with IPA data evaluation. Dark blue color: treatment with silybin; light blue color: treatment with 70% (V/V) ethanolic Silybi mariani fructus extract. Effects were considered statistically significant, if values were exceeding a threshold of p > -log 1.31.

Transcriptional regulation of SLC transporters:

Gene expression of solute carrier transporters was investigated more in detail (tab. 9).

Tab. 9: Relative quantities of solute carrier transporters mRNA expression. Data are shown as a fold change and obtained by microarray analysis. HepG2 cells were exposed to 70% (V/V) Silybi mariani fructus extract (silymarin: 9.6 µg/ml) and silybin (9.6 µg/ml).

	Silybi mariani fructus	Silybin
SLC16A6	4.22	3.44
SLC24A4	-2.95	
SLC7A11		2.84

It was shown that three different transporters were significantly regulated for at least one condition. Solute carrier family 16 member A6 (SLC16A6) was upregulated for both conditions. Solute carrier family 24 member A4 (SLC24A4) was downregulated after exposure to Silybi mariani fructus. Exposure to silybin induced the gene expression of solute carrier family 7 member A4 (SLC7A4).

qRT-PCR:

Data obtained by microarray were verified by qRT-PCR for selected genes (fig. 23). Results of qRT-PCR displayed minor differences in fold changes but confirmed the results obtained by microarray analysis.

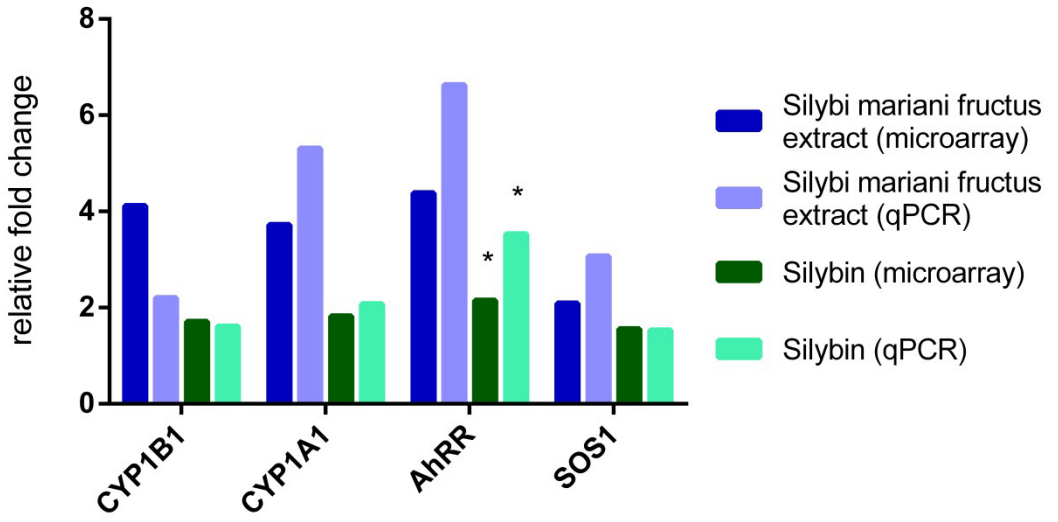


Fig. 23: Fold changes in gene expression measured with microarray and qRT-PCR. Data obtained with qRT-PCR were normalized to GAPDH and set into relation to the solvent control (AhRR: aryl-hydrocarbon receptor repressor, Cyp1A1: Cytochrome P450 family 1 member A1, Cyp1B1: Cytochrome P450 family 1 member B1, SOS1: Son of sevenless homolog 1) * means statistically not significant.

5.5 Discussion

The complexity of the Silybi mariani fructus extracts was demonstrated in the ^{1}H-NMR profile. According to the signals obtained in the ^{1}H-NMR spectrum, it can be suggested that phytochemical constituents like terpenoids, steroids, sugars, flavonoids and flavonolignans were extracted. Moreover, it was demonstrated with RP-HPLC-DAD that silymarin in a concentration of 4.8 mg/ml was obtained in the extract.

Cellular effects of Silybi mariani fructus and silybin were investigated. A corresponding concentration of silymarin and silybin was applied to HepG2 cells, respectively (9.6 µg/ml silymarin in the Silybi mariani fructus extract, 9.6 µg/ml silybin as a pure compound).

Investigations of HepG2 cell proliferation indicated no major antiproliferative effects for either condition. Previous studies have suggested an IC_{50} value of 150 µg/ml after 24 hours of exposure in HepG2 cells (Ashtiani H., 2013; Vakili M., 2012). Since the cell proliferation rate of HepG2 cells exposed to silybin and the Silybi mariani fructus extract did not display major differences, it can be suggested that the extracted constituents, aside from silymarin, did not exhibit antiproliferative effects.

Transcriptomics-based investigations were performed in this study, as it had already been shown that gene expression profiling may be beneficial for the understanding of the underlying mechanisms of phytopharmaceuticals (Ulrich-Merzenich G., 2007).

Associated with toxicities, the impairment of mitochondria in HepG2 cells after exposure to silybin were indicated by microarray experiments. The increase in the mitochondrial-permeability transition is a mechanism that causes mitochondrial failure, which can lead to necrosis from ATP depletion or caspase-dependent apoptosis if ATP depletion does not fully occur (Jaeschke H., 2002; Piret J., 2004).

Furthermore, exposure of HepG2 cells to silybin led to the activation of the transforming growth factor β (TGF-β) signaling, and TGF-β was significantly downregulated under this condition (data not shown). In earlier studies, it had already been demonstrated that silymarin influenced the expression of TGF-β (Jeong D., 2005; Jia J., 2001; Kim S., 2009). TGF-β has various functions and plays a role in growth, differentiation, apoptosis and tissue homeostasis (Massague J., 1998), but it has also been shown that alterations in the TGF-β expression are also involved in hepatoprotective effects (He Q., 2002). Previous studies with mast cells supported that silymarin has shown anti-fibrotic and anti-inflammatory effects associated with the activation of hepatic stellate cells through the regulation of TGF-β (Jeong D.H., 2005). Hepatic stellate cells may be activated by hepatotoxins thus eventually leading to liver fibrosis.

One of the major findings of microarray analysis was the differential expression of biotransformation enzymes upon exposure of HepG2 cells to the Silybi mariani fructus extract and silybin. In this study, the aryl hydrocarbon receptor (AhR) was involved in signal cascades upon exposure to Silybi mariani fructus extract. AhR is a transcription factor that plays a role in the transcriptional regulation of several key enzymes involved in the metabolism of xenobiotic substances. This transcription factor can be activated by a wide range of herbal compounds, such as flavonoids, alkaloids, phenols and catechins (Denison M., 2003). Subsequently, from the results obtained in the present study, it can be suggested that mRNA expression of the cytochrome P450s CYP1A1 and CYP1B1 may be regulated by AhR. Despite the role in the biotransformation of AhR, it further functions in many cellular processes, like cell growth, apoptosis, cell adhesion, immune responses and cell matrix metabolism (Puga A., 2009; Vogel C., 2014).

Previous investigations have indicated that silymarin has inhibited the activity of cytochrome P450 CYPA1, CYP2E1 and CYP2D6 (Dvorak Z., 2006a; Kiruthiga P., 2013; Zuber R., 2002). In this regard, the induction of Cyp1A1 was obtained for HepG2 cells exposed to Silybi mariani fructus extract based on the transcriptional level.

Interestingly, exposure to silybin as a chemically-defined substance activated the pregnane X receptor/retinoid X receptor (PXR/RXR) signal cascade, which was not achieved after exposure to Silybi mariani fructus extract. PXR in conjunction with RXR display receptors with a central role in drug metabolism. Various phytochemicals and clinical drugs are metabolized by the cytochrome P450 CYP3A4, which is inducible with PXR/RXR heterodimer receptors. Additional important roles of PXR/RXR are associated with drug efflux pumps, lipid metabolism and the excretion synthesis and metabolism of bile acid (Moreau A., 2008; Schuetz E., 2001; Teng S., 2003). Thus, various important physiological and protective functions are assignable to PXR/RXR. Interestingly, PXR/RXR was shown to be involved in the expression of the organic anion transporting polypeptide 2 (Oatp2) (Staudinger J., 2001), which is suggested to be involved in the antidote action of silymarin after *Amanita phalloides* poisoning (Fehrenbach T., 2003). A possible role of silybin for the PXR and the induction of cytochrome P450 CYP3A4 has been described previously (Dvorak Z., 2006a; Xie Y., 2013), supporting the PXR response obtained in the current study.

The biotransformation of silybin and Silybi mariani fructus indicated that the multicomponent mixture activated more and different genes associated with xenobiotic metabolism. According to the results of cytochrome P450s CYP1A1 and CYP1B1, it can be concluded that the induction is primarily not received by silybin, but rather by other secondary metabolites of the Silybi mariani fructus extract.

In Germany, silybin is approved for the treatment of *Amanita phalloides* poisoning. The meta-analysis of clinical data has encouraged the supportive therapy of silymarin for this indication (Saller R., 2008). For the toxin blockade of silymarin, it has been suggested that SLC transporters (SLC21A6) may be involved (Fehrenbach T., 2003). However, results described in previous studies have indicated that SCL21A6 is not present in HepG2 cells (Cui Y., 2003). With regard to this finding, no alterations were shown for SLC21A6 on the transcriptional level in this study. Therefore, the expression of other SLC transporters in HepG2 cells exposed to the Silybi mariani fructus extract and silybin was investigated in the present study. The results obtained in the current study indicate the regulation of SLC16A6, SLC24A4 and SLC7A11. Previous studies have indicated a putative role of SLC16A6 and SLC7A11 in drug resistance (Januchowski R., 2013). Since ABC transporters may also play a role in this phenomenon, the microarray results of this study were screened for ABC transporter expression but showed no significant gene expression.

The transferability of results obtained from *in vitro* experiments to *in vivo* experiments is challenging. The relevance of data obtained from *in vitro* experiments under controlled conditions for clinical practice has to be considered carefully. Results obtained with *in vitro* experiments may be beneficial for the first prediction of biotransformation and possible toxic

effects. Discrepancies between *in vitro* results and clinical studies have been observed, thus, the clinical relevance should always be proven in clinical trials (Goey A., 2013). Several reports describe the low bioavailability of silymarin; hence, it is difficult to estimate and assess the human-relevant concentration in plasma.

Gene expression profiling and systems biology may facilitate the unravelling of the cellular and putative synergistic effects of herbal mixtures.

6 Interlaboratory comparison of microarray gene expression analyses of HepG2 cells exposed to different Chelidonii herba extracts

6.1 Abstract

Background: DNA/RNA microarray technologies are frequently applied to investigate transcriptional changes. The objective of the present study was the analysis of reproducibility and affected signal cascades of different Agilent microarray platforms performed in different laboratories. The herbal substance Chelidonii herba was used as a model.

Materials and Methods: Identical samples, extracted in the same laboratory (BfArM) were used for microarray analysis. The labeling and hybridization procedure was performed according to a standardized procedure. In total, four replicates were used for each experimental condition and the vehicle control, respectively. Two different microarray platforms (SurePrint G3 Human Gene Expression 8x60K Microarray Kit [Miltenyi Biotec] and SurePrint G3 Human Gene Expression 8x60K v2 Microarray Kit [BfArM]) were used. The data were evaluated using Genespring 12.5 software (Agilent Technologies).

Results: In total, 30,746 biological features were common for both microarray platforms. After exposure to a dichloromethane and an ethanolic Chelidonii herba extract, 150 and 211 genes were significantly regulated represented for both microarray platforms, respectively. The gene expression profile revealed 108 and 35 significantly regulated genes in common for the ethanolic 50% (V/V) and the aqueous extract, respectively. Additionally, microarray analysis demonstrated regulated genes only for one microarray platform although they were represented for both microarray platforms. At least 64.4-86% thereof showed fold changes ≤ 3 and ≥ -3. The comparison of common significantly regulated genes indicated less variability. Microarray data showed a quite good correlation of affected signal cascades.

Discussion and Conclusion: The reproducibility of microarray is reasonable, especially for genes with fold changes ≥ 3 and ≤ -3. Because of a good correlation of affected signal cascades, it can be suggested that results obtained by microarray analysis can be applied for the comprehensive investigation of gene expression profiling. As some controversial gene regulations were observed, genes of special interest should be validated by qRT-PCR. Nevertheless, differences in the regulation of genes cannot be excluded.

6.2 Introduction

Since the 1990s microarrays have emerged and their usage has continuously been enhanced. For DNA/RNA microarrays various applications are known, such as the analysis of gene expression (Afshari C., 2011; Brazhnik P., 2002; de la Fuente A., 2002; Fedorov A., 2014; te Pas M., 2013; van Dyk E., 2014) or the detection of single nucleotide polymorphisms (SNPs) (Nguyen T., 2013). Protein Chips are used to reveal interactions or activities of proteins or to determine protein functions (Melton L., 2004). A specific form of a protein microarray is an antibody microarray used for diagnostic applications or to detect protein expressions (Rho J., 2013). Small-molecule microarrays have risen to areas ranging from protein profiling to the discovery of therapeutic leads (Uttamchandani M., 2005).

Currently, different microarray platforms from different companies are commercially available. It is known that interlaboratory and interplatform differences may contribute to variability in evaluation of microarray data. Various researchers investigated the comparability of data obtained by the application of different platforms. Although variability in the microarray data obtained with different platforms and laboratories occurred, standardized protocols and data evaluation strategies reduced variability (Dobbin K., 2005; Dumur C., 2008).

The objective of the present study was an interlaboratory comparison with very similar platforms. RNA samples generated in the same laboratory were analyzed by different Agilent whole genome microarrays by different experimenters in our laboratory and at Miltenyi Biotec. Miltenyi Biotec is a company which commercially provided microarray services. Data from both laboratories were statistically evaluated using the same strategy.

6.3 Materials and Methods

Cell culture and exposure to the different Chelidonii herba extracts:

HepG2 cells were cultivated as described before (chapter 3.3).

RNA extraction:

The isolation of total RNA was performed as described before (chapter 3.3).

Microarray labelling and hybridization:

Identical RNA samples were used for the comparison of the different microarray experiments. Labeling and subsequent hybridization of microarrays were performed consistently in the laboratories of BfArM and Miltenyi Biotec.

In both laboratories the quality of RNA was examined by the RIN-values (Agilent 2100 Bioanalyzer). The concentration and purity of extracted RNA was measured using an UV-visible spectrometer (NanoDrop 1000; Thermo Fisher Scientific, Waltham, MA) by absorption at wavelengths of 260 and 280 nm. RNA samples with a 260/280 nm absorption ratio > 1.8 and RIN > 9.5 were used in a subsequent microarray analysis. Microarray experiments were performed with 100 ng of total RNA and cRNA was generated using reverse transcriptase. In both laboratories one-colour labeling was performed with Cy3-dCTP during the amplification reaction. The Cy3 dye incorporation efficiency was evaluated using a NanoDrop 1000 spectrophotometer and showed efficiencies of ≥17 fmol/ng.

After fragmentation (60 °C, 30 min in BfArM and 65 °C, 30 min at Miltenyi Biotec), the single colour cRNA samples were hybridized to a DNA chip (Whole Genome Gene Expression Microarrays v2, Agilent Technologies, 8x60K in BfArM and Whole Genome Gene Expression Microarrays, Agilent Technologies, 8x60K at Miltenyi Biotec) at 65 °C for 17 hours in a hybridization oven (Agilent Technologies). Four independent experiments were performed under each experimental condition.

Different microarray platforms were used for the microarray experiments (tab. 10) and the samples were labeled by the same method. The biological features investigated by the different microarray platforms differed. SurePrint G3 Human Gene Expression 8x60K v2 Microarray Kit a recently developed microarray from Agilent Technologies showed an update of mRNA and long intergenic non-coding RNA (lincRNA) probes from the Broad Institute (www.genomics.agilent.com/article.jsp?crumbAction=push&pageId=1516/; 9.2.2014).

Tab. 10: Details and specifications of microarrays used for interlaboratory comparison in our laboratory and at Miltenyi Biotec.

	Miltenyi	Own laboratory
Labeling	SurePrint G3 Human Gene Expression 8x60K Microarray Kit	SurePrint G3 Human Gene Expression 8x60K v2 Microarray Kit
Format	8 x 60 K	8 x 60 K
Manufacturing	Agilent 60-mer SurePrint technology	Agilent 60-mer SurePrint technology
Biological features	Target 27,958 Entrez Gene RNAs 7,419 lincRNAs	50,599
Positive controls	96 x 10 ERCC control probes 10 x 32 E1A spike-in control probes	96 x 10 ERCC control probes 10 x 32 E1A spike-in control probes
Composition	Design based on: • RefSeq Build 36.3 • Ensemble Release 52 • Unigene Build 216 • GenBank (April 2009) • Plus: novel content for lincRNAs (long intergenic noncoding RNAs)	Design based on: • RefSeq Build 50 • Ensemble Release 52 • Unigene Build 216 • GenBank (April 2009) • Broad Institute Human lincRNA catalog (Nov 2011) • Broad Institute TUCP transcripts catalog (Nov 2011)

Microarray data analysis:

Hybridized DNA chip slides were scanned using an Agilent Scanner (Agilent Technologies) with Feature Extraction Software (Agilent Technologies). Fluorescence intensity data from both laboratories were imported into GeneSpring GX version 12.5 (Agilent Technologies) and normalized using quantile scaling. In a first step serving quality control, data were filtered to exclude low-quality data. Biological features with lower intensities than the threshold and biological features with saturated intensities were excluded. Finally, biological features with at least twofold regulation were used for the statistical data analysis. The statistical analysis was performed with a *t*-test corrected using the Benjamini and Hochberg false discovery rate (Benjamini Y., 1995). Genes were considered as significantly altered with an at least twofold regulation and $p < 0.05$.

6.4 Results

RNA quality and labeling results:

RIN values in both laboratories indicated RNA of good quality (RIN ≥ 9.5). Cy-3 dye incorporation efficiency was higher in our laboratory than at Miltenyi Biotech. According to the recommendations from Agilent Technologies, both Cy-3 incorporation rates were adequate for microarray hybridization.

Interplatform correlations:

A common gene annotation (Entrez Gene ID) was used to match the gene identities represented for both microarrays for the comparison of the different platforms. In total, 30,746 biological features were common for both microarray platforms (fig. 24).

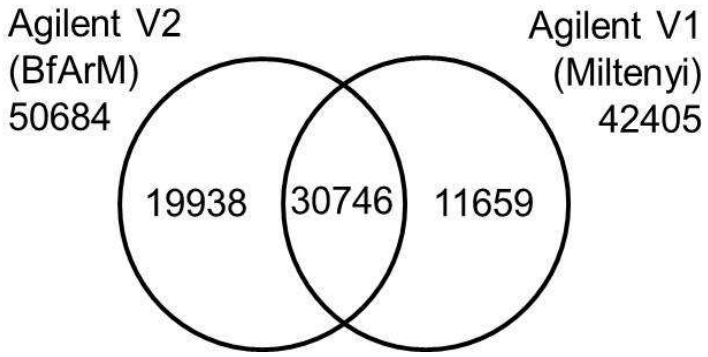


Fig. 24: The number of Entrez Gene identifiers represented by both Agilent microarray platforms. The Venn diagram shows that 30,746 biological features are represented by both platforms.

The statistical data evaluation revealed a different number of differently regulated biological features after exposure to the different Chelidonii herba extracts, respectively (fig. 25). As expected, a large proportion of the biological features was unaltered after exposure of HepG2 to the different Chelidonii herba extracts for both types of microarray platforms.

Most regulated biological features for both array platforms were observed for gene expression profiles of the ethanolic extract (fig. 25). Microarray experiments with the dichloromethane extract indicated 150 significantly regulated genes for both array platforms. The gene expression profiles obtained after exposure to the ethanolic 50% (V/V) extract revealed 108 commonly regulated genes while less commonly regulated genes were shown for the aqueous extract of Chelidonii herba.

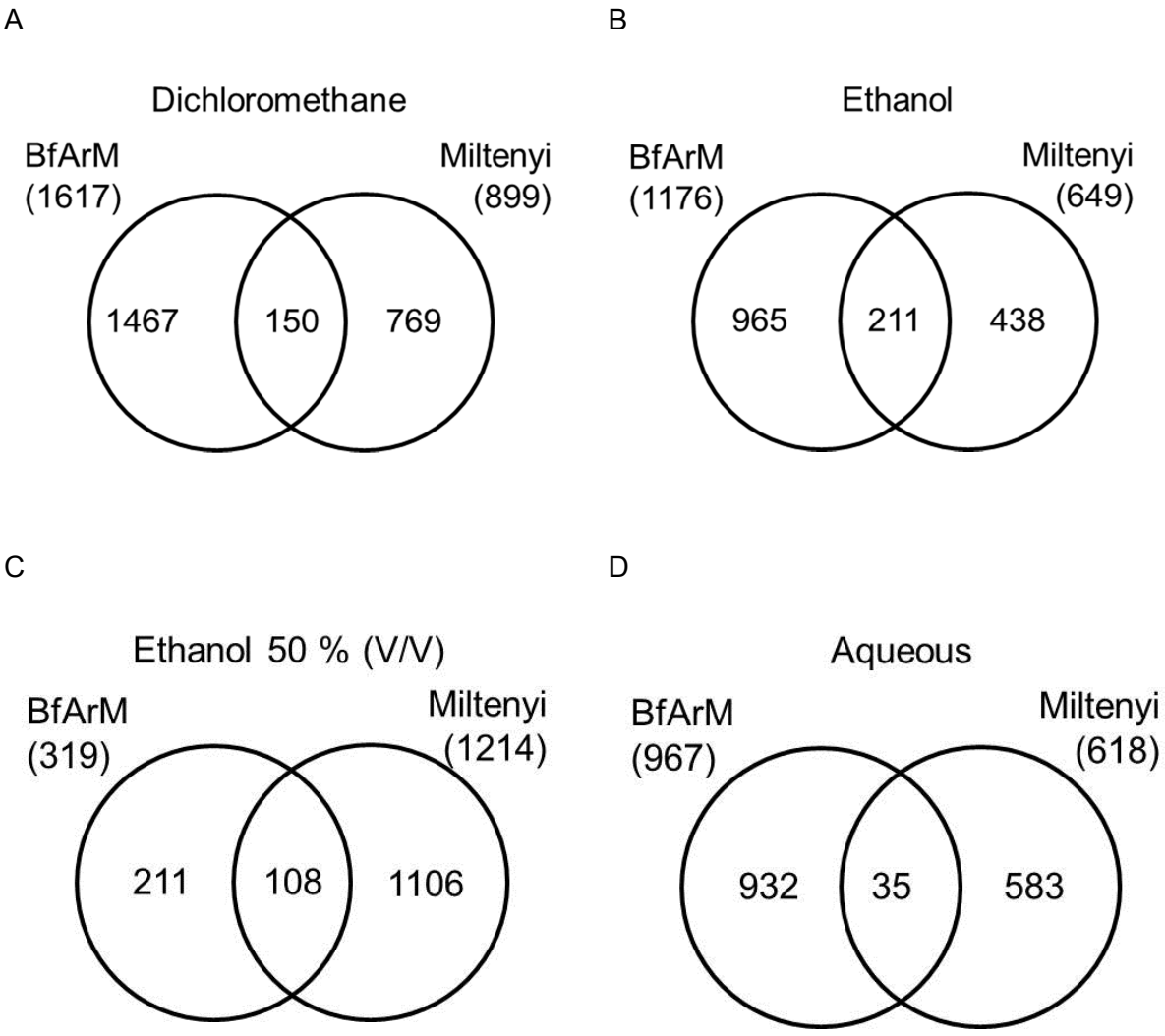


Fig. 25: Overlap of significantly (p< 0.01, fold change ≥ 2 and ≤ -2) affected genes by the different Chelidonii herba extracts, identified by the different Agilent platforms. Experiments with the dichloromethane (A), ethanol (B), ethanol 50% (V/V) and aqueous (D) Chelidonii herba extracts were compared.

Several genes represented for both microarray platforms were shown to be significantly regulated just by one microarray platform. A more detailed analysis of regulated genes just by one microarray platform revealed that most of them were regulated less than threefold (tab. 11).

Tab. 11: Percentage of regulated genes (fold change ≤ 3 and ≥ -3, p ≤ 0.01) represented for both microarray platforms.

Experimental condition	BfArM (%)	Miltenyi (%)
Dichloromethane extract	70.3	64.4
Ethanolic extract	86.0	68.7
Ethanolic 50% (V/V) extract	84.4	69.0
Aqueous extract	83.0	60.0

Technical reproducibility:

In the present study the experimental reproducibility was accessed by those genes that were represented for both microarray platforms. Several genes were regulated in common on both microarray platforms (tab. 12). The analysis of the gene expression data demonstrated a few genes that were differently regulated on the two microarray platforms. For exposure of HepG2 cells to the dichloromethane extract 15 genes (10.0%) were identified that were controversially regulated while for exposure to the ethanolic extract 17 (8.1%) of the commonly regulated genes were differentially regulated. Exposure to the ethanolic 50% (V/V) and the aqueous extract demonstrated four (3.7%) and eight (22.9%) controversially regulated genes, respectively. The correlation of differentially regulated genes with an at least threefold regulation indicated higher correlation results. After exposure to the dichloromethane extract, eight genes (5.3%) and for the ethanolic extract five genes (2.4%) were differentially regulated. After exposure to the ethanolic 50% (V/V) and the aqueous extracts two genes (1.9% and 5.7%, respectively) were differently regulated.

Tab. 12: Correlation between the expression data of technical replicates analyzed by different microarray platforms.

Experimental condition	Commonly regulated genes	Differently regulated genes
Dichloromethane extract	135	15
Ethanolic extract	194	17
Ethanolic 50% (V/V) extract	104	4
Aqueous extract	27	8

Affected biological pathways:

To identify affected biological pathways across both microarray platforms Genespring 12.5 software (Agilent Technologies) was used. The analysis of significantly affected pathways and signal cascades was performed with significantly regulated genes common to both microarray platforms.

The highest number of regulated pathways was obtained for exposure to the ethanolic Chelidonii herba extract (fig. 26), 25 signal cascades being commonly regulated. After exposure to the aqueous Chelidonii herba extracts 13 commonly regulated biological pathways were observed. The exposure of the dichloromethane and the ethanolic 50% (V/V) extracts induced ten and eleven biological pathways in common, respectively.

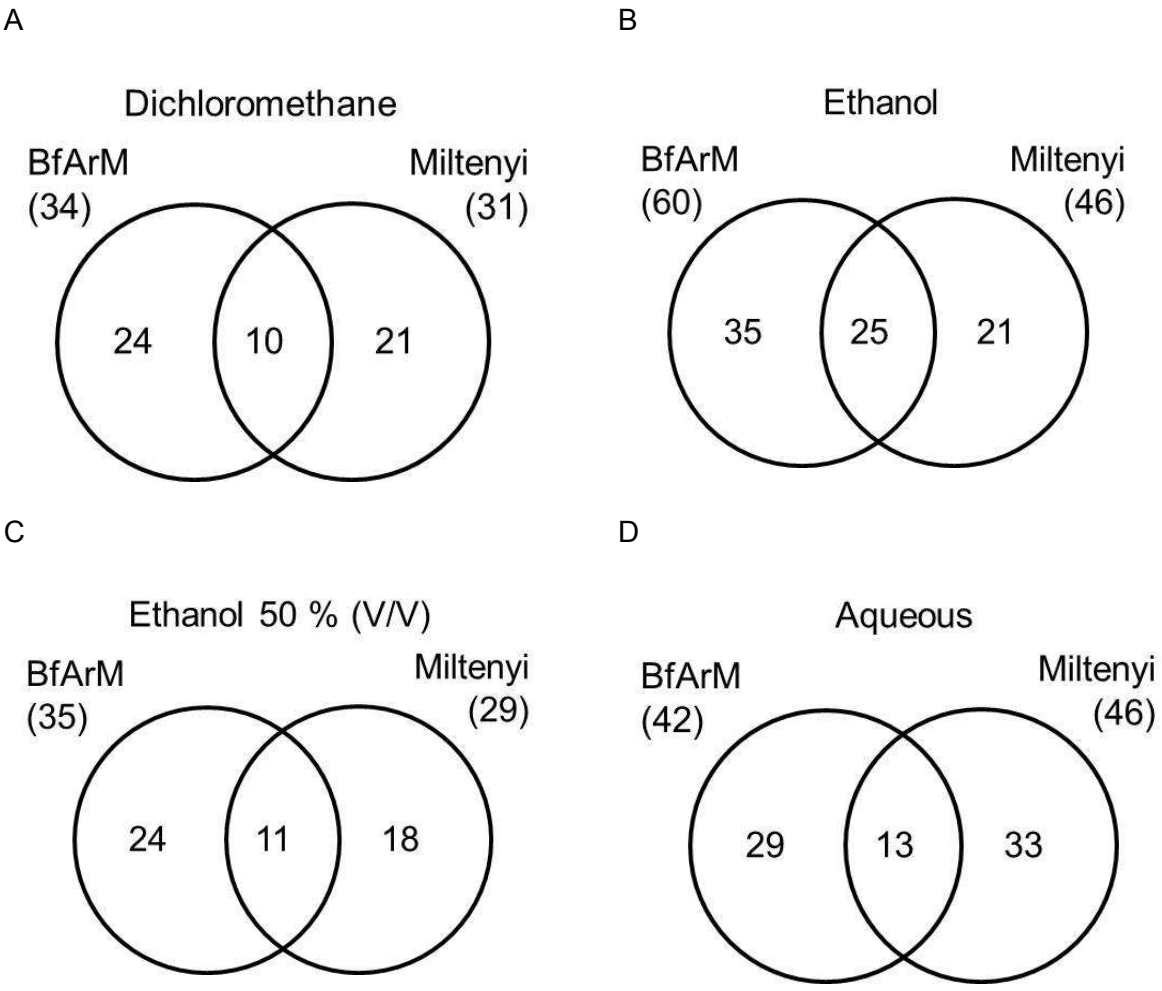


Fig. 26: Venn diagrams showing the overlap of biological processes significantly affected ($p<0.005$) by the different Chelidonii herba extracts, identified by the different Agilent platforms. Comparisons were made for experiments with the dichloromethane (A), ethanol (B), ethanol 50% (V/V) and aqueous (D) Chelidonii herba extracts.

6.5 Discussion

Because of increasing application of microarray technology to analyze the whole transcriptome there is necessary to assess the comparability of microarray data obtained in different laboratories or by different platforms. In the present study, identical RNA samples were investigated to analyze the reproducibility and biological significance. Hence, biological variation can nearly be excluded to be responsible for variation. Only the period of storage has to be considered as a parameter for the biological variation. In previous studies, it was shown that prolonged storage may influence results obtained by microarray results (Hatzis C., 2011).

The correlation of genes obtained in the present study, represented for both microarray platforms, indicated that at least 77% of genes showed the same direction of fold change. A more detailed analysis revealed that mostly genes with lower expression levels (threefold regulation) were responsible for the variability. Previous studies already indicated higher variance for small fold changes (Baum M., 2003; Dobbin K., 2005).

In the literature there are controversial reports on the assessment of reproducibility of interplatform and interlaboratory experiments. A previous study that compared interplatform and interlaboratory differences showed that laboratory effects had a larger impact on the precision of microarray data than interplatform effects (Irizarry R., 2005). In contrast, the MicroArray Quality Control (MAQC) project showed a good concordance of interplatform data (Guo L., 2006). Recently, gene expression profiles investigated with microarray techniques and RNA sequencing methods, different in terms of technology, showed good concordance (Arino J., 2013; Guo Y., 2013), thus indicating the advancements in microarray technology.

In conclusion, it was shown that variability in the gene expression profiles depend on different laboratories, experimenters and microarray platforms. Fold changes in mRNA expression were relatively low. However, genes altered threefold indicated higher congruency. The comparison of data may lead to the suggestion that genes with higher fold changes should be received for systems biological data evaluation. However, current microarray studies frequently use statistically significant and twofold regulated genes for further analysis.

7 Authentication of different *Artemisia* sp. by complementary methods

7.1 Abstract

Background: For quality control and safety of medicinal plants it is indispensable to identify and authenticate herbal substances. In this study, multidisciplinary methods were investigated for their applicability in complementary quality assessment of herbal substances. As a model, different *Artemisia* sp. (Artemisiae annuae herba, Artemisiae scopariae herba, Artemisiae argyi folium) were analyzed, which are also used in traditional Chinese medicine (TCM). Furthermore, *A. scoparia* Waldst. & Kit. samples from different provenances were examined. ^{1}H-NMR analysis together with principal component analysis was applied and PCR-based methods were used to identify herbal substances.

Using the metabolomics-based approach, different *Artemisia* sp. and *Artemisia scoparia* samples from different provenances were extracted using dichloromethane in triplicates. ^{1}H-NMR fingerprints were recorded and evaluated by PCA. Using a PCR-based approach, the internal transcribed spacer region (ITS) was analyzed. Additionally, for *A. scoparia* the external transcribed spacer region (ETS) was used and investigated using NCBI BLAST database.

Results: Clustering results were obtained for the different *Artemisia* sp. by ^{1}H-NMR fingerprint and PCA. Using the PCR-based approach with ITS primers, according to NCBI BLAST, *A. annua* and *A. argyi* were identified. Furthermore, by PCA *A. scoparia* samples from different geographical origins were discriminated. By the analyses of both, ITS and ETS sequence data, *Artemisia scoparia* samples were unambiguously identified.

Conclusion: Investigations of *Artemisia* sp. have illustrated that ^{1}H-NMR analysis in combination with PCA are useful in the characterization of herbal substances and their preparations. Identification of herbal substances by analysis of DNA marker regions presents a considerable potential for identification of herbal substances complementary to macroscopic, microscopic and chromatographic fingerprinting methods. To unambiguously identify herbal substances at least two marker regions should be used.

7.2 Introduction

In Europe there is an increasing trade with herbal substances and products derived thereof traditionally used in China and India (Patwardhan B., 2005). The study of macroscopic and microscopic characters of the herbal substances is not always sufficient and the distinction of taxonomically closely related herbal substances can be challenging. Recent progress based on metabolomic and molecular approaches offers a set of powerful methods investigated in the present study.

Beside mass-spectrometric methods, ^{1}H-NMR spectroscopy is an approach to characterize the metabolome. By ^{1}H-NMR spectroscopy it is possible to detect all proton-bearing compounds within one sample. For plant extracts most “organic” compounds are covered: carbohydrates, amino acids, fatty acids, amines, ethers, esters and lipids (Ward J., 2003). Other approaches used to identify herbal substances are molecular methods. In terms of quality control, molecular methods may complete analytical testing, but usually they do not replace methods like TLC or HPLC. Most of the molecular approaches are PCR-based and often include sequencing of DNA of marker regions.

The genus *Artemisia* comprises about 350 to 500 species (Riggins C., 2012). In the Chinese Pharmacopoeia 2010, four *Artemisia* sp. are monographed: Artemisiae argyi folium (*Artemisia argyi* Levl. et Vant. [Aiye]), Artemisiae scopariae herba (*A. scoparia* Waldst. & Kit. and *Artemisia capillaris* Thunb. [Yinchen]) and Artemisiae annuae herba (*Artemisia annua* L. [Qinghao]).

Recent phytochemical research on *A. scoparia* resulted in the detection of different secondary metabolites, including essential oil constituents like the monoterpene hydrocarbons α-myrcene, *p*-cumene, geranyl acetate and acetylisoeugenol, furthermore sequiterpenes, such as caryophyllene oxide and aromatic compounds (phenylmethanal and capillin) (Cha J., 2005; Sharopov F., 2011; Singh H., 2010). Additionally, flavones and flavonoids like eupatolitin glycosides, quercetin, scoparon, and scopoletin have been isolated (Chandrasekharan I., 1981).

A. annua is an herb from which the sesquiterpene lactone artemisinin was isolated, which is an antiplasmodial compound (White N., 1997). Further constituents identified in Artemisiae annuae herba are artemisia ketone, α-pinene, and 1,8-cineole (Radulovic N., 2013). Furthermore, some flavone compounds like artemetin (Elford B., 1987; Weathers P., 2012), scopoletin (Melillo de M., 2012), and scopoline (van der Kooy F., 2013) were identified for this herbal substance.

A. argyi contains flavones like quercetin (Tan R., 1992), scopoletin and isoscopoletin (Adams M., 2006), a number of essential oils like borneol (Li N., 2008), sesquiterpene ketones (Yoshikawa M., 1996) and the monoterpenes α-myrcene, α-pinene, and limonene (Li N., 2008).

The aim of this study was to evaluate the potential of complementary methods to identify and authenticate herbal substances. As a model, different *Artemisia* sp. that are monographed in the Chinese Pharmacopoeia 2010 (Artemisiae argyi folium, Artemisiae scopariae herba and Artemisiae annuae herba) were analyzed. In addition, *A. scoparia* samples from different provenances were investigated (fig. 27). Regarding the cultivation area, the phenotype from the different samples widely differed.

A B

Fig. 27: *Artemisia scoparia* from Chinese import (A) and cultivated in Germany (B), adopted from (Scherübl R., 2012).

With an analytical approach, a metabolomic fingerprint of different *Artemisia* sp. with ^{1}H-NMR analysis combined with PCA was investigated. The potential to identify herbal substances by marker sequences was investigated by analysis of the ITS. Moreover, *A. scoparia* samples from different geographical origins were additionally investigated by the analysis of ETS.

7.3 Materials and Methods

Herbal substance material:

Artemisia sp.*:*

For the investigation of the metabolome and ITS Artemisiae scopariae herba, Artemisiae argyi folium and Artemisiae annuae herba were obtained from different pharmacies and suppliers (tab. 13).

Tab. 13: Herbal substances and herbal medicinal product and sources used in this study sing PCR-based approach and metabolomics based method.

Herbal substance	Source
Artemisiae annuae herba	Caesar & Loretz GmbH (Hilden, Germany)
	Complemedis AG (Schönenwerd, Switzerland)
	(Granulate)
	Kaiser pharmacy (Bonn, Germany)
Artemisiae argyi folium	Complemedis AG (Schönenwerd, Switzerland)
	Kaiser pharmacy (Bonn, Germany)
Artemisiae scopariae herba	Complemedis AG (Schönenwerd, Switzerland)
	Galke (Bad Grund, Germany)
	Kaiser pharmacy (Bonn, Germany)

Additionally, further herbal substances were investigated using ^{1}H-NMR analysis in combination with PCA (tab. 14). The herbal substance Artemisiae annuae herba from Complemedis AG (tab. 13) was excluded as it was a granulated herbal medicinal product.

Tab. 14 Herbal substances used in this study using the metabolomics-based method.

Herbal substance	Source
Artemisiae annuae herba	Herba Sinica Hilsdorf GmbH (Rednitzhembach, Germany) PharmaChin GmbH (Berlin, Germany) Pharmacy (Singapore) SinoPhytoMed GmbH (Waldsassen, Germany)
Artemisiae argyi folium	Caesar & Loretz GmbH (Hilden, Germany)
Artemisiae scopariae herba	Herba Sinica Hilsdorf GmbH (Rednitzhembach, Germany) SinoPhytoMed GmbH (Waldsassen, Germany)

Artemisia scoparia from different provenances:

In total, 18 *A. scoparia* samples from different provenances were investigated in this thesis (tab. 15). Eight of the plants were cultivated in Germany (Steinach [Straubing] and Forstwiesen [Ingolstadt]). Plant material was harvested in spring at a height of 60-80 cm, with the exception of one flowering sample collected in autumn. The plant was identified by DNA sequencing (Heuberger H., 2010).

In China, ten herbal samples were cultivated investigated in this thesis. The aerial parts of *A. scoparia* from China were purchased from the suppliers Arobemed, Lian, Pharmachin, Phytax and one sample was provided by the Heilongjiang University of Chinese Medicine, Harbin China. Three more samples were obtained from the manufacturer Yong Quan and imported from Chengdu and Shanghai.

Tab. 15 Artemisiae scopariae herba from China and from Germany investigated in the present study.

Provenance	Source
Germany	Lfd 14
	Lfd 15
	Lfd 16
	Lfd 17
	Lfd 18
	Lfd 19
	Lfd 20
	Lfd 21
China	Arobemed (Emmendingen, Germany)
	Hangzhou (local pharmacy)
	Herba Sinica Hilsdorf GmbH (Rednitzhembach, Germany)
	Lian (Wollerau, Switzerland)
	PharmaChin GmbH (Berlin, Germany)
	Phytax (Schlieren, Switzerland)
	Yong Quan (Shanghai 2001) (Ennepetal, Germany)
	Yong Quan (Shanghai 2005) (Ennepetal, Germany)
	Yong Quan (Chengdu 2009) (Ennepetal, Germany)
	University of Chinese medicine (Harbin, China)

Extraction of Artemisia herbal substances:

The herbal substance was powdered by a mill and 300 mg were extracted with 10 ml dichloromethane for 15 min. After filtering, the solvent was eliminated under reduced pressure and the dried extract was resolved in 500 µl deuterated acetone. Three replicates of each of the herbal preparations were prepared and analyzed by ^{1}H-NMR in the same manner.

^{1}H-NMR analysis and PCA:

^{1}H-NMR spectra of extracts were recorded with a Bruker advance 300 DPX instrument (temperature 25 °C, 64 scans). Calibration of spectra was performed according to signals of incomplete deuterated solvents. Data were processed using TOPSPIN software and evaluation by PCA was performed with AMIX software (Bruker). Spectral data from δ 10 to δ 0 ppm were included for PCA. The region from δ 7.1 to δ 7.4 ppm was excluded for PCA because of signals from residual acetone in the extract. The bucket width was 0.05 ppm and data were integrated according to the sum of intensities.

Extraction of DNA:

DNA extraction was performed using a PSP Spin Stool DNA Kit (Stratec, Berlin, Germany) according to the manufacturer's protocol. 50 mg of the powdered herbal substance was used for DNA extraction.

PCR:

ITS was amplified using previously established universal plant specific ITS primers (TKA-for: 5'-GAACCTGCGGAAGGATCATTG-3', TKA-rev: 5'-GCTTAAACTCAGCGGGTAGTCC-3') or (TKB-for: 5'-GCAGGTTCACCTACGGAAAC-3'; TKB-rev: 5'- CGCGACCCCAGGTCA-3') (ITS1: 5'-TCCGTAGGTGAACCTGCGG-3; ITS4: 5'-TCCTCCGCTTATTGATATGC -3) (Kersten T., 2013). Primers applied to amplify ETS were specific for *A. scoparia* (ETS Artemisia for: 5´-GTGCGGCAAGGCTTGTATCG-3´; ETS Artemisia rev: 5´-ACCGCGTCAAGGTTCACCTC-3´). PCR was performed in a total volume of 25 µl consisting of 1×PCR buffer, 0.5 µl template DNA, 0.1 µM of each primer (Eurofins MWG Operon, Germany) and 0.25 µl DNA polymerase (Promega, Germany). PCR included an initial denaturation step at 95 °C for 2 min, followed by 35 cycles for 30 s at 94 °C, 20 s at 60 °C and 30 s with 72 °C; and a final extension period of 4 min at 72 °C. PCR products were analyzed by agarose gel electrophoresis.

PCR sample clean up and sequencing reaction:

Purification of PCR fragments was performed by using a QIAquick Gel Extraction Kit or QIAquick PCR Purification Kit according to the manufacturer´s instructions (Qiagen GmbH).

Sequencing results were obtained by GATC Biotech AG (Konstanz, Germany). To improve the quality of sequencing data, amplicons were ligated using pGEM-T vector system (Promega, Mannheim, Germany) and transformed with competent *E. coli* XL1 blue. The transformants were plated on LB-ampicillin plates and selected colonies were cultivated in LB medium containing ampicillin (100 µg/ml) overnight (37 °C). Plasmid DNA was isolated

using PureYield Plasmid Miniprep System (Promega, Germany) according to the manufacturer's protocol.

Sequence-data analysis:

By NCBI BLAST alignments (National Center for Biotechnology, www.ncbi.nlm.nih.gov/) a sequence similarity search was performed to identify sequences with similarity.

7.4 Results

^{1}H-NMR analysis:

NMR-fingerprint analysis of different Artemisia sp.:

In a previous research project, the ^{1}H-NMR spectra of some *Artemisia* sp. were already analyzed (Daniel C., 2009). In the present study, the data were evaluated by PCA to evaluate the ^{1}H-NMR-based approach more in detail. In total, three different *Artemisia* sp. were analyzed: Artemisiae argyi folium, Artemisiae annuae herba and Artemisiae scopariae herba.

PCA demonstrated differences between the three different *Artemisia* samples (fig. 28). Samples of Artemisiae annuae herba, Artemisiae argyi folium and Artemisiae scopariae herba were classified, respectively. The distribution of the clusters indicated that Artemisiae argyi folium and Artemisiae scopariae herba represented the highest variability.

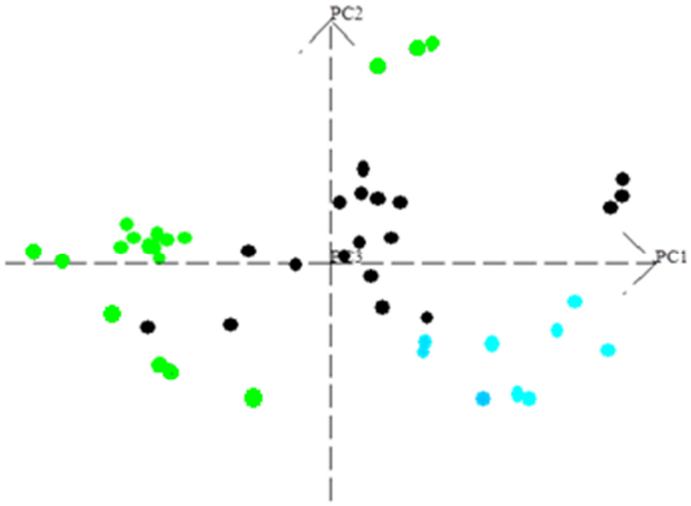


Fig. 28: Score plot of PCA of PCs of ^{1}H-NMR spectra of dichloromethane extracts of Artemisiae annuae herba (black), Artemisiae argyi folium (blue) and Artemisiae scopariae herba (green). The extracts were analyzed in acetone-d6.

Examination of the loading plot (fig. 29) showed that the first and second principle component (PC) explained the variance associated with aliphatic constituents since high loading values were observed in this region of the ^{1}H-NMR-spectra. The main differences between the extracts were found in the region from about δ 3.5 to δ 0.5 ppm, where most of the protons associated with terpenoids, steroids, and organic acids resonate. In addition, the loading plot of PC1 and PC2 illustrated a few positive and negative regions of the spectra at about δ 4.0 ppm. In this region, most of the protons of carbohydrates, amino acids, and glycosides resonate. Signals between δ 6.0 and δ 5.5 ppm indicate that molecules associated with flavonoids, phenylpropanoids, phenolics, and tannins were less determining for the variance of the different extracts compared to signals observed in the upfield region.

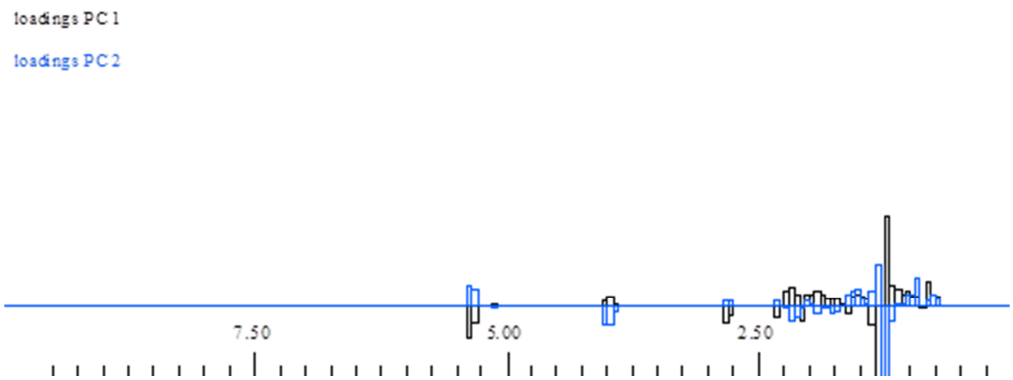


Fig. 29: 1D loadings for PC1 (black) and PC2 (blue) of different *Artemisia* sp. (Artemisiae annuae herba, Artemisiae argyi folium and Artemisiae scopariae herba). Scaling corresponds to the chemical shift (ppm) of ^{1}H-NMR spectra.

NMR-fingerprint analysis of A. scoparia from Germany and China

In this part of the study, extracts were prepared from samples of *A. scoparia*. ^{1}H-NMR spectra were analyzed in analogy to the approach used for the investigation of different *Artemisia* sp.. The score plot of PCA (fig. 30) showed discriminable cluster formation of the *A. scoparia* samples from China and Germany, respectively.

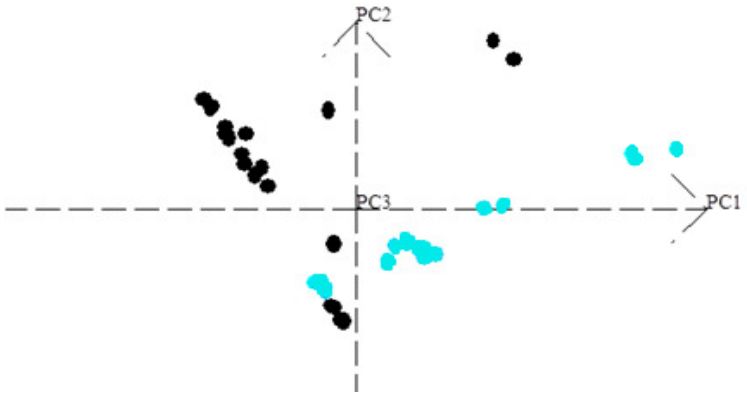


Fig. 30: Score plot of PCA of the first three PCs of ^{1}H-NMR spectra of dichloromethane extracts of *A. scoparia* from China (blue) and Germany (black). The extracts were analyzed in acetone-d6.

The loading plot of PC1 and PC2 (fig. 31) illustrated that most positive and negative regions of the spectra were located between δ 3.5 and δ 0 ppm, a region associated with protons from terpenoids, steroids and organic acids. Signals in the spectra between δ 5.0 and δ 6.0 ppm associated with protons from flavonoids, phenylpropanoids and phenolics indicated a minor impact of variance compared to the upfield region.

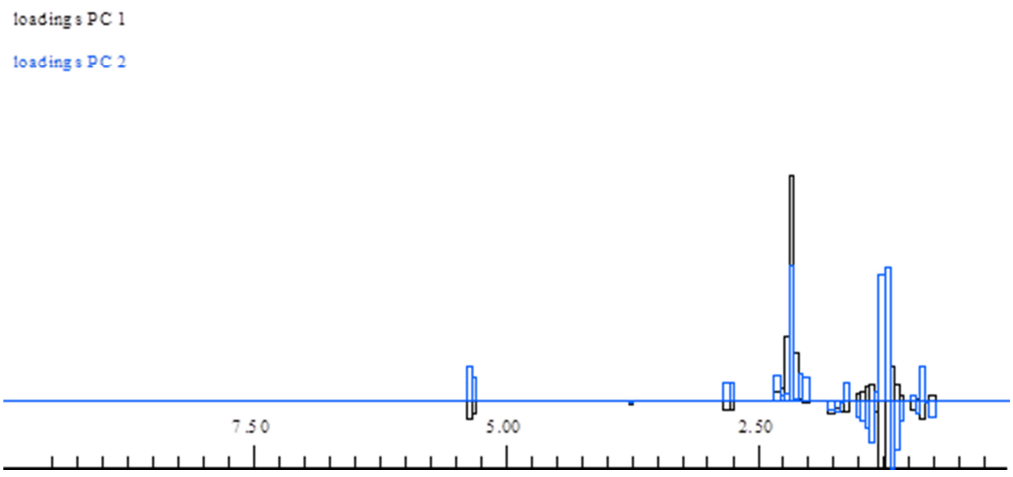


Fig. 31: 1D loadings for PC1 (black) and PC2 (blue) of *A. scoparia* from China and Germany. Scaling corresponds to the chemical shift (ppm) of ^{1}H-NMR spectra.

Analyses of ITS and ETS:

ITS sequences obtained for the different *Artemisia* sp. were investigated. Some data were already received in a previous research project in our group (Daniel C., 2009). In the present study, they were re-evaluated by NCBI BLAST analysis (tab. 16).

Three different Artemisiae annuae herba samples were investigated. Sequencing results were obtained for 5´→3´direction and for 3´→5´direction using the ITS1, ITS4, TKB forward, and TKB reverse primer, respectively. According to NCBI BLAST, the herbal substances were identified as *Artemisia annua*, respectively.

Additionally, two Artemisiae argyi folium samples were investigated and sequence identity showed 100%; thereby, the NCBI BLAST hit result confirmed the identity of *A. argyi* L..

According to NCBI BLAST, the analyzed Artemisiae scopariae herba samples corresponded to *Artemisia* sp.. However, one exception was observed, as one sample indicated higher similarity to *Artemisia japonica.* The investigated ITS sequences obtained for *A scoparia* were highly similar.

Tab. 16: Different *Artemisia* sp. investigated in the present study.

	NCBI accession code	Identified species (NCBI BLAST)	PCR and sequencing deoxynucleotides
Artemisia annua			
Artemisiae annuae herba, Complemedis	AY548199.1	*Artemisia annua*	ITS1
Artemisiae annuae herba, Complemedis	GU724282.1	*Artemisia annua*	ITS4
Artemisiae annuae herba, Kaiser pharmacy	AY548199.1	*Artemisia annua*	ITS1
Artemisiae annuae herba, Kaiser pharmacy	GU724282.1	*Artemisia annua*	ITS4
Artemisiae annuae herba, Caesar & Loretz	JQ230972.1	*Artemisia annua*	TKB for
Artemisiae annuae herba, Caesar & Loretz	JQ230972.1	*Artemisia annua*	TKB rev
Artemisia argyi			
Artemisiae argyi folium, Complemedis	GU724270.1	*Artemisia argyi*	TKB for
Artemisiae argyi folium, Kaiser pharmacy	GU724270.1	*Artemisia argyi*	TKB for
Artemisia scoparia			
Artemisiae scopariae herba, Kaiser pharmacy	GU724303.1	*Artemisia scoparia*	ITS1
Artemisiae scopariae herba, Complemedis	AY548200.1	*Artemisia japonica*	ITS1
Artemisiae scopariae herba, Galke	GU724303.1	*Artemisia scoparia*	TKA forw
Artemisiae scopariae herba, Galke	GU724303.1	*Artemisia scoparia*	TKA rev

Additionally, ITS sequences from *A. scoparia* samples from different provenances were investigated using TKA deoxynucleotides and specific ETS deoxynucleotides for identification of herbal samples. The alignment of the different sequences showed no major differences in the analyzed ITS and ETS sequences, respectively. According to the NCBI BLAST alignment of the obtained ITS sequences, one sample showed more similarity to *Artemisia japonica*. For the remaining *A. scoparia* samples, the highest accordance was shown for *Artemisia* sp..

ETS sequences were additionally investigated to assure the identity of the *A. scoparia* samples. According to NCBI BLAST alignment, all samples showed the highest similarity to *A. scoparia* (tab. 17).

Tab. 17: Sequencing results of ITS and ETS of *A. scoparia* samples from China and Germany.

	ITS		ETS	
	NCBI BLAST result	**NCBI accession code**	**NCBI BLAST result**	**NCBI accession code**
Germany				
Lfd 14	*A. scoparia*	FJ528303.1	*A. scoparia*	DQ028857.1
Lfd 15	*A. scoparia*	FJ528303.1	*A. scoparia*	DQ028857.1
Lfd 16	*A. scoparia*	FJ528303.1	*A. scoparia*	DQ028857.1
Lfd 17	*A. scoparia*	FJ528303.1	*A. scoparia*	DQ028857.1
Lfd 18	*A. scoparia*	FJ528303.1	*A. scoparia*	DQ028857.1
Lfd 19	*A. scoparia*	FJ528303.1	*A. scoparia*	DQ028857.1
Lfd 20	*A. scoparia*	FJ528303.1	*A. scoparia*	DQ028857.1
Lfd 21	*A. scoparia*	FJ528303.1	*A. scoparia*	DQ028857.1
China				
University of Chinese medicine	*A. scoparia*	FJ528303.1	*A. scoparia*	DQ028857.1
Herba Sinica	*A. scoparia*	FJ528303.1	*A. scoparia*	DQ028857.1
Lian	*A. scoparia*	FJ528303.1	*A. scoparia*	DQ028857.1
PharmaChin	*A. japonica*	GU724289.1	*A. scoparia*	DQ028857.1
Yong Quan (Shanghai 2001)	*A. scoparia*	FJ528303.1	*A. scoparia*	DQ028857.1
Yong Quan (Shanghai 2005)	*A. scoparia*	FJ528303.1	*A. scoparia*	DQ028857.1
Phytax	*A. scoparia*	FJ528303.1	*A. scoparia*	DQ028857.1
Yong Quan (Chengdu 2009)	*A. scoparia*	FJ528303.1	*A. scoparia*	DQ028857.1
Arobemed	*A. scoparia*	FJ528303.1	*A. scoparia*	DQ028857.1
Hangzhou (pharmacy)	*A. scoparia*	FJ528303.1	*A. scoparia*	DQ028857.1

7.5 Discussion

The appropriate quality and safety of herbal medicinal products is obligate for licensing. Fundamental steps for a safe use have to include the identification and authentication of the plant species. Therefore, complementary methods are of special interest for the analyses of herbal substances and preparations thereof, also from non-European origin. *Artemisia* sp. have been used in health care for centuries in various Asian countries (Kalemba D., 2002; Kordali S., 2005). Thus, it is an interesting model object to test the applicability of complementary methods for quality control of medicinal plants.

In the present study, *Artemisia* sp. monographed in the Chinese Pharmacopoeia 2010, were investigated by ^{1}H-NMR fingerprinting. PCA differenciated Artemisiae argyi folium, Artemisiae scopariae herba and Artemisiae annuae herba into different groups indicating that this method can adequately discriminate different herbal substances. Secondary metabolites, mainly responsible for differences in sample composition were mainly associated with terpenoids, steroids and organic acids. In previous studies, terpenoids were identified as phytochemical constituents of *Artemisia* sp. (Harada R., 1982; Ahmad A., 1994; Singh H., 2009; Cavar S., 2012).

Artemisinin is a sesquiterpene endoperoxide lactone identified for *A. annua* frequently used in the treatment of malaria. The structure of artemisinin using ^{1}H-NMR and ^{13}C-NMR assignments previously was investigated (Blaskó G., 1988; Wallaart T., 1999). In the present study, for preparations containing Artemisiae annuae herba, small signals were obtained at δ 5.86 ppm and δ 1.44 ppm in the ^{1}H-NMR spectra that may be associated with artemisinin. However, multitudes of partly overlapping signals were observed in the ^{1}H-NMR spectra in the region from δ 2.5 to δ 0.5 ppm. Therefore, it is difficult to identify molecules exactly responsible for the classification of preparations containing *Artemisia*.

Furthermore, the metabolomics-based approach was used to characterize morphologically different *Artemisia scoparia* samples from different provenances. It was previously shown that environmental factors may have an important influence on the metabolome (Brunetti C., 2013). For *A. annua* it was already shown that its antimalarial compound artemisinin varies depending on the time of harvest (Wallaart T., 2000). Moreover, in previous studies, it was shown that the provenance strongly influences the secondary metabolite profile (Sellami H., 2013). The loading plot indicated that secondary metabolites responsible for the classification of samples were mainly associated with steroids, terpenoids, organic acids, and to minor amounts with flavonoids, phenoloics and tannins. In a previous study, identical *A. scoparia* samples from Germany and China were investigated (Scherübl R., 2014). It was shown by

standard methods that the flavonoid quercitrin was exclusively present in the German samples. The essential oils capillene (10.1-46.0%), spathulenol (3.5-17.0%), and hexadecanoic acid (1.0-37.6%) were identified as the major constituents represented in *A. scoparia* from both provenances. Furthermore, in the study performed by R. Scherübl et al., it was shown that fatty acids were present in higher amounts in *A. scoparia* from China than from Germany. The samples from Germany revealed higher amounts of monoterpenes. Further differences were identified among the sesquiterpenes. Bisabolane-type derivatives were identified predominately within the samples originating from Germany, whereas cadinane-type ones accumulated mostly in the Chinese samples (Scherübl R., 2014). Interestingly, in the present study, the loading plot indicated variance between the extracts from different provenances associated with terpenoids, steroids and organic acids, flavonoids, phenylpropanoids and phenolics. Hence, for classification the same groups of phytochemical constituents were identified.

Before herbal preparations are marketed in Europe, the identity has to be determined as specified in the European Pharmacopeia or in a national pharmacopoeia. For this purpose, macroscopic, microscopic, and fingerprinting techniques are frequently used. Additionally, the content of specific marker compounds is often determined. However, chromatographic techniques and the use of marker compounds showed limitations. Some rare and expensive medicinal plants were adulterated with morphological related species or spiked with marker substances (Joshi K, 2004). Hence, a DNA-based approach may be useful to overcome these problems. The marker sequence ITS2 was determined as a useful DNA marker to identify species of the Asteraceae family (Gao T., 2010). This may lead to the suggestion that the usage of ITS might be an appropriate a marker region for the identification of *Artemisia* sp.

The results of the present study showed that the analysis of ITS sequences was useful for the identification of different *Artemisia* sp.. However, it was shown that minor changes in the sequence may lead to differing NCBI BLAST results. According to NCBI BLAST alignment, one *A. scoparia* sample showed more similarity to the related species *A. japonica*. By the usage of a second marker sequence, the herbal substance was confirmed to be *Artemisia scoparia*. Hence, it is very useful to consult at least two marker sequences for unambiguous identification, as it is also proposed by various previous studies (CBOL Plant Working Group, 2009; Kuzmina M., 2012; Mankga L., 2013; Zhang W., 2013).

In conclusion, the metabolomics-based approach enabled comprehensive assessment of complex herbal substances. The applicability of this approach has already been positively evaluated in previous studies (Chauthe S., 2012; Daniel C., 2008; Frederich M., 2011; Kim H., 2005; Mattoli L., 2006; Politi M., 2009; van der Kooy F., 2009). In the present study, different *Artemisia sp.* with a traditional use in Asia were authenticated by ^{1}H-NMR analysis combined with PCA. Additionally, differences in the metabolome of the medicinal plant *A. scoparia* from two provenances were displayed by this approach. Interestingly, differences in the composition of herbal constituents confirmed previously published results that were obtained by standard methods (TLC, HPLC/MS, GC/MS) (Scherübl R., 2014). The identity of different *Artemisia* samples was revealed by the PCR-based approach and the application of two marker sequences. Especially for herbal substances belonging to TCM this is a very meaningful approach. However, herbal substances from non-European origin are often processed. Subsequently, DNA may be degraded and PCR-based techniques are not suitable (Kersten T., 2008).

Thus, the comprehensive application of both approaches is a useful strategy to identify and authenticate herbal substances and preparations thereof. Overall, the present study clearly proposes ^{1}H-NMR fingerprinting along with the statistical method PCA to authenticate herbal substances in combination with PCR-based methods for the identification of herbal substances.

8 Overall discussion

8.1 Bridging: chemical profile and functional activity

The quality of herbal substances and their preparations is of paramount importance for their safety and efficacy. Therefore, this study investigated the potential of recently developed methodologies, such as metabolomic profiling and gene expression profiling, to complement standard methods for characterization of herbal substances and their preparations.

Herbal substances and their preparations are complex in nature due to their high number of constituents; hence, the appropriate chemical characterization is indispensable. Therefore, a strategy was chosen that included the chemical characterization based on a metabolomics-based approach (Daniel C., 2009). In addition, herbal preparations were analyzed by pharmacopeial monographs. The biological functions were investigated by the characterization of cellular responses using gene expression profiling and analysis of cell proliferation.

Three different herbal substances were investigated. Two of them, Chelidonii herba and Cimicifugae racemosae rhizoma, are associated with liver toxicity. The third investigated herbal substance, Silybi mariani fructus, has traditionally been used to treat toxic liver damage and also for the supportive treatment of chronic inflammatory liver diseases. The project was designed to investigate a broad spectrum of herbal preparations, but not to focus on herbal medicinal products available on the market. Some extracts were prepared similar to commercially available herbal preparations, because respective extraction solvents used are known to be potent in extracting the active substance/s. However, other extraction solvents were used to obtain extracts with different composition. This strategy was shown to be very useful in the complementary investigation of herbal substances.

The alkaloids sanguinarine and chelerythrine represented in Chelidonii herba were suggested to be liver-toxic (Dvorak Z., 2006b; Kosina P., 2004; Ulrichová J., 1996). Four different herbal extracts with different patterns of constituents were investigated. In finished herbal medicinal products containing Chelidonii herba, a mixture of ethanol and water is a frequently used extraction solvent. Accordingly, ethanol and ethanol 50% (V/V) were included in the present study. In addition, water and dichloromethane were used as these solvents are of different polarity.

The correlation of the chemical profile and cell proliferation suggested that antiproliferative effects were not exclusively associated with the alkaloid content. Gene expression data and systems biological data evaluation revealed that the different constituents in the respective

extracts activated different signal cascades. Major differences were demonstrated in signal cascades related to biotransformation and liver toxicity.

Preparations derived from Cimicifugae racemosae rhizoma are widely used in the population; however, cases of liver toxicity have been reported. Three different preparations containing Cimicifugae racemosae rhizoma were investigated and were analyzed in analogy to preparations derived from Chelidonii herba. Isopropanol and mixtures of ethanol and water are frequently used solvents used in commercially available preparations containing Cimicifugae racemosae rhizoma. Hence, in the present study, ethanol and 50% ethanol were used as extraction solvents and additionally, dichloromethane was used. Results obtained for the aqueous extract of Chelidonii herba showed that only few phytochemical constituents were extracted. In order to save resources for microarray investigations, aqueous extracts were not included. For the same reason, extracts prepared with isopropanol were excluded as the study was not intended to match the regulatory background of Cimicifugae racemosae rhizoma. The correlation of the chemical profile to HepG2 cell proliferation showed that triterpene glycosides may be involved in antiproliferative effects. However, in accordance with previous studies, cinnamic acid esters are suggested to exhibit cytotoxic activities (Hostanska K., 2004a; Hostanska K., 2004b). The ethanolic and dichloromethane extract derived from Cimicifugae racemosae rhizoma exhibited the highest content of triterpene glycosides. Both herbal preparations exhibited a significant activation of several signal cascades associated with cholesterol biosynthesis and fatty acid metabolism. Less triterpene glycosides were demonstrated for the 50% (V/V) ethanolic extract containing Cimicifugae racemosae rhizoma. Cells exposed to the latter extract showed the induction of less signal cascades related to cholesterol biosynthesis and fatty acid metabolism. Hence, it may be suggested that triterpene glycosides might be responsible for cellular responses related to these signal cascades in our model. Similar results were obtained for signal cascades attributed to liver toxicity. The 50% (V/V) ethanolic Cimicifugae racemosae rhizoma extract did not affect liver-toxic signal cascades. Differences for extracts rich in triterpene glycoside were mainly displayed in signal cascades related to drug metabolism.

In a third approach, a preparation containing Silybi mariani fructus and silybin, a chemically-defined substance thereof with suggested pharmacological activity, were investigated. The objective was to identify altered genes and activated signal cascades in HepG2 cells of an herbal substance, which is not associated with liver toxicity. Moreover, experiments performed in this subproject specifically addressed the question in how far gene expression profiling can generate information to develop hypotheses on synergy and multitarget activity. Preparations containing Silybi mariani fructus are traditionally used for the treatment of liver diseases and furthermore, silymarin is approved for the treatment of *Amanita phalloides*

poisoning. A preparation containing Silybi mariani fructus was obtained using 70% (V/V) ethanol. Ethanol is often used for the preparation of finished herbal medicinal products containing Silybi mariani fructus.

Microarray data analysis revealed that more and different signal cascades related to the biotransformation were altered for HepG2 cells after exposure to Silybi mariani fructus compared to the exposure to silybin. Commonly affected signal cascades identified for HepG2 cells after both treatments were liver damage and liver regeneration. However, results related to liver toxicity also showed differences, such as signal cascades associated with liver necrosis, exclusively activated after exposure to silybin; whereas the signal cascade “liver fibrosis” was exclusively induced after exposure to the 70% (V/V) ethanolic Silybi mariani fructus extract, respectively. These findings demonstrate that complex gene expression profiling technology can detect differences in cellular responses to an herbal preparation versus a chemically-defined substance. Moreover, significantly regulated genes associated with SLC transporters were identified. SLC transporters have been assigned to play a role in drug resistance (Januchowski R., 2013; Webb A., 2013). On the basis of gene expression profiling, the results demonstrate that a complex herbal preparation induced an entirely different mode of action compared to an individual chemically-defined substance thereof. Nevertheless, the question for genes responsible for the mode of action remains.

Conclusively, the results obtained in the study on Chelidonii herba and Cimicifugae racemosae rhizoma with differently prepared herbal preparations indicated that a preparation-specific gene expression fingerprint was obtained. Moreover, the results obtained for Chelidonii herba and Cimicifugae racemosae rhizoma clearly underline the value of the existing concept of a clearly defined herbal substance and specifications for the manufacturing process to obtain consistently safe herbal medicinal products. Variations in the composition of herbal extracts may not only lead to toxic effects; interactions of drugs may also play a role in defining the safety of herbal medicinal products. On the basis of gene expression data, minor differences in the composition of constituents may result in a divergent regulation of cytochromes. These enzymes play a major role in drug metabolism. With regard to extracts representing different phytochemical constituents, different gene expression patterns were obtained, providing that preparation-specific gene expression signatures were obtained (fig. 32).

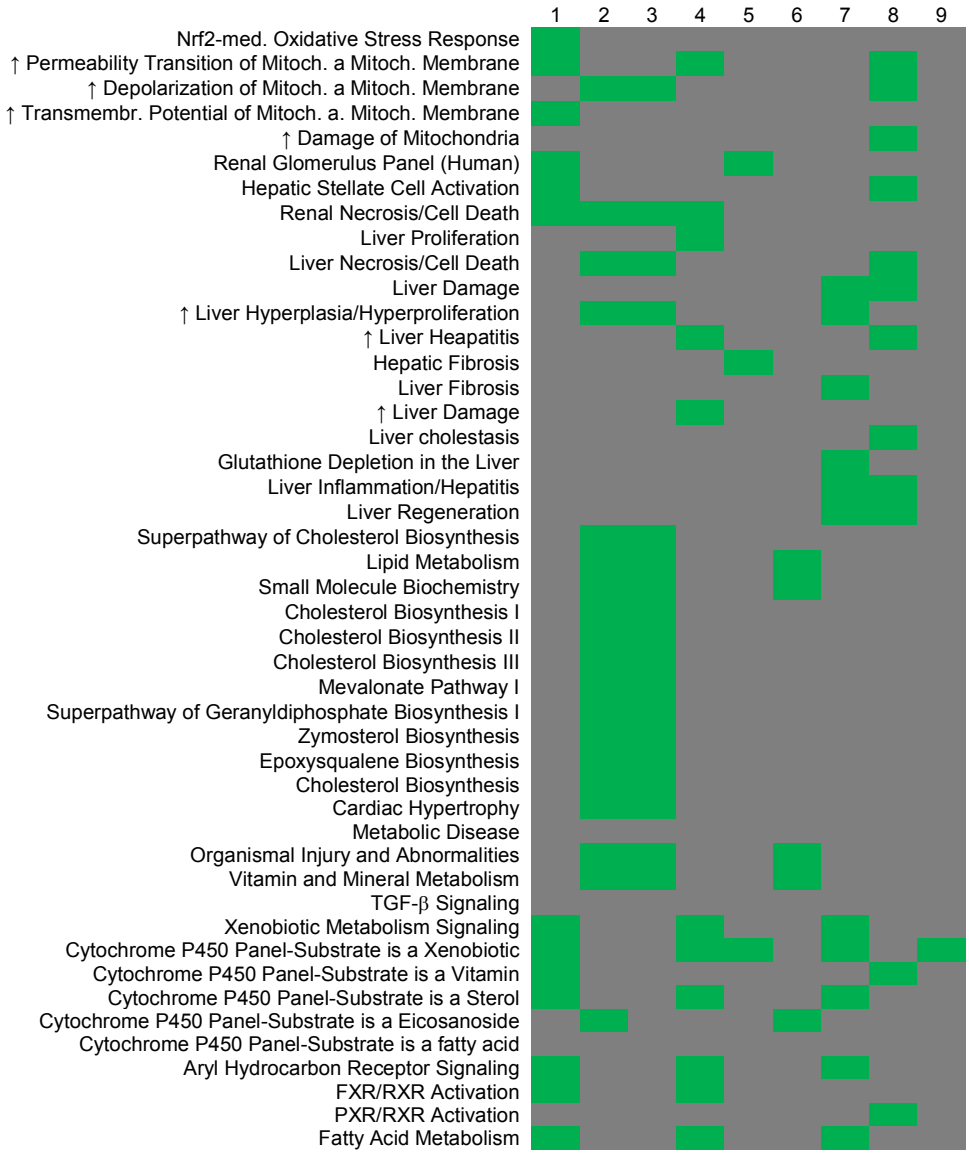


Fig. 32: Summary of activated (green) and unaffected (grey) signal cascades obtained with IPA microarray data evaluation. 1: ethanolic extract Chelidonii herba (58%), 2: ethanolic Cimicifugae racemosae rhizoma extract (61%), 3: dichloromethane Cimicifugae racemosae rhizoma extract (72%), 4: 50% (V/V) ethanolic Chelidonii herba extract (73%), 5: dichloromethane Chelidonii herba extract (77%), 6: 50% (V/V) ethanolic Cimicifugae racemosae rhizoma extract (81%), 7: 70% ethanolic Silybi mariani fructus extract (98%), 8: silybin (98%), 9: aqueous Chelidonii herba extract (105%). Values in brackets show relative cell proliferation of HepG2 cells after six hours of exposure to the extracts, respectively.

8.2 Validity of microarray data

In the present study, microarray results were validated interlaboratorily by different experimenters using different microarray platforms. Identical RNA samples were investigated. Therefore, regarding biological variability it can be assumed that there is only a minor impact on variability of gene expression profiles. The coverage of genes with a less than threefold change was lower compared to genes with higher fold changes. The coverage of affected biological pathways showed increased congruency, another indicator of reliability of the data. Alternatively to a twofold change in gene expression, threefold changed genes could be used for the investigation of signal cascades. Regarding gene expression profiles in the present study, most genes showed smaller fold changes. Therefore, using threefold changed genes, a lot of information would have been ignored. Furthermore, qRT-PCR was used to validate selected genes. The results indicated good reliability, although minor differences in fold changes were observed.

8.3 Gene expression profiling in the assessment of liver toxicity

For the assessment of safety of herbal substances and herbal preparations clinical data are limited. The approach using gene expression profiling of HepG2 cells after exposure to different herbal preparations is a first step to increase knowledge, but concerning relevance *in vivo*, results have to be considered with caution. Concentration of the active substance in the herbal preparations used in cell culture experiments did not consider concentrations of herbal substances applied in therapy. Taken these limitations into account, the potential of gene expression profiling and systems biological data evaluation based on the performed experiments is roughly estimated for the use in the assessment of liver toxicity in the following paragraph.

All signal cascades presented in this study were used for this evaluation, which showed most significant results with microarray data evaluation. However, other signal cascades were less significantly activated not shown in the present study.

The correlation of antiproliferative effects and relative activated signal cascades and relative activated signal cascades related to toxicity indicates for most treatment conditions a correlation between antiproliferative effects and activated signal cascades (tab. 18).

Tab. 18 Antiproliferative effects in HepG2 cells and activation of signal cascades. Results of HepG2 cell proliferation were obtained after six hours of exposure of the different extracts. Activated signal cascades were set into relation to all activated signal cascades at least under one condition with respect to the respective experiment. Activated signal cascades related to toxicity were set into relation to all activated signal cascades associated with toxicity at least for one condition with respect to the respective experiment.

	Antiproliferative effect of HepG2 cells	Relative activated signal cascades (%)	Relative activated signal cascades related to toxicity (%)
***C. majus* ethanolic extract**	58	70.6	63.6
***C. racemosa* ethanolic extract**	61	100.0	100.0
***C. racemosa* dichloromethane extract**	72	90.5	100.0
***C. majus* 50% (V/V) ethanolic extract**	73	58.8	54.6
***C. majus* dichloromethane extract**	77	17.8	18.2
***C. racemosa* 50% (V/V) ethanolic extract**	81	28.6	28.6
***S. marianum* 70% ethanolic extract**	98	52.4	35.7
Silybin	98	61.9	71.4
***C. majus* aqueous extract**	105	5.9	0

Extracts that provoked stronger antiproliferative effects in HepG2 cells activated a higher percentage of activated signal cascades. Regarding relatively activated signal cascades or relatively activated signal cascades related to toxicity, no major differences were identified. However, differences were observed in relative activated signal cascades of cells exposed to 70% (V/V) ethanolic Silybi mariani fructus extract and silybin. For these two conditions no major antiproliferative effects were observed, even after prolonged exposure under both conditions. However, the number of activated signal cascades related to toxicities is quite high. These contrary results clearly underline that data obtained with gene expression profiling and systems biological data evaluation have to be regarded critically for biological

relevance. Nevertheless, in our model, effects may have been observed at the transcriptional level, which could not be elucidated with other methods.

8.4 Current status of gene expression profiling: pros and cons

The investigated gene expression profiling approach has shown the potential to be useful in the comprehensive characterization of herbal substances and preparations thereof.

- The transcriptomics approach using microarray technique is very useful in the analysis of regulated genes. Specific gene expression “fingerprints” after exposure of HepG2 cells to herbal preparations were observed.

- Regarding ethical considerations, *in vitro* studies better embrace animal protection and welfare than *in vivo* studies. Moreover, with regard to environmental influences, a well-characterized cell line may provide results with higher reproducibility. HepG2 cells were shown to be a suitable model for the investigation of liver-toxic effects and they are frequently applied for toxicogenomics studies. HepG2 cells are of great relevance to detect cytotoxic and genotoxic substances and by extension cytoprotective, antigenotoxic and co-genotoxic agents (Mersch-Sundermann V., 2004). In addition, HepG2 cells show metabolic activity represented by various biotransformation enzymes. For the characterization of herbal substances and their preparations, metabolic activity is an important characteristic. The metabolism of drugs and other xenobiotics is exemplified by cytochrome P450-mediated oxidation to more hydrophilic compounds. During biotransformation, some functional groups are oxidized, which can result in transient reactive intermediates. Phytochemical components, such as pyrrolizidine alkaloids require metabolic activation mediated by cytochrome P450 enzymes to generate reactive pyrrolic metabolites that react with cellular proteins and DNA leading to hepatotoxicity and genotoxicity (Fu P., 2004).

However, limitations of the transcriptomics-based have also been shown.

- Statistical data evaluation has shown to have a large impact on the results obtained by microarray analysis. Recent publications with systems biological data evaluation selected genes with the lowest threshold of at least twofold change (Abd A., 2013; Filali H., 2014; Guo Y., 2013; Marshall A., 2013). However, in the current study, genes with threefold change showed higher congruency and reproducibility. Hence, the use of genes with a threefold increase may be more robust. The results of the present study represented rather low fold changes of gene expression. Only a few genes were significantly regulated tenfold or even higher. Therefore, also genes with a twofold expression were included for systems biology based data evaluation.

- By using gene expression profiling, all experiments performed showed the activation of several signal cascades. On the level of gene expression data it is difficult to estimate signal cascades or networks responsible for the mode of action. Therefore, further investigations are necessary to clarify cellular responses. However, gene expression profiling may be beneficial to generate hypotheses for subsequent investigations.

- The interpretation by systems biological approaches has to be considered carefully. Systems biology is an interdisciplinary science, using the combination of experimental procedure and (mathematical) models, which have contributed to the understanding of complex biological systems. But there is still an ongoing progress to unravel the association of gene regulation and protein formation; furthermore, systems biology-based data evaluation is challenged by limited knowledge of the interplay of protein networks and the large number of regulatory feedback loops (Draghici S., 2007; Kaltenbach H., 2009; Klipp E., 2009; Maier T., 2009; Savageau M., 2009). By identifying activated signal cascades only the set of gene is considered, the position yet is ignored (Draghici S., 2007).

- In the present study, HepG2 cells have shown limitations for the functional characterization of the investigated herbal substances and their preparations. For silymarin it was previously shown that SLC transporters (SLC21A6) may be involved in the proposed toxin blockade (Fehrenbach T., 2003). The SLC21A6 protein has not been identified in HepG2 cells (Cui Y., 2003). Furthermore, HepG2 cells lack

estrogen receptor α (ERα), therefore postulated estrogenic effects of Cimicifugae racemosae rhizoma could not be investigated.

- Results obtained by *in vitro* methods and microarrays should be considered with caution for relevance *in vivo*. By using *in vitro* technologies, important issues can be addressed, such as the activation of (specific) biotransformation enzymes (Masimirembwa, 2001). However, currently, *in vivo* methods can not be fully replaced.

8.5 Outlook: Combined approach bridging of data about the chemical profile and the functional activity

The approach of the combined investigation of the chemical profile and the functional characterizations by gene expression profiling seems to be very promising. Current findings obtained in the present study established a basis for further studies.

Mixtures of complex herbal substances and herbal preparations are frequently applied. Therefore, regarding gene expression profiling experiments, it would be highly interesting to investigate putative synergistic effects of such mixtures.

As an extension of the approach investigated, additional analysis of the proteome would offer the opportunity to further investigate cells exposed to herbal substances and herbal preparations.

8.6 Metabolomic fingerprinting

In previous research projects of the BfArM, an approach related to metabolomics was investigated for its future potential in regulatory affairs (Daniel C., 2009). The approach targeted on the authentication of herbal substances. In this study, the approach was further tested on applicability, possibilities and limitations in the investigation of herbal substances related to Asian therapeutic system.

In an earlier study, three different species of the genus *Artemisia* were investigated (Daniel C., 2009). In the present study, data were re-evaluated and clustering results were obtained. In cooperation with Prof. Heilmann from the University of Regensburg, 18 *A. scoparia* samples from different provenances were investigated additionally, also displaying clusters in PCA. The potential of this technique was clearly shown, as discriminating groups of

constituents were shown to be identical by the metabolomics-based approach and standard methods.

8.7 Potential of metabolomics-based approach

According to the results achieved in this study, the importance of marker substances in assessment of herbal preparations should be reconsidered. But investigations of the present study with differently prepared Chelidonii herba extracts showed that potential toxic components like alkaloids were not involved in the classification of herbal preparations. Therefore, the metabolomics-based approach cannot be used to completely substitute standard methods that quantify toxicity-related herbal compounds. For the appropriate authentication of herbal substances with ^{1}H-NMR analysis in combination with PCA, a database with an adequate number of samples has to be constructed.

8.8 PCR-based approach

Regarding the identification of herbal substances, sequence data from three different *Artemisia sp*. obtained in a previous study in our group were re-evaluated (Daniel C., 2009). The results of the PCR-based approach using ITS-sequencing are summarized (tab. 19).

Tab. 19: Summary of results obtained for different *Artemisia sp.* with the PCR-based method.

	A. argyi	***A. annua***	***A. scoparia***
DNA extraction	✓	✓	✓
PCR	✓	✓	✓
Sequencing results	✓	✓	✓
Species identification by NCBI BLAST database	✓	✓	

The herbal material investigated was dried but unprocessed. The extraction of DNA and subsequent PCR with universal plant specific primers established in the research project (Kersten T., 2013) were successful for all samples analyzed. The resulting PCR-fragments were sequenced successfully and results showed species-specific homologies. For *A. argyi*

and *A. annua*, the herbal origin was clearly identified by evaluation of the obtained ITS-sequences. The results obtained for *A. scoparia* ITS sequences corresponded mostly to *Artemisia sp*, a sequencing result provided to the NCBI database by C. Daniel and W. Knöß in a former research project. By retracing of the sequencing results, *A. scoparia* was identified as the plant origin. The sequencing results of the PCR-fragment obtained from one *A. scoparia* sample showed minor differences in the sequence, although no major morphological differences could be identified. The ^{1}H-NMR analysis and subsequent PCA did not demonstrate major differences. According to the NCBI BLAST alignment, differences in the sequence led to a divergent classification of the herbal origin. Hence, the identification of herbal origin was ensured by the usage of a second marker region using ETS sequence.

8.9 Potential of marker regions to identify herbal substances

Basically, the PCR-based approach is a suitable method to identify herbal substances. The investigation of one marker region showed to be valuable as a simple method for a first orientation. At least two herbal marker regions should be recruited to obtain reliable results, which display statements of current reports on the literature (Erickson D., 2008; CBOL Plant Working Group, 2009). For future prospects, a second universal marker region should be established to unambiguously identify herbal substances.

Furthermore, the results of NCBI database should be obtained carefully. As it is an open access database, the reliability of sequences has to be considered carefully. According to the number of reference data available, ITS seems to be a powerful marker region (tab. 20). In the future, collection of reliable sequences from marker regions of medicinal plants should be continued.

Tab. 20: Number of entities of DNA sequences for land plants in the database NCBI (www.ncbi.nlm.nih.gov; 10. 2. 2014). Multiple entries for the identical plant are possible for the marker sequence, respectively.

Marker	Entries for land plants
ITS	175,843
ETS	19,048
matK	95,060
rbcL	107,437
psbA	64,298
trnH	61,423
Cox1	1,225

A combined approach, using the ^{1}H-NMR fingerprints together with the analysis of marker substances is of great interest for a first characterization of herbal substances and herbal preparations. Investigations showed that *Artemisia* sp. was a good example for the characterization by these complementary methods; hence, it may be suggested that this combined approach is useful in the characterization of the herbal substances derived from the genus *Artemisia*. PCA is promising in the analysis of quality of herbal preparations and the application of marker substances is valuable for identification.

The present study indicated that standard methods can be complemented by recently developed methods. Gene expression profiling in combination with the metabolomics-based approach may be beneficial for the assessment of quality, analysis of global gene response and the identification of putative mechanisms of action. Metabolomics-based approaches are highly valuable for the classification of multicomponent mixtures as results obtained in the present study clearly show. The application of marker substances was proven to be suitable for retracing of the origin of herbal substances. In summary, new methodologies are suitable to contribute to characterize herbal substances and herbal preparations; however, current knowledge is limited and further studies are needed to consider routine application with respect to regulatory affairs.

9 Summary

Herbal substances and preparations thereof have been applied and used worldwide throughout human history. Regarding regulatory affairs, the assessment of quality of herbal substances and their preparations is mandatory. Quality control is challenging, as herbal substances and their preparations are always complex mixtures. Furthermore, herbal substances and their preparations are suggested to act on multiple targets; therefore, a comprehensive characterization of biological function is of major impact.

The focus of the present thesis was to bridge data about the chemical profile and the biological activity of herbal preparations (fig. 33).

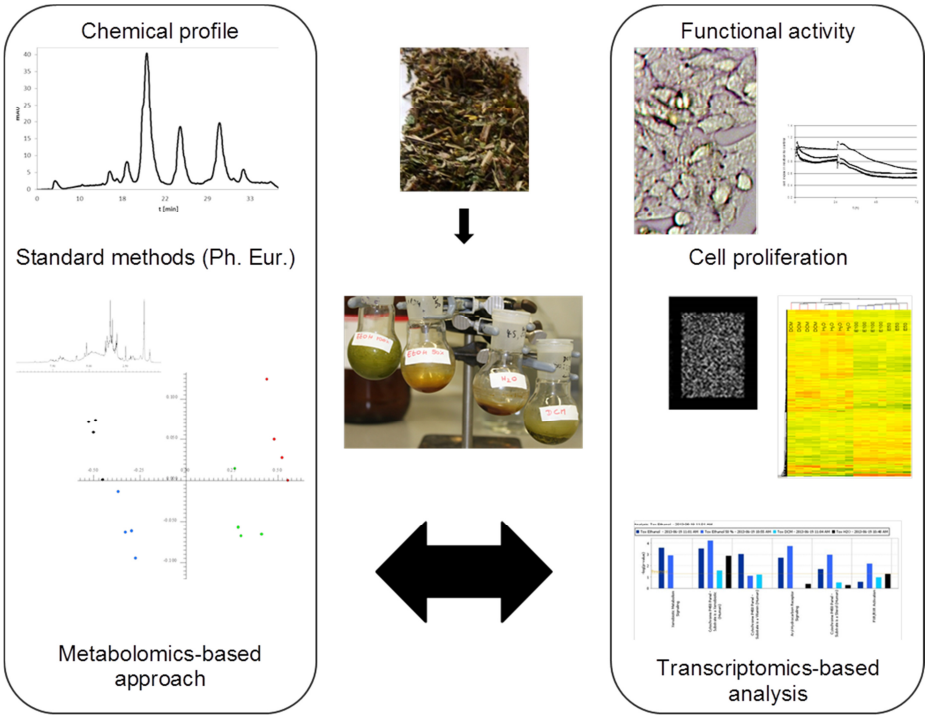


Fig. 33: Strategy for the characterization of herbal preparations.

Recently emerging omics-techniques have been shown to be useful in the characterization of herbal substances and their preparations. Regarding quality of complex mixtures comprising various phytochemical constituents, ^{1}H-NMR analysis in combination with principal component analysis (PCA) is a highly valuable approach. In the present study, different herbal preparations were chemically characterized by their metabolome. Additionally, standard methods of the European Pharmacopeia were applied for the quantification of selected marker compounds. With respect to characterization of biological activities, a transcriptomics-based approach was used. HepG2 cells were exposed to different herbal preparations and gene expression profiling was used to comprehensively characterize global cellular responses. Cell proliferation after exposure to different extracts was investigated.

Cellular effects were characterized by gene expression profiling and by systems biological data evaluation; regulated genes were attributed to affected signal cascades. Selected differently regulated genes were verified by qRT-PCR.

In the present study, three different herbal substances and one isolated constituent thereof were comprehensively investigated. For Chelidonii herba and Cimicifugae racemosae rhizoma several cases associated with liver toxicities have been reported. Herbal preparations with different patterns of phytochemical components were obtained by extraction with solvents of different polarities. The third herbal substance, Silybi mariani fructus, has traditionally been used for treatment of diseases related to liver and gallbladder.

The results of the investigations with different extracts containing Chelidonii herba demonstrated that the extraction solvent used had an impact on the composition of constituents. These differences could be discriminated by PCA. Standard methods showed characteristic patterns of alkaloids for the different extracts. Differences in the composition of herbal constituents also provoked different antiproliferative effects on HepG2 cells. The ethanolic extract marked the strongest antiproliferative effects. By correlating these results to the chemical profile it could be suggested that the total alkaloid content may not exclusively be responsible for liver toxicity. Systems biological data evaluation showed that most significantly induced signal cascades associated with liver toxicity were identified for HepG2 cells exposed to the ethanolic extract. Moreover, differences in cellular responses were mainly associated with drug metabolism.

Three herbal preparations derived from Cimicifugae racemosae rhizoma could be clearly discriminated by PCA of ^{1}H-NMR fingerprinting. Quantification of triterpene glycosides revealed varying contents thereof in the extracts. The ethanolic extract represented the highest amount of triterpene glycosides and provoked highest antiproliferative effects on HepG2 cells. The content of triterpene glycosides bridged to HepG2 cell proliferation could lead to the hypothesis that these phytochemical constituents were mainly responsible for antiproliferative effects in the used model. Moreover, as demonstrated by microarray experiments a high number of affected signal cascades were related to liver toxicity, e. g. liver necrosis and cell death were identified for extracts containing high amounts of triterpene glycosides such as the ethanolic and the dichloromethane Cimicifugae racemosae rhizoma extract. Additionally, a higher number of significantly activated signal cascades associated with cholesterol biosynthesis and lipid metabolism were identified for preparations derived from Cimicifugae racemosae rhizoma representing high contents of triterpene glycosides. Differences in signal cascades related to biotransformation were identified for HepG2 cells after exposure to herbal preparations showing a similar content of triterpene glycosides.

Hence, this may lead to the suggestion that various groups of phytochemical constituents are involved in activation of genes related to drug metabolism.

For Silybi mariani fructus, investigation was focusing on applying the methodology on an herbal preparation and on silybin, a defined natural constituent of this preparation. ^{1}H-NMR fingerprinting indicated a characteristic pattern of various phytochemical constituents in the herbal preparation. Silymarin was quantified using standard methods. HepG2 cells were either exposed to this herbal preparation or to silybin in similar concentrations related to silymarin. Cell proliferation was rather not affected after exposure to both treatment conditions. Gene expression profiles revealed major differences affecting signal cascades related to toxicity, which were more frequently induced in HepG2 cells after exposure to silybin. Signal cascades related to biotransformation were differentially activated. Additionally, regulated genes were identified which are suggested to play a role in drug resistance.

The reproducibility of the methodology using microarrays was investigated. Identical RNA samples were investigated by different microarray platforms in our laboratory and by a company which commercially provides microarray services. Highest variability of the data was identified for significantly regulated genes with low fold changes. A higher congruency was obtained for at least threefold regulated genes. This comparison indicated differences in the gene expression profiles; however, a higher correlation of affected signal cascades was shown. Various studies apply genes with significant twofold regulated genes for microarray data evaluation, although reproducibility of gene expression improves with threefold change. In the present study, at least twofold significantly regulated genes were included to follow the strategy frequently applied in microarray experiments; moreover, in the present study, only a limited amount of genes represented high fold changes.

A subproject of this thesis evaluated in detail the potential of the metabolomics-based method and a polymerase chain reaction (PCR)-based approach, applied for the identification of herbal substances. This subproject based on methods previously established in our group. Investigations of *Artemisia scoparia* Waldst. & Kit. samples were performed in cooperation (Prof. Heilmann and Prof. Franz from the University of Regensburg) to directly compare data about the chemical profile by standard methods and the metabolomics-based approach. For this purpose, *A. scoparia* samples from different provenances (China and Germany) were investigated. Using the metabolomics-based approach together with PCA, discrimination of *Artemisia* sp. was obtained. Regarding phytochemical groups of constituents responsible for the classification, mainly terpenoids, but also phenolics, flavonoids and phenylpropanoids were identified. Standard methods revealed that terpenoids (essential oil) and flavonoids widely varied between the herbal samples from different

provenances. Thereby, it was clearly shown that the metabolomics-based approach is highly valuable for a global classification of herbal preparations. Furthermore, the potential of this approach was positively evaluated as classification of different *Artemisia* sp., monographed in the Chinese Pharmacopeia, could be achieved at the species level. The analysis of at least two marker sequences is recommended to unambiguously identify the herbal origin.

The comprehensive characterization of complex herbal mixtures by a metabolome-based approach is very useful for authentication of herbal substances and reliable for the discrimination of major constituents. However, regarding phytochemical metabolites present in small amounts, limitations of the method were shown. For the identification of herbal substances, the PCR-based approach by ITS sequencing is suitable.

The present thesis provided new insights by bridging data about the chemical profile and gene expression profiles of herbal preparations. The investigation and correlation of both the chemical profile and functional activities is promising to comprehensively characterize herbal substances and their preparations. Regarding specificity, the metabolomics-based approach and gene expression profiling clearly showed characteristic signatures depending on the herbal preparation used. These results show the high importance of a defined manufacturing procedure. However, knowledge in gene regulation and systems biology is still limited and data about signal cascades affected in an *in vitro* system are only a starting point for further investigations.

10 References

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